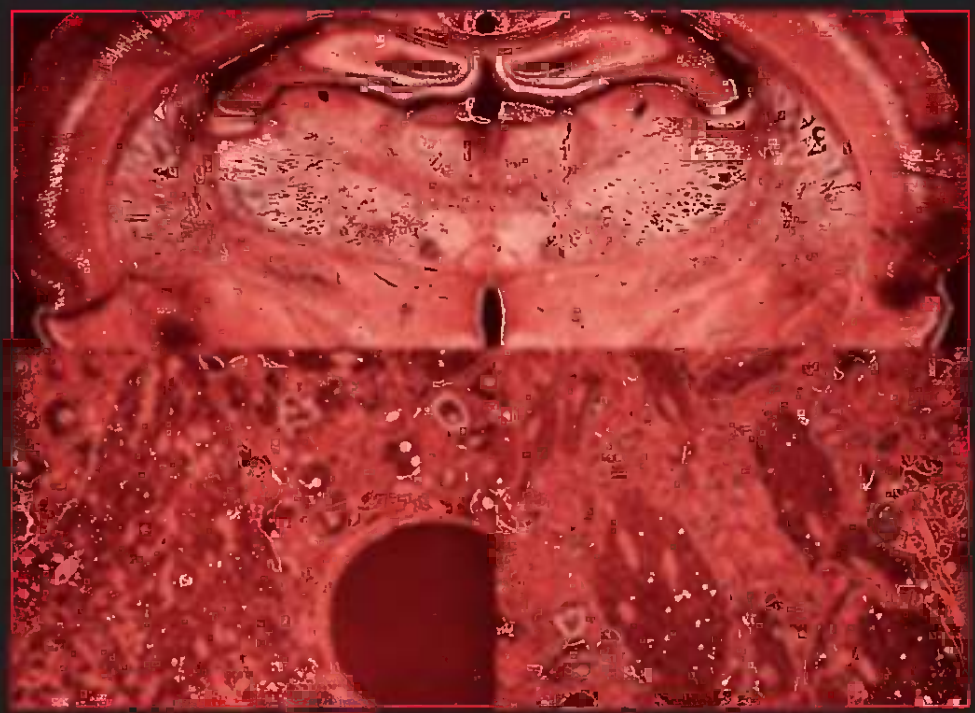
The Serotonin Receptors

From Molecular Pharmacology to Human Therapeutics

Edited by

Bryan L. Roth, MD, PhD



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The Serotonin Receptors

The Receptors

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Preface

It has been nearly 20 years since the last Humana Press book devoted to serotonin (5-hydroxytryptamine; 5-HT) receptors has appeared. Since then, the field of 5-HT receptors has undergone a revolution due to the discovery of many additional 5-HT receptors. Although 5-HT was chemically elucidated in 1948 by Page and colleagues (Rapport et al., 1948) and 5-HT receptors initially classified in 1957 (Gaddum and Picarelli, 1957), the complexity of 5-HT pharmacology was not fully appreciated until the late 1970s and early 1980s when many putative 5-HT receptors were identified by radioligand binding studies (e.g., 5-HT1A, 5-HT2, 5-HT1E and so on) (Leysen et al., 1979; Hamon et al., 1980; Peroutka et al., 1981; Leonhardt et al., 1989). The first 5-HT receptors were cloned in 1988 (Fargin et al., 1988; Julius et al., 1988) and the discovery of 14 distinct human 5-HT receptors since then ushered in the era of 5-HT receptor molecular biology (Kroeze et al., 2003). The cloning and sequencing of 5-HT receptors has also revealed the presence of post-transcriptionally modified mRNA species (RNA editing) (Burns et al., 1997) as well as naturally occurring mutations and their relations to various diseases (e.g., single nucleotide polymorphisms; SNPs) (Arranz et al., 1995).

The identification of the amino acid sequences of 5-HT receptors has allowed us to predict how 5-HT and related agonists bind to and activate 5-HT receptors (Shapiro et al., 2000; Shapiro et al., 2002). The hope has been that this information will lead, eventually, to the development of novel, subtype-selective 5-HT receptor agonists and antagonists (Kroeze et al., 2002).

The first several chapters of *The Serotonin Receptors: From Molecular Pharmacology to Human Therapeutics* are aimed at reviewing our knowledge of the molecular and structural biology of 5-HT receptors, followed by our current understanding of 5-HT receptor pharmacology. The elucidation of the sequences of 5-HT receptors has also facilitated the development of highly selective tools for mapping the distribution of 5-HT receptors. These tools include selective 5-HT receptor antibodies and hybridization probes. The use of these biochemical probes has revealed an unexpected complexity in both the cellular and subcellular distribution of 5-HT receptors.

The next few chapters describe the anatomical, cellular, and subcellular distribution of 5-HT receptors. Because of the plethora of receptors and receptor subtypes, however, it has been exceedingly difficult to identify the physiological role of various 5-HT receptors using pharmacological tools. A powerful approach

to elucidating the physiological role of 5-HT receptors was to use mice in which 5-HT receptors were deleted (e.g., knockout mice); the first 5-HT receptor knockouts were reported in 1994 (Saudou et al., 1994) and, since then, nearly all 5-HT receptors have been "knocked-out"—typically with novel phenotypes (Tecott et al., 1995; Brunner et al., 1999).

The final chapters review our understanding the physiological role(s) of 5-HT receptors based mainly on studies performed in genetically engineered mice. This book represents our collective attempts to provide the reader with a "snapshot" of the 5-HT receptor field circa 2006. The scope of the book is vast, proceeding from the genomic to the therapeutic. Because it is unlikely that any reader will devote the time to reading the entire book cover-to-cover, each chapter has been designed to represent a complete review of the particular field. Thus, each chapter begins with a short introduction to 5-HT receptors and then proceeds to review the particular subfield in depth. Not surprisingly, therefore, the enterprising reader will find some overlap between various introductory sections.

Acknowledgments

I would like to especially thank Mr. Jon Evans who has tirelessly collected, edited, and collated the finished chapters and who has done most of the "leg work" associated with this book. Without Jon's devotion to this task, the book would never have been completed. Any omissions and errors are my sole responsibility. I would also like to thank my wife Judith and my daughter Rachel for their warmth and understanding during the gestation of this book. Lastly, I dedicate this book to "beings throughout the ten directions—hands palm-to-palm."

Bryan L. Roth, MD, PhD

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1

Molecular Biology and Genomic Organization of G Protein–Coupled Serotonin Receptors

Wesley K. Kroeze and Bryan L. Roth

Summary

Among animals with nervous systems, serotonin (5-hydroxytryptamine; 5-HT) is a ubiquitous neurotransmitter, and numerous classes and subclasses of G protein–coupled 5-HT receptors have evolved to transduce extracellular 5-HT signals to the intracellular milieu. In this chapter, we summarize naturally occurring variation in serotonin receptor sequences. These sequences vary by species and by class and subclass and are further modified from their canonical sequences by RNA editing, alternative splicing, and the existence of single-nucleotide polymorphisms. By the presence of 5-HT receptors in such relatively simple organisms as *Caenorhabditis elegans*, it can be inferred that serotonergic signaling as a means of intracellular communication arose fairly early in evolutionary history. The multiple subclasses of 5-HT receptors and the various means to further modify receptor sequences, such as splicing and editing, presumably point to a biological requirement for very delicate "fine-tuning" of serotonergic signaling. How this fine-tuning is accomplished is likely to occupy and intrigue biologists for many years.

Key Words: Serotonin; 5-hydroxytryptamine; receptor; sequence; database.

1. Introduction

The G protein–coupled serotonin (5-hydroxytryptamine; 5-HT) receptors are typical group A rhodopsin-like G protein–coupled receptors (GPCRs) in that they are predicted to possess seven transmembrane spanning helices, three intracellular and three extracellular loops, an extracellular amino-terminus, and

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an intracellular carboxy-terminus. The true structures of these receptors remain unknown, although the crystallization of the bovine rhodopsin receptor *(1)* provides promise for the solution of the structures of the G protein–coupled 5-HT receptors in the near future. Functionally, the transmembrane regions serve to bind ligands, especially the endogenous ligand serotonin, the intracellular domains couple these receptors to various intracellular functions, and for the most part, the extracellular domains have uncertain functional roles (reviewed in ref. *2*). The endogenous ligand is the neurotransmitter serotonin, and the presence of serotonin and its receptors in a variety of invertebrates argues for a relatively early evolutionary origin of these receptors. Since our most recent review on this topic *(2)*, several genome sequencing projects have been completed or nearly completed, and new 5-HT receptors have continued to be added to the public databases. In addition, new insights have been gained on the relationship among sequence, structure, and function on many fronts. In this chapter, we will summarize aspects of protein and nucleic acid sequence variation among the GPCRs, with emphasis on newer findings.

In mammals, there are six classes of G protein–coupled 5-HT receptors, namely 5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7. These classes are further subdivided as follows. The 5-HT_1 receptor class contains the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptor subclasses. The 5-HT_2 receptor class contains the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. The 5-HT_5 receptor class contains the 5-HT_{5A}, and 5-HT_{5B} receptor subclasses. For the most part, the 5-HT_4, 5-HT_6, and 5-HT_7 classes do not contain subclasses of receptors *per se*, and sequence diversity in these classes is provided mainly by alternative mRNA splicing, which will be the subject of a subsequent chapter. The organization of classes and subclasses of receptors in mammals is not completely conserved, even among other vertebrates, as, for example, the pufferfish *Tetraodon* apparently has two subclasses of 5-HT_7 receptors.

Additional sequence diversity is provided by RNA editing in some subclasses of 5-HT receptors, and numerous single-nucleotide polymorphisms (SNPs) and splice variants are known to exist in many subclasses of these receptors. Editing of 5-HT receptors will be reviewed in this chapter, and SNPs and splice variants will be reviewed in subsequent chapters.

In addition to the 5-HT receptors identified from mammals, many 5-HT receptors have also been cloned from nonmammalian vertebrates and from several invertebrate species. Study of these 5-HT receptors should provide additional information on the nature of the residues essential for binding of at least the natural ligand, serotonin, to 5-HT receptors and might provide insight into how these receptors evolved. For example, residues that are completely conserved among all 5-HT receptors are likely to have essential roles in the function of the receptor, whether that be ligand binding, interaction with signaling

or scaffolding proteins, or maintenance of the three-dimensional structure of the protein. Residues that are conserved among one or a few subclasses of 5-HT receptors might, for example, contribute to the subtype selectivity of certain medications or explain coupling to restricted subsets of Gα-subunits. The wealth of data now available regarding naturally occurring receptor sequences suggests that methods such as the "evolutionary trace" method of the Lichtarge group *(4)* could also be very useful in further unraveling the relationships among sequence, structure, and function in these proteins.

Notwithstanding the progress that has been made in the sequencing of various genomes and cloning of new receptors, much remains to be done to gain a full appreciation of the natural variation in sequence of the 5-HT receptors, even in relatively frequently studied animal species. For example, of the 13 likely G protein–coupled 5-HT receptors in mammalian genomes, only 2 have been described from rabbits, 5 from pigs and dogs, 7 from guinea pigs, and 3 from hamsters. Table 1 lists the G protein–coupled 5-HT receptors known to date.

2. Database Annotation of 5-HT Receptors

With ongoing efforts in various genome sequencing projects, as well as the cloning of individual genes, 5-HT receptors are continually being added to the public databases. Although these additions to the databases provide a richness of new information for the investigator, a word of caution is in order. Functional annotation is now being provided by automated or semiautomated algorithms; thus, sequences can be annotated as 5-HT receptors without pharmacologic or functional data. Automated annotation of new sequences is generally accurate when there are very clear homologies with previously known receptors, as with newly cloned mammalian receptors (Table 1). However, when the database sequences of newly cloned or sequenced, putative 5-HT receptors diverge significantly from previously known 5-HT receptor sequences, as with some of the newly discovered invertebrate and nonmammalian vertebrate receptors (Table 1), the annotations should be considered to be tentative until pharmacologic or functional data are obtained.

In addition to automated functional annotation of putative 5-HT receptor sequences, determination of open reading frames (ORFs), start codons, and intron/exon boundaries from genomic sequences is now also automated. The absence of expert human curation in such automated annotation can potentially lead to the presence of incorrect sequence data in the databases that has arisen from misidentification of ORFs, start codons, and intron/exon boundaries in raw genomic sequences. For example, the sequences of several chimpanzee 5-HT receptors have recently been added to the GenBank databases, but the sequences given for these receptors indicate that one or several of the seven transmembrane (TM) helices are not present in the sequence, although

Table 1
G Protein–Coupled 5-HT Receptors Known to Date

Receptor	Species	Probable second messenger[a]	Accession number	Ref.
Invertebrates				
5-HT_{B1}	*Aplysia* sea slug	PI	Q16950	*48*
5-HT_{B2}	*Aplysia* sea slug	PI	Q16951	*48*
5-HT_7-Ae	*Aedes* mosquito		AAG49292	*49*
5-HT_7-ANO	*Anopheles* mosquito		XP–313129	Database only
5-HT-DRO	*Drosophila* fruit fly	+AC	AAA28305	*50*
5-HT-PLAN1	*Dugesia* planarian		BAA22404	*51*
5-HT-PLAN4	*Dugesia* planarian		BAA22403	*51*
5-HT_7-He	*Helisoma* snail		AAQ84306	Database only
5-HT-Ser7	*C. elegans* nematode		AAB04582	Database only
5-HT-DRO2	*Drosophila* fruit fly		CAA57429	*52*
5-HT_{2A}-ANO	*Anopheles* mosquito		XP–307953	Database only
5-HT-Ser1	*C. elegans* nematode		AAC15827	*39*
5-HT-ASC	*Ascaris* nematode	PI	AAC78396	*53, 54*
5-HT-LYM2	*Lymnaea* snail	PI	AAC16969	*55*
5-HT-LOB	*Panulirus* lobster	PI	AAS57919	*56*
5-HT-DRO1A	*Drosophila* fruit fly	+AC	CAA77570	*57*
5-HT_{1A}-ANO	*Anopheles* mosquito		EAA04158	Database only
5-HT-HEL	*Heliothis* moth	–AC	CAA64863	*58*
5-HT-BUT	*Papilio* butterfly		BAD72868	*59*
5-HT-DRO1B	*Drosophila* fruit fly	–AC	CAA77571	*57*
5-HT-TICK	*Boophilus* tick	–AC	AAQ89933	*60*
5-HT-BEE	*Apis* honeybee		XP–393915	Database only
5-HT-BOM	*Bombyx* moth	–AC	CAA64862	*58*
5-HT_{1B}-ANO	*Anopheles* mosquito		XP–317820	Database only
5-HT-LYM	*Lymnaea* snail		AAA29290	*61*
5-HT-1He1	*Helisoma* snail		AAQ95277	Database only
5-HT-AP1	*Aplysia* sea slug	–AC	AAM46088	*62*
5-HT-AP2	*Aplysia* sea slug	–AC	AAC28786	*63*
5-HT-Ser4	*C. elegans* nematode	–AC	NP–497452	*64*
5-HT-HAEM	*Haemonchus* nematode		AAO45883	*65*
5-HT-BARN	*Balanus* barnacle		BAA12013	*66*
5-HT-SH	*Metapenaeus* shrimp		AAS05316	Database only
5-HT-Ser2	*C. elegans* nematode	–AC	NP–741936	*67*
5-HT-Ser3	*C. elegans* nematode		NP–491954	Database only
5-HT	*C. elegans* nematode		NP–508238	Database only

Table 1 *(continued)*

Receptor	Species	Probable second messenger[a]	Accession number	Ref.
Vertebrates				
5-HT_{1A}-α	*Fugu* fish	–AC	CAA65175	*68*
5-HT_{1A}-β	*Fugu* fish	–AC	CAA65176	*68*
5-HT_{1A}-α	*Tetraodon* fish	–AC	CAF93441	Database only
5-HT_{1A}-β	*Tetraodon* fish	–AC	CAF91711	Database only
5-HT_{1A}	*Tilapia* fish	–AC	AAP83427	Database only
5-HT_{1A}	Chicken	–AC	XP–429136	Database only
5-HT_{1A}	Human	–AC	NP–000515	*69, 70*
5-HT_{1A}	Rat	–AC	NP–036717	*71*
5-HT_{1A}	Gorilla	–AC	BAA94490	Database only
5-HT_{1A}	Orangutan	–AC	BAA94491	Database only
5-HT_{1A}	Mouse	–AC	NP–032334	*72*
5-HT_{1A}	Chimpanzee	–AC	BAA94489	Database only
5-HT_{1A}	Fox	–AC	AAP12466	*73*
5-HT_{1A}	Dog	–AC	AAN08044	*74*
5-HT_{1A}	Frog	–AC	CAA69208	*75*
5-HT_{1B}	Chimpanzee	–AC	BAA94456	Database only
5-HT_{1B}	Human	–AC	AAH69065	*76–84*
5-HT_{1B}	Gorilla	–AC	BAA94457	Database only
5-HT_{1B}	Dog	–AC	AAP12469	*73, 85*
5-HT_{1B}	Fox	–AC	AAP12468	*73*
5-HT_{1B}	Pig	–AC	NP–999463	*86*
5-HT_{1B}	Mouse	–AC	NP–034612	*87*
5-HT_{1B}	Rat	–AC	NP–071561	*88*
5-HT_{1B}	Rabbit	–AC	CAA90531	*89*
5-HT_{1B}	Golden hamster	–AC	AAK25827	Database only
5-HT_{1B}	Mole rat	–AC	AAB82748	Database only
5-HT_{1B}	Chinese hamster	–AC	CAA60175	Database only
5-HT_{1B}	Guinea pig	–AC	AAB58500	*90*
5-HT_{1B}	Opossum	–AC	AAA17567	*91*
5-HT_{1B}	*Tetraodon* fish	–AC	CAF89927	Database only
5-HT_{1B}	Chicken	–AC	XP–419875	Database only
5-HT_{1D}	Chimpanzee	–AC	XP–524604	Database only
5-HT_{1D}	Human	–AC	NP–000855	*76, 92*
5-HT_{1D}	Pig	–AC	NP–999323	*93*
5-HT_{1D}	Rabbit	–AC	CAA90530	*89*
5-HT_{1D}	Guinea pig	–AC	CAA64210	*94*
5-HT_{1D}	Rat	–AC	NP–036984	*79, 88*

(continued)

Table 1 *(continued)*

Receptor	Species	Probable second messenger[a]	Accession number	Ref.
5-HT_{1D}	Mouse	–AC	NP–032335	*82, 95*
5-HT_{1D}	Dog	–AC	CAA32207	*96–98*
5-HT_{1D}	*Fugu* fish	–AC	CAA58745	*68*
5-HT_{1D}	*Tilapia* fish	–AC	AAP83428	Database only
5-HT_{1E}	Chimpanzee	–AC	XP–527443	Database only
5-HT_{1E}	Human	–AC	NP–000856	*81, 84, 99*
5-HT_{1E}	Gorilla	–AC	BAA94460	Database only
5-HT_{1E}	Orangutan	–AC	BAA94461	Database only
5-HT_{1E}	Guinea pig	–AC	AAR05654	*100*
5-HT_{1F}	Chimpanzee	–AC	XP–526246	Database only
5-HT_{1F}	Human	–AC	NP–000857	*101, 102*
5-HT_{1F}	Gorilla	–AC	BAA90455	Database only
5-HT_{1F}	Orangutan	–AC	BAA90456	Database only
5-HT_{1F}	Mouse	–AC	NP–032336	*103*
5-HT_{1F}	Pig	–AC	NP–999266	*104*
5-HT_{1F}	Guinea pig	–AC	AAB58496	*105*
5-HT_{1F}	Rat	–AC	NP–068629	*101*
5-HT_{1F}	Chicken	–AC	XP–425535	Database only
5-HT_{1F}	*Tetraodon* fish	–AC	CAG09227	Database only
5-HT_{2A}	Cow	PI	NP–001001157	Database only
5-HT_{2A}	Dog	PI	NP–001005869	*85*
5-HT_{2A}	Pig	PI	NP–999382	*106, 107*
5-HT_{2A}	Human	PI	NP–000612	*108–113*
5-HT_{2A}	Rhesus	PI	AAB34691	*106*
5-HT_{2A}	Orangutan	PI	CAH93260	Database only
5-HT_{2A}	Rat	PI	NP–058950	*108, 114*
5-HT_{2A}	Chinese hamster	PI	CAA37800	*115*
5-HT_{2A}	Mouse	PI	NP–766400	*116*
5-HT_{2A}	Chicken	PI	XP–425628	Database only
5-HT_{2A}	Chimpanzee	PI	XP–522752	Database only
5-HT_{2B}	Human	PI	NP–000858	*117, 118*
5-HT_{2B}	Mouse	PI	NP–032337	*119*
5-HT_{2B}	Rat	PI	NP–058946	*120*
5-HT_{2B}	Frog	PI	CAD71264	*121*
5-HT_{2B}	*Tetraodon* Fish	PI	CAC85912	*122*
5-HT_{2C}	Chicken	PI	XP–426265	Database only
5-HT_{2C}	Dog	PI	NP–001006649	*85*

(continued)

Table 1 *(continued)*

Receptor	Species	Probable second messenger[a]	Accession number	Ref.
5-HT_{2C}	Rat	PI	NP–036897	*123*
5-HT_{2C}	Human	PI	NP–000859	*109*, *124*
5-HT_{2C}	Mouse	PI	NP–032338	*125*, *126*
5-HT_{2C}	Chimpanzee	PI	XP–529113	Database only
5-HT_{4}	Pig	+AC	NP–001001267	*107*
5-HT_{4}	Guinea pig	+AC	CAA73912	Database only
5-HT_{4}	Human	+AC	CAC22248	*107*, *127–131*
5-HT_{4}	Mouse	+AC	CAA70775	*128*, *129*, *132*
5-HT_{4}	Rat	+AC	NP–036985	*37*, *107*, *129*
5-HT_{4}	Chicken	+AC	XP–414481	Database only
5-HT_{4}	*Tetraodon* fish	+AC	CAF95370	Database only
5-HT_{4}	Chimpanzee	+AC	XP–518024	Database only
5-HT_{5A}	Guinea pig		CAD59057	*133*
5-HT_{5A}	Rat		NP–037280	*134*
5-HT_{5A}	Human		NP–076917	135
5-HT_{5A}	Mouse		NP–032340	*136*, *137*
5-HT_{5A}	Chicken		XP–425970	Database only
5-HT_{5A}	Zebrafish		NP–001007122	Database only
5-HT_{5A}	*Tetraodon* fish		CAG04298	Database only
5-HT_{5A}	Chimpanzee		XP–519477	Database only
5-HT_{5B}	Mouse		NP–034613	*136*
5-HT_{5B}	Rat		XP–341112	*134*, *138*
5-HT_{6}	Human	+AC	NP–000862	*107*, *139*
5-HT_{6}	Chimpanzee	+AC	XP–524584	Database only
5-HT_{6}	Rat	+AC	NP–077341	*140*, *141*
5-HT_{6}	Mouse	+AC	NP–067333	*142*
5-HT_{7}	Guinea pig	+AC	AAA83015	*143*
5-HT_{7}	Rat	+AC	NP–075227	*144–148*
5-HT_{7}	Pig	+AC	NP–999250	*149*
5-HT_{7}	Human	+AC	NP–000863	*144*, *145*, *150*
5-HT_{7}	Chimpanzee	+AC	XP–521556	Database only
5-HT_{7}	Dog	+AC	CAF31500	Database only
5-HT_{7}	Mouse	+AC	NP–032341	*151*
5-HT_{7}	Chicken	+AC	XP–420880	Database only
5-HT_{7}	*Tetraodon* fish	+AC	CAG05134	Database only
5-HT_{7x}	*Tetraodon* fish	+AC	CAG09680	Database only

[a]+AC = stimulates adenylate cyclase activity; −AC = inhibits forskolin-stimulated adenylate cyclase activity; PI = stimulates phosphatidylinositol (PI) hydrolysis.

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5HT2A-CHIMP     MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGC
5HT2A-HUMAN     MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGC
                ************************************************************

5HT2A-CHIMP     LSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIAD
5HT2A-HUMAN     LSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIAD
                ************************************************************

5HT2A-CHIMP     MLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNP
5HT2A-HUMAN     MLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNP
                ************************************************************

5HT2A-CHIMP     IHHSRFNSRTKAFLKIIAVWTISVEMPEKEKKIIGEKSGKLKKGRNPSRTIFERGTPAHC
5HT2A-HUMAN     IHHSRFNSRTKAFLKIIAVWTISVG----------------------------------
                ************************

5HT2A-CHIMP     PNYSSYKSCRISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYF
5HT2A-HUMAN     ----------ISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYF
                          ************************************************

5HT2A-CHIMP     LTIKSLQKEATLCVSDLGTRAKLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSI
5HT2A-HUMAN     LTIKSLQKEATLCVSDLGTRAKLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSI
                ************************************************************

5HT2A-CHIMP     SNEQKACKVLGIVFFLFV------------------------------------------
5HT2A-HUMAN     SNEQKACKVLGIVFFLFVVMWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAV
                ******************

5HT2A-CHIMP     ---------------------------------LILVNTIPALAYKSSQLQMGQKKNSKQD
5HT2A-HUMAN     NPLVYTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQD
                                                 ****************************

5HT2A-CHIMP     AKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV
5HT2A-HUMAN     AKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV
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Fig. 1. Alignment of the putative chimpanzee 5-HT_{2A} receptor sequence (accession number XP–522752) with the human 5-HT_{2A} receptor sequence (accession number NP–000612), illustrating an example of differences between the receptor sequences of the two species probably caused by faulty database annotation.

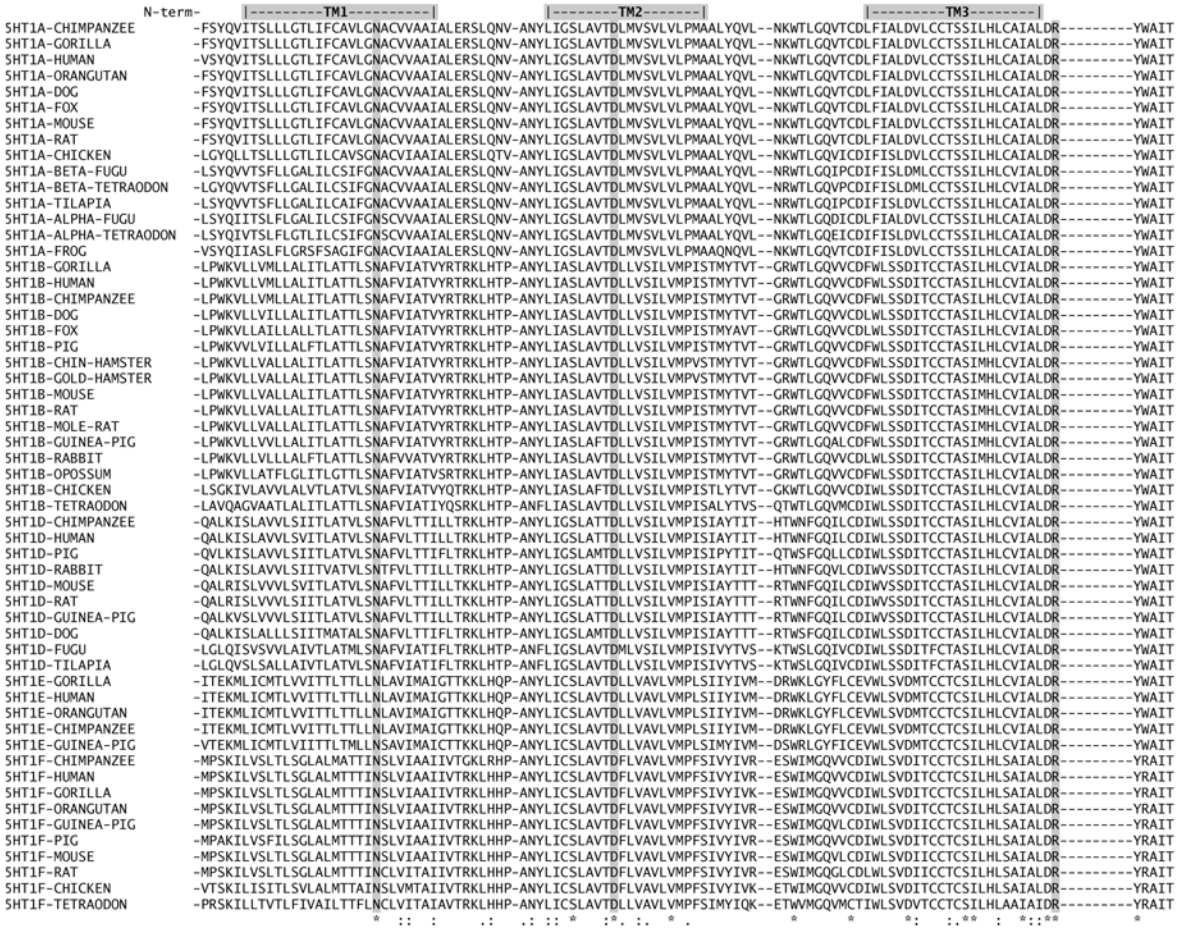
obviously, for these receptors to be functional, all seven TM helices must be present. Thus, for example, the chimpanzee 5-HT_{2A} receptor (accession number XP–522752) has an approx 50-residue insertion in transmembrane helix 4 (TM4) and a deletion that removes most of TM6 and TM7, although the distal end of the C-terminus is present in the database sequence (Fig. 1). Similarly, the first 35 residues of the chimpanzee 5-HT_{2C} receptor (accession number XP–529113) as given in the database bear no resemblance to the human 5-HT_{2C} receptor, and the "true" 5-HT_{2C} receptor sequence of the chimpanzee protein as listed in the database begins about halfway through TM4. In the chimpanzee 5-HT_4 receptor sequence (accession number XP–518024), the "Y" of the highly conserved DRY motif at the intracellular end of TM3, the i2 intracellular loop, and TM4 are missing from the database sequence. The chimpanzee 5-HT5A

receptor sequence (accession number XP–519477) is incomplete and contains a run of "X"s that preclude alignment with the other known 5-HT receptors; additionally, although both the N-terminus and C-terminus are present in the sequence, only the N-terminus is similar to known 5-HT receptors, and the C-terminal sequence, following the run of "X"s, bears no resemblance to any known 5-HT receptor by BLAST search. Although we have presented the chimpanzee 5-HT6 receptor sequence (accession number XP–524584) in the alignment of all vertebrate 5-HT receptors shown in Fig. 2, the amino-terminus of the sequence in the database is likely to be incorrect because it is much longer in the chimpanzee sequence than in any other known 5-HT receptor sequence. A possible explanation for this is that the DNA sequence for this region is very GC rich, which may have made DNA sequencing difficult, and, thus, that an error in the reading of this sequence may have resulted in the abnormally long amino-terminus. Obviously, many of these chimpanzee sequences as listed would represent nonfunctional receptors. Therefore, it remains an open question (1) whether these represent aberrant splicing isoforms among the chimpanzee 5-HT receptors that do not exist in humans, (2) whether chimpanzee 5-HT receptors are truly different than those from other mammals, or (3) whether further investigation of the genomic sequences with expert curation will reveal 5-HT receptor sequences more like those from other primate or mammalian species. Of these three possibilities, the third seems most likely.

The chicken 5-HT_{2C} receptor sequence in the database (accession number XP–426265) is 1337 residues long, as compared to 458 residues for the human receptor (accession number NP–000859). Much of this "extra" sequence resides in the amino-terminus, which in the chicken sequence has the highly conserved "GN" motif in TM1 at positions 459–460, as opposed to positions 70–71 in the human 5-HT_{2C} receptor sequence. In the chicken sequence, the e1 extracellular loop is also very long and the highly conserved "DRY," motif is given as "DRC," although many other features of 5-HT_{2C} receptors are retained. It is possible that 5-HT_{2C} receptors of birds are in fact very different from the 5-HT_{2C} receptors of other species, as the current sequence data imply, or that the chicken 5-HT_{2C} receptor sequence in the database is incorrect. Which of these alternatives is found to be true awaits further research.

As can be seen from the alignment shown in Fig. 2, the sequence given in the database for the *Tetraodon* 5-HT_4 receptor (accession number CAF95370) is unusually short, beginning with a methionine in TM1. This "ATG" is unlikely to be the true "start" codon for this receptor because this results in part of TM1 being absent, but examination of the genomic sequence shows a "TAG" stop codon immediately upstream of the "ATG" methionine codon. Whether this is

(text continued on p. 18)


N-term-
TM1
TM2
TM3
5HT1A-CHIMPANZEE -FSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-GORILLA -FSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-HUMAN -VSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-ORANGUTAN -FSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-DOG -FSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-FOX -FSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-MOUSE -FSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-RAT -FSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-CHICKEN -LGYQLLTSLLLGTLILCAVSGNACVIAAIALERSLQTV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQVICDIFISLDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-BETA-FUGU -LSYQVVTSFLLGALILCSIFGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NRWTLGQIPCDIFISLDMLCCTSSILHLCVIALDR---------YWAIT
5HT1A-BETA-TETRAODON -LGYQVVTSFLLGALILCSIFGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NRWTLGQVPCDIFISLDMLCCTSSILHLCVIALDR---------YWAIT
5HT1A-TILAPIA -LSYQVVTSFLLGALILCAIFGNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NRWTLGQIPCDIFISLDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-ALPHA-FUGU -LSYQIITSLFLGALILCSIFGNSCVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQDICDLFIALDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-ALPHA-TETRAODON -LSYQIVTSLFLGTLILCSIFGNSCVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL--NKWTLGQEICDIFISLDVLCCTSSILHLCAIALDR---------YWAIT
5HT1A-FROG -VSYQIIASLFLGRSFSAGIFGNACVIAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAAQNQVL--NKWTLGQVTCDIFISLDVLCCTSSILHLCAIALDR---------YWAIT
5HT1B-GORILLA -LPWKVLLVMLLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-HUMAN -LPWKVLLVMLLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-CHIMPANZEE -LPWKVLLVMLLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-DOG -LPWKVLLVILLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDLWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-FOX -LPWKVLLAILLALLTLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYAVT--GRWTLGQVVCDLWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-PIG -LPWKVVLVILLALFTLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-CHIN-HAMSTER -LPWKVLLVALLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPVSTMYTVT--GRWTLGQVVCDFWLSSDITCCTASIMHLCVIALDR---------YWAIT
5HT1B-GOLD-HAMSTER -LPWKVLLVALLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPVSTMYTVT--GRWTLGQVVCDFWLSSDITCCTASIMHLCVIALDR---------YWAIT
5HT1B-MOUSE -LPWKVLLVALLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASIMHLCVIALDR---------YWAIT
5HT1B-RAT -LPWKVLLVALLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASIMHLCVIALDR---------YWAIT
5HT1B-MOLE-RAT -LPWKVLLVALLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASIMHLCVIALDR---------YWAIT
5HT1B-GUINEA-PIG -LPWKVLLVVLLALITLATTLSNAFVIATVYRTRKLHTP-ANYLIASLAFTDLLVSILVMPISTMYTVT--GRWTLGQALCDFWLSSDITCCTASIMHLCVIALDR---------YWAIT
5HT1B-RABBIT -LPWKVLLVLLLALFTLATTLSNAFVVATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDLWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-OPOSSUM -LPWKVLLATFLGLITLGTTLSNAFVIATVSRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT--GRWTLGQVVCDFWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-CHICKEN -LSGKIVLAVVLALVTLATVLSNAFVIATVYQTRKLHTP-ANYLIASLAFTDLLVSILVMPISTLYTVT--GKWTLGQVVCDIWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1B-TETRAODON -LAVQAGVAATLALITLATTLSNAFVIATIYQSRKLHTP-ANFLIASLAVTDLLVSILVMPISALYTVS--QTWTLGQVMCDIWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-CHIMPANZEE -QALKISLAVVLSIITLATVLSNAFVLTTILLTRKLHTP-ANYLIGSLATTDLLVSILVMPISIAYTIT--HTWNFGQILCDIWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-HUMAN -QALKISLAVVLSVITLATVLSNAFVLTTILLTRKLHTP-ANYLIGSLATTDLLVSILVMPISIAYTIT--HTWNFGQILCDIWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-PIG -QVLKISLAVVLSIITLATVLSNAFVLTTIFLTRKLHTP-ANYLIGSLAMTDLLVSILVMPISIPYTIT--QTWSFGQLLCDIWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-RABBIT -QALKISLAVVLSIITVATVLSNTFVLTTILLTRKLHTP-ANYLIGSLATTDLLVSILVMPISIAYTIT--HTWNFGQVLCDIWVSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-MOUSE -QALRISLVVVLSVITLATVLSNAFVLTTILLTKKLHTP-ANYLIGSLATTDLLVSILVMPISIAYTTT--RTWNFGQILCDIWVSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-RAT -QALRISLVVVLSIITLATVLSNAFVLTTILLTKKLHTP-ANYLIGSLATTDLLVSILVMPISIAYTTT--RTWNFGQILCDIWVSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-GUINEA-PIG -QALKVSLVVVLSIITLATVLSNAFVLTTILLTRKLHTP-ANYLIGSLATTDLLVSILVMPISIAYTTT--RTWNFGQILCDIWVSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-DOG -QALKISLALLLSIITMATALSNAFVLTTIFLTRKLHTP-ANYLIGSLAMTDLLVSILVMPISIAYTTT--RTWSFGQILCDIWLSSDITCCTASILHLCVIALDR---------YWAIT
5HT1D-FUGU -LGLQISVSVVLAIVTLATMLSNAFVIATIFLTRKLHTP-ANFLIGSLAVTDMLVSILVMPISIVYTVS--KTWSLGQIVCDIWLSSDITFCTASILHLCVIALDR---------YWAIT
5HT1D-TILAPIA -LGLQVSLSALLAIVTLATVLSNAFVIATIFLTRKLHTP-ANFLIGSLAVTDLLVSILVMPISIVYTVS--KTWSLGQIVCDIWLSSDITFCTASILHLCVIALDR---------YWAIT
5HT1E-GORILLA -ITEKMLICMTLVVITTLTTLLNLAVIMAIGTTKKLHQP-ANYLICSLAVTDLLVAVLVMPLSIIYIVM--DRWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDR---------YWAIT
5HT1E-HUMAN -ITEKMLICMTLVVITTLTTLLNLAVIMAIGTTKKLHQP-ANYLICSLAVTDLLVAVLVMPLSIIYIVM--DRWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDR---------YWAIT
5HT1E-ORANGUTAN -ITEKMLICMTLVVITTLTTLLNLAVIMAIGTTKKLHQP-ANYLICSLAVTDLLVAVLVMPLSIIYIVM--DRWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDR---------YWAIT
5HT1E-CHIMPANZEE -ITEKMLICMTLVVITTLTTLLNLAVIMAIGTTKKLHQP-ANYLICSLAVTDLLVAVLVMPLSIIYIVM--DRWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDR---------YWAIT
5HT1E-GUINEA-PIG -VTEKMLICMTLVIITTLTMLLNSAVIMAICTTKKLHQP-ANYLICSLAVTDLLVAVLVMPLSIMYIVM--DSWRLGYFICEVWLSVDMTCCTCSILHLCVIALDR---------YWAIT
5HT1F-CHIMPANZEE -MPSKILVSLTLSGLALMATTINSLVIAAIIVTGKLRHP-ANYLICSLAVTDFLVAVLVMPFSIVYIVR--ESWIMGQVVCDIWLSVDITCCTCSILHLSAIALDR---------YRAIT
5HT1F-HUMAN -MPSKILVSLTLSGLALMTTTINSLVIAAIIVTRKLHHP-ANYLICSLAVTDFLVAVLVMPFSIVYIVR--ESWIMGQVVCDIWLSVDITCCTCSILHLSAIALDR---------YRAIT
5HT1F-GORILLA -MPSKILVSLTLSGLALMTTTINSLVIAAIIVTRKLHHP-ANYLICSLAVTDFLVAVLVMPFSIVYIVK--ESWIMGQVVCDIWLSVDITCCTCSILHLSAIALDR---------YRAIT
5HT1F-ORANGUTAN -MPSKILVSLTLSGLALMTTTINSLVIAAIIVTRKLHHP-ANYLICSLAVTDFLVAVLVMPFSIVYIVR--ESWIMGQVVCDIWLSVDITCCTCSILHLSAIALDR---------YRAIT
5HT1F-GUINEA-PIG -MPSKILVSLTLSGLALMTTTINSLVIAAIIVTRKLHHP-ANYLICSLAVTDFLVAVLVMPFSIVYIVR--ESWIMGQVLCDIWLSVDITCCTCSILHLSAIALDR---------YRAIT
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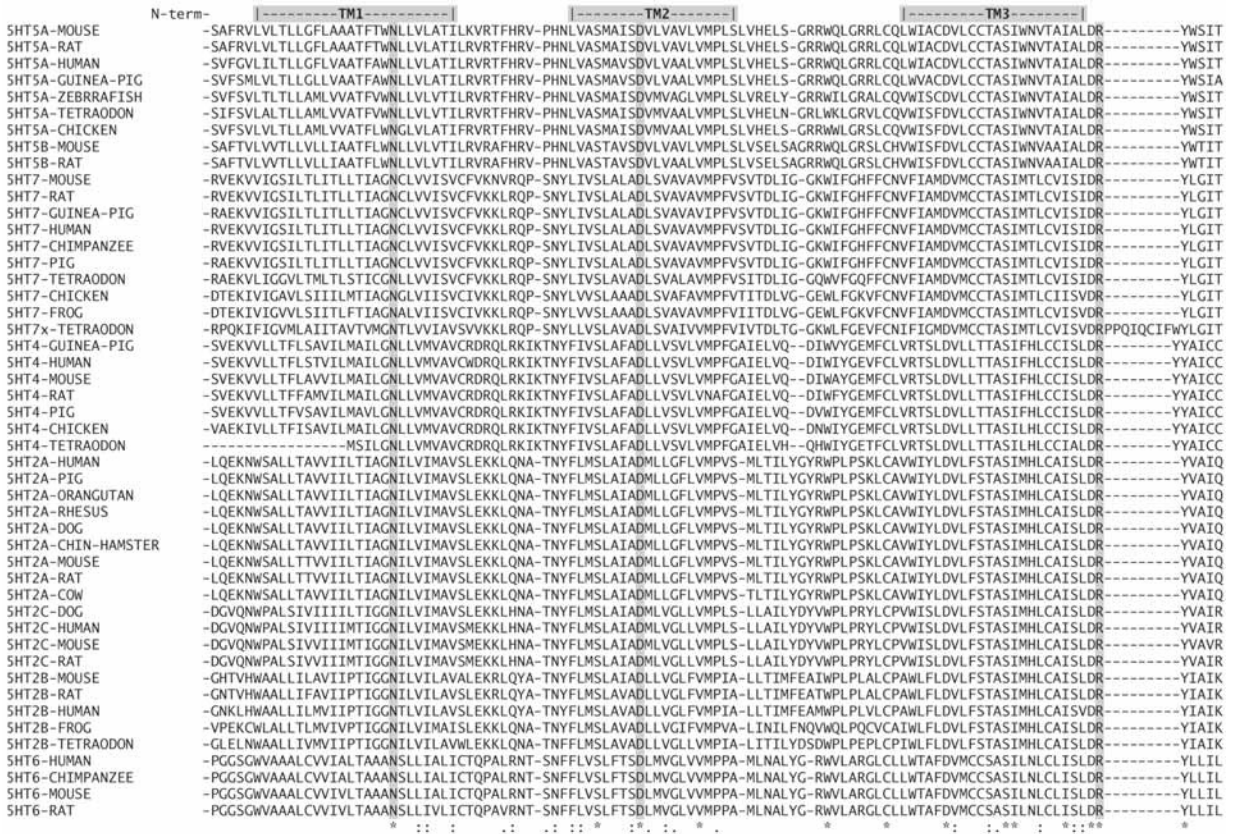
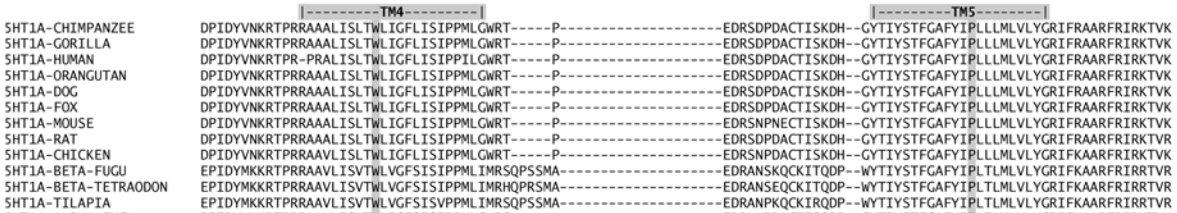


Fig. 2. Clustal W (1.82) multiple sequence alignment of 104 vertebrate serotonin receptors. Amino-terminus and carboxy-terminus have been removed for clarity. Completely conserved residues are marked with an asterik (*) under the alignment.

(continued)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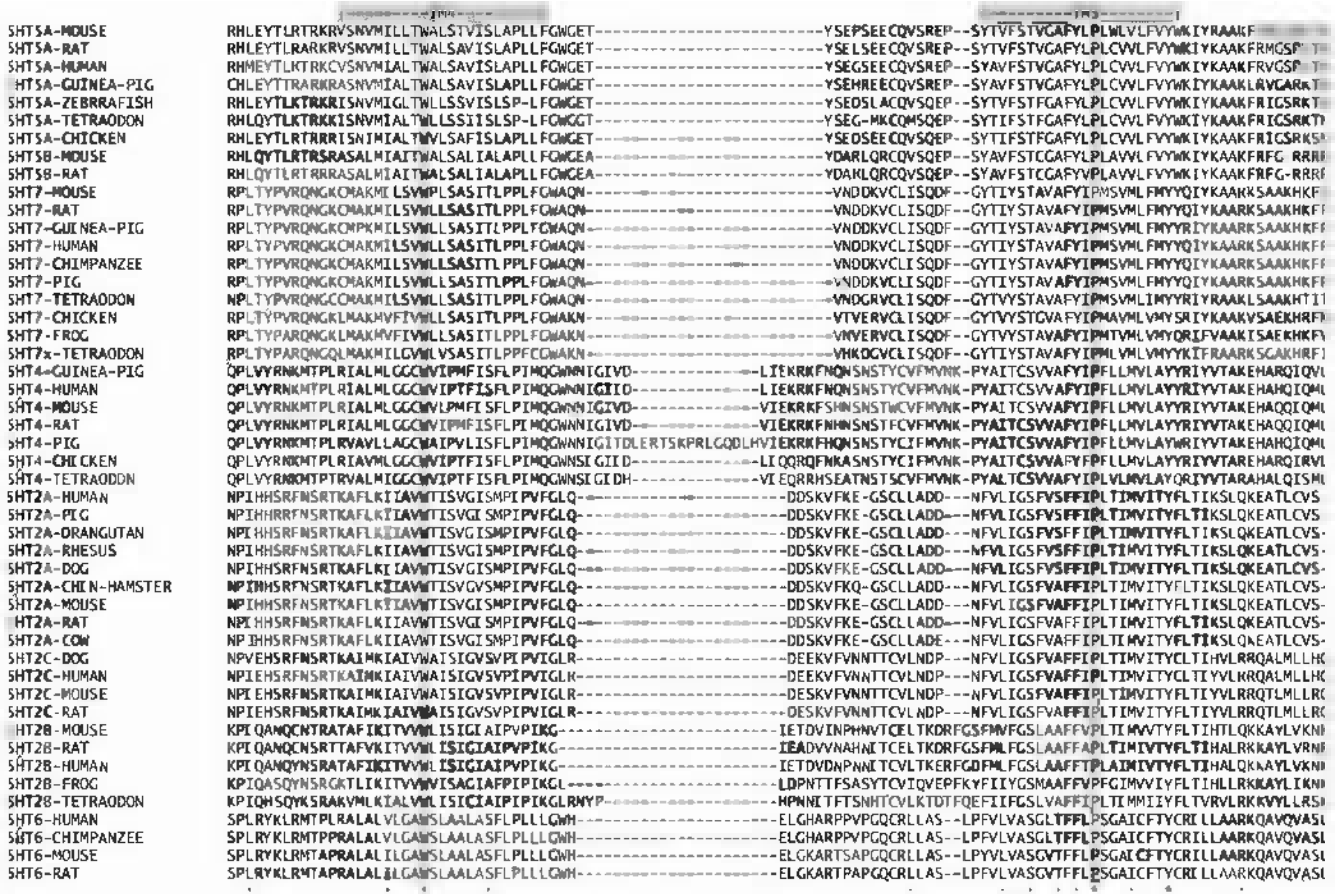

Fig. 2. *(continued)*

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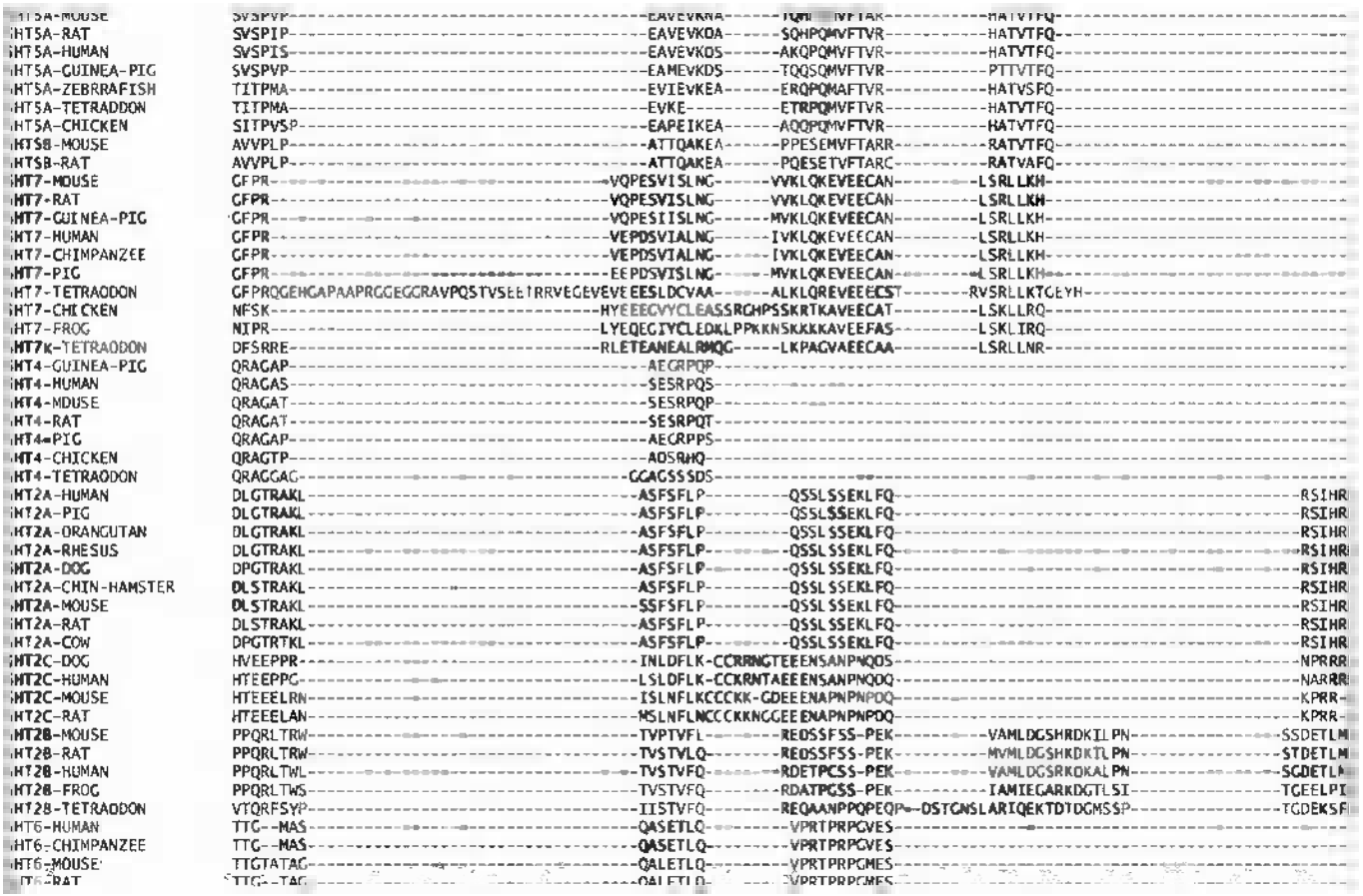
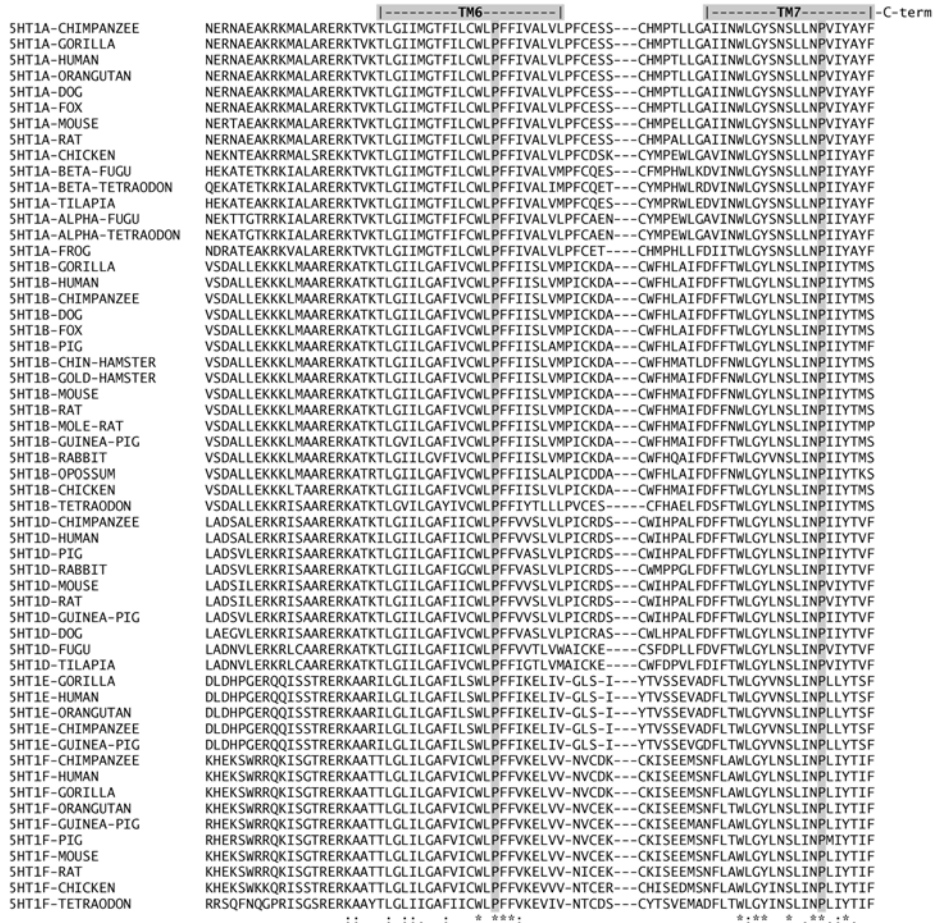


Fig. 2. *(continued)*


|---------TM6---------|
|--------TM7--------|-C-term
5HT1A-CHIMPANZEE NERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPTLLGAIINWLGYSNSLLNPVIYAYF
5HT1A-GORILLA NERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPTLLGAIINWLGYSNSLLNPVIYAYF
5HT1A-HUMAN NERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPTLLGAIINWLGYSNSLLNPVIYAYF
5HT1A-ORANGUTAN NERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPTLLGAIINWLGYSNSLLNPVIYAYF
5HT1A-DOG NERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPTLLGAIINWLGYSNSLLNPVIYAYF
5HT1A-FOX NERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPTLLGAIINWLGYSNSLLNPVIYAYF
5HT1A-MOUSE NERTAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPELLGAIINWLGYSNSLLNPVIYAYF
5HT1A-RAT NERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS---CHMPALLGAIINWLGYSNSLLNPVIYAYF
5HT1A-CHICKEN NEKNTEAKRRMALSREKKTVKTLGIIMGTFILCWLPFFIVALVLPFCDSK---CYMPEWLGAVINWLGYSNSLLNPIIYAYF
5HT1A-BETA-FUGU HEKATETKRKIALARERKTVKTLGIIMGTFILCWLPFFIVALVMPFCQES---CFMPHWLKDVINWLGYSNSLLNPIIYAYF
5HT1A-BETA-TETRAODON QEKATETKRKIALARERKTVKTLGIIMGTFILCWLPFFIVALIMPFCQET---CYMPHWLRDVINWLGYSNSLLNPIIYAYF
5HT1A-TILAPIA HEKATEAKRKIALARERKTVKTLGIIMGTFILCWLPFFIVALVMPFCQES---CYMPRWLEDVINWLGYSNSLLNPIIYAYF
5HT1A-ALPHA-FUGU NEKTTGTRRKIALARERKTVKTLGIIMGTFIFCWLPFFIVALVLPFCAEN---CYMPEWLGAVINWLGYSNSLLNPIIYAYF
5HT1A-ALPHA-TETRAODON NEKATGTKRKIALARERKTVKTLGIIMGTFICWLPFFIVALVLPFCAEN---CYMPEWLGAVINWLGYSNSLLNPIIYAYF
5HT1A-FROG NDRATEAKRKVALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCET----CHMPHLLFDIITWLGYSNSLLNPIIYAYF
5HT1B-GORILLA VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHLAIFDFFTWLGYLNSLINPIIYTMS
5HT1B-HUMAN VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHLAIFDFFTWLGYLNSLINPIIYTMS
5HT1B-CHIMPANZEE VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHLAIFDFFTWLGYLNSLINPIIYTMS
5HT1B-DOG VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHLAIFDFFTWLGYLNSLINPIIYTMS
5HT1B-FOX VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHLAIFDFFTWLGYLNSLINPIIYTMS
5HT1B-PIG VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLAMPICKDA---CWFHLAIFDFFTWLGYLNSLINPIIYTMF
5HT1B-CHIN-HAMSTER VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHMATLDFFNWLGYLNSLINPIIYTMS
5HT1B-GOLD-HAMSTER VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHMAIFDFFNWLGYLNSLINPIIYTMS
5HT1B-MOUSE VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHMAIFDFFNWLGYLNSLINPIIYTMS
5HT1B-RAT VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHMAIFDFFNWLGYLNSLINPIIYTMS
5HT1B-MOLE-RAT VSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDA---CWFHMAIFDFFNWLGYLNSLINPIIYTMP
5HT1B-GUINEA-PIG VSDALLEKKKLMAARERKATKTLGVILGAFIVCWLPFFIISLVMPICKDA---CWFHMAIFDFFTWLGYLNSLINPIIYTMS
5HT1B-RABBIT VSDALLEKKKLMAARERKATKTLGIILGVFIVCWLPFFIISLVMPICKDA---CWFHQAIFDFFTWLGYVNSLINPIIYTMS
5HT1B-OPOSSUM VSDALLEKKKLMAARERKATRTLGIILGAFIVCWLPFFIISLALPICDDA---CWFHLAIFDFFNWLGYLNSLINPIIYTKS
5HT1B-CHICKEN VSDALLEKKKLTAARERKATKTLGIILGAFIVCWLPFFIISLVLPICKDA---CWFHMAIFDFFTWLGYLNSLINPIIYTMS
5HT1B-TETRAODON VSDALLEKKRISAARERKATKTLGVILGAYIVCWLPFFIYTLLLPVCES-----CFHAELFDSFTWLGYLNSLINPIIYTMS
5HT1D-CHIMPANZEE LADSALERKRISAARERKATKTLGIILGAFIICWLPFFVVSLVLPICRDS---CWIHPALFDFFTWLGYLNSLINPIIYTVF
5HT1D-HUMAN LADSALERKRISAARERKATKTLGIILGAFIICWLPFFVVSLVLPICRDS---CWIHPALFDFFTWLGYLNSLINPIIYTVF
5HT1D-PIG LADSVLERKRISAARERKATKTLGIILGAFIICWLPFFVASLVLPICRDS---CWIHPALFDFFTWLGYLNSLINPIIYTVF
5HT1D-RABBIT LADSVLERKRISAARERKATKTLGIILGAFIGCWLPFFVASLVLPICRDS---CWMPPGLFDFFTWLGYLNSLINPIIYTVF
5HT1D-MOUSE LADSILERKRISAARERKATKTLGIILGAFIICWLPFFVVSLVLPICRDS---CWIHPALFDFFTWLGYLNSLINPVIYTVF
5HT1D-RAT LADSILERKRISAARERKATKTLGIILGAFIICWLPFFVVSLVLPICRDS---CWIHPALFDFFTWLGYLNSLINPVIYTVF
5HT1D-GUINEA-PIG LADSVLERKRISAARERKATKTLGIILGAFIVCWLPFFVVSLVLPICRDS---CWIHPALFDFFTWLGYLNSLINPIIYTVF
5HT1D-DOG LAEGVLERKRISAARERKATKTLGIILGAFIVCWLPFFVASLVLPICRAS---CWLHPALFDFFTWLGYLNSLINPIIYTVF
5HT1D-FUGU LADNVLERKRLCAARERKATKTLGIILGAFIICWLPFFVTLVWAICKE----CSFDPLLFDVFTWLGYLNSLINPVIYTVF
5HT1D-TILAPIA LADNVLERKRLCAARERKATKTLGIILGAFIVCWLPFFIGTLVMAICKE----CWFDPVLFDIFTWLGYLNSLINPVIYTVF
5HT1E-GORILLA DLDHPGERQQISSTRERKAARILGLILGAFILSWLPFFIKELIV-GLS-I---YTVSSEVADFLTWLGYVNSLINPLLYTSF
5HT1E-HUMAN DLDHPGERQQISSTRERKAARILGLILGAFILSWLPFFIKELIV-GLS-I---YTVSSEVADFLTWLGYVNSLINPLLYTSF
5HT1E-ORANGUTAN DLDHPGERQQISSTRERKAARILGLILGAFILSWLPFFIKELIV-GLS-I---YTVSSEVADFLTWLGYVNSLINPLLYTSF
5HT1E-CHIMPANZEE DLDHPGERQQISSTRERKAARILGLILGAFILSWLPFFIKELIV-GLS-I---YTVSSEVADFLTWLGYVNSLINPLLYTSF
5HT1E-GUINEA-PIG DLDHPGERQQISSTRERKAARILGLILGAFILSWLPFFIKELIV-GLS-I---YTVSSEVGDFLTWLGYVNSLINPLLYTSF
5HT1F-CHIMPANZEE KHEKSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NVCDK---CKISEEMSNFLAWLGYLNSLINPLIYTIF
5HT1F-HUMAN KHEKSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NVCDK---CKISEEMSNFLAWLGYLNSLINPLIYTIF
5HT1F-GORILLA KHEKSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NVCDK---CKISEEMSNFLAWLGYLNSLINPLIYTIF
5HT1F-ORANGUTAN KHEKSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NVCEK---CKISEEMSNFLTWLGYLNSLINPLIYTIF
5HT1F-GUINEA-PIG RHEKSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NVCEK---CKISEEMANFLAWLGYLNSLINPLIYTIF
5HT1F-PIG RHERSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NVCEK---CKISEEMSNFLTWLGYLNSLINPMIYTIF
5HT1F-MOUSE KHEKSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NVCEK---CKISEEMSNFLAWLGYLNSLINPLIYTIF
5HT1F-RAT KHEKSWRRQKISGTRERKAATTLGLILGAFVICWLPFFVKELVV-NICEK---CKISEEMSNFLAWLGYLNSLINPLIYTIF
5HT1F-CHICKEN KHEKSWKKQRISSTRERKAATTLGLILGAFVICWLPFFVKEVVV-NTCER---CHISEDMSNFLAWLGYINSLINPLIYTIF
5HT1F-TETRAODON RRSQFNQGPRISGSRERKAAYTLGLIIGAFIICWLPFFVKEVIV-NTCDS---CYTSVEMADFLTWLGYINSLINPLIYTIF

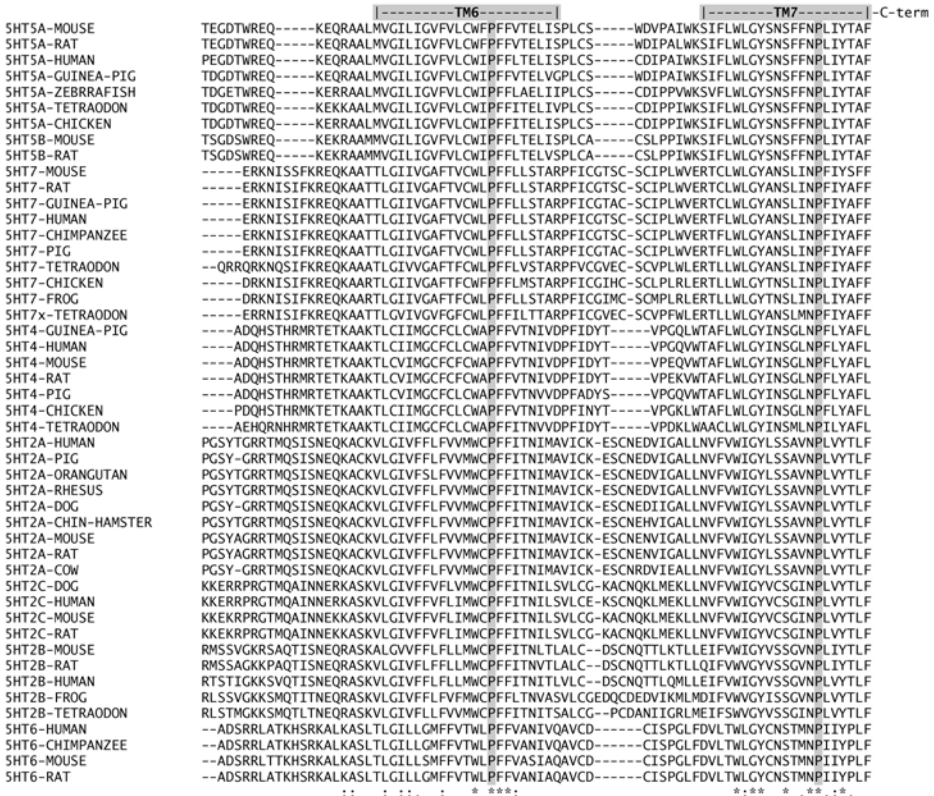


Fig. 2. *(continued)*

the "true" sequence for this receptor or whether the 5-HT4 receptor in *Tetraodon* is a pseudogene (see below) also awaits the results of further research.

Among invertebrate receptors, for example, the sequences of the N-termini of the 5-HT_{1A} and 5-HT_{1B} receptors from the *Anopheles* mosquito (accession numbers EAA04158 and XP–317820, respectively) are unusually short. The *Anopheles* 5-HT_{1A} receptor has only 18 residues upstream of the highly conserved "GN" motif in TM1; based on a comparison with all the other known 5-HT receptor sequences, the methionine that is 18 residues upstream of the "GN" motif is unlikely to be the true start codon. An even more extreme example is seen with the *Anopheles* 5-HT_{1B} receptor, which has only 43 residues upstream of the highly conserved "DRY" motif at the cytoplasmic end of TM3; certainly this is not sufficient for three transmembrane helices, the first extracellular and intracellular loops, and the N-terminus. The methionine annotated as the "start" methionine of the *Anopheles* 5-HT_{1B} receptor is more likely to be located near the cytoplasmic end of TM2; therefore, the sequence of the receptor upstream of this point remains unknown. Thus, the sequences of these receptors as shown in the databases must be considered as partial sequences, and knowledge of the full sequences of these receptors awaits future updates of the database and perhaps even further cloning and sequencing efforts.

3. Evolutionary Considerations

G Protein–coupled receptors as a group have been identified from relatively simple organisms such as bacteria (bacteriorhodopsin) and yeast (mating factor receptors). However, 5-HT receptors are not known from bacteria or single-celled eukaryotes, which is not surprising because they lack nervous systems and, therefore, neurotransmitters and their receptors. One can imagine circumstances under which such simple organisms (e.g., those living in the intestine) could use 5-HT receptors to sense the presence of 5-HT in their environment, but to date this has not been reported. There is one archaeal sequence from *Thermoplasma acidophilum* in the GenBank database (accession number NP–393638) that is annotated as a "serotonin receptor related protein"; however, this "receptor" lacks the "DRY" motif at the cytoplasmic end of TM3, the "NPxxY" motif in TM7, and other highly conserved features associated with 5-HT receptors. Additionally, BLAST searching with the archaeal sequence does not result in any 5-HT receptor "hits." Therefore, this archaeal sequence is probably incorrectly annotated and is unlikely to be a 5-HT receptor.

Nematodes and other invertebrates with relatively simple nervous systems have receptors for 5-HT and other neurotransmitters in their genomes (*see* Table 1). Interestingly, most of the invertebrate 5-HT receptors discovered to date resemble either the mammalian 5-HT_{1A} receptors or the 5-HT_7 receptors, although

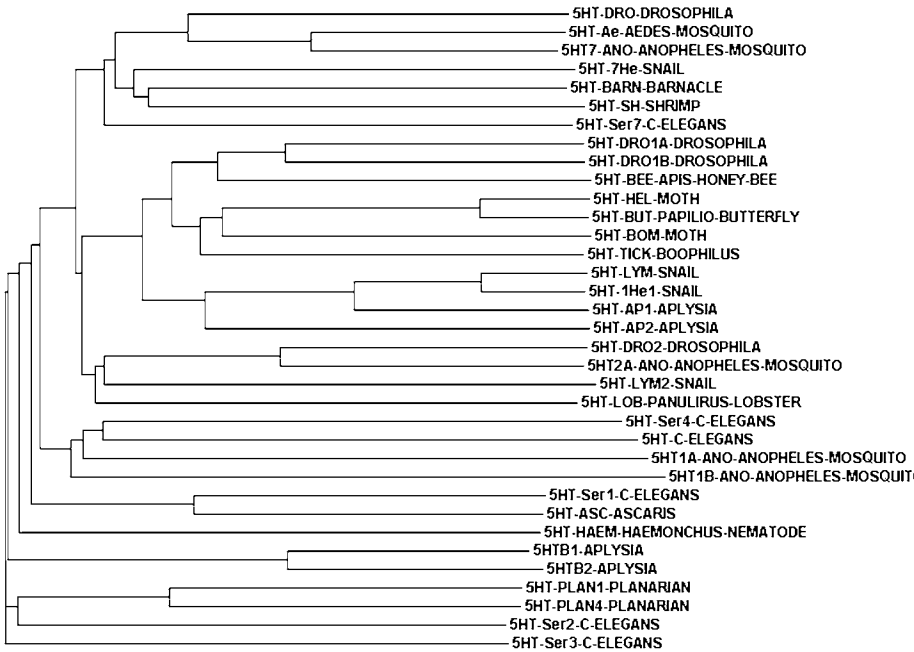


Fig. 3. Dendrogram showing the relationships among 5-HT receptors known from invertebrates.

some are not clearly similar to any of the known mammalian 5-HT receptor subclasses. This suggests the speculative hypothesis that either the 5-HT_{1A} or the 5-HT_7 receptor subclass represents the ancestral archetypical 5-HT receptor.

The need for diversity in 5-HT receptor-mediated signaling must have arisen relatively early in evolutionary history, as some invertebrate species have multiple 5-HT receptor subtypes. For example, at least four subclasses of 5-HT receptors are known from the *Anopheles* mosquito, *Aplysia*, and *Drosophila*, and at least seven from the nematode *Caenorhabditis elegans*. Additional diversity in signaling is provided by alternative splicing (*see* subsequent chapter).

In Fig. 3 is shown the relationships among the currently known 5-HT receptors from invertebrates, and in Fig. 4, a dendrogram showing the relationships of the vertebrate 5-HT receptors is shown. These trees are based on alignments done using the Clustal W algorithm available at the webpage of the European Bio-informatics Institute (http://www.ebi.ac.uk/clustalw/), after trimming of the amino-terminus and carboxy-terminus, as we have done previously *(5)*. Among the invertebrate receptors (Fig. 3), very few discrete groupings of receptors are seen, except for a group of 5-HT_7-like receptors from a variety of organisms (seen at the top of the dendrogram).

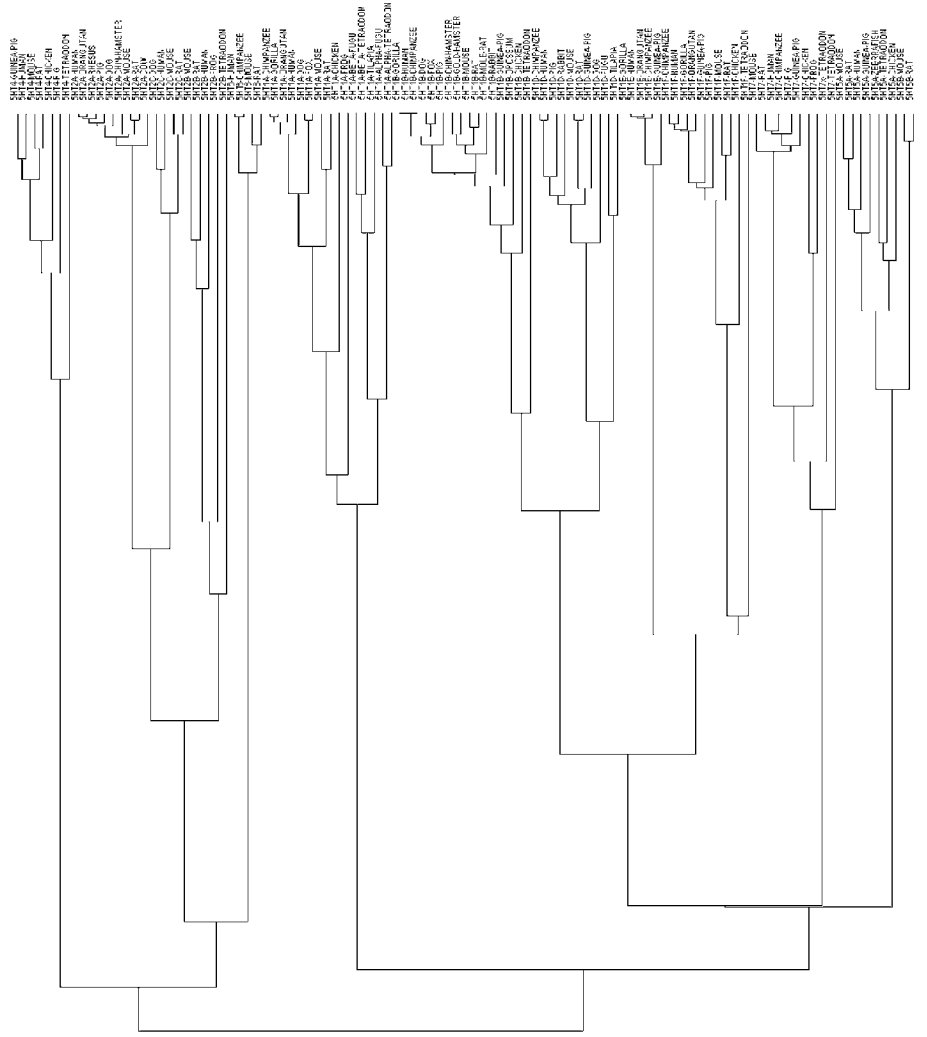


Fig. 4. Dendrogram showing the relationships among 5-HT receptors known from vertebrates.

The relationships among the vertebrate 5-HT receptor subclasses can be seen in Fig. 4. The 5-HT_{2A} and 5-HT_{2C} receptors of vertebrates are more similar to each other than either is to the 5-HT_{2B} receptors. The 5-HT_6 receptors are more similar to the 5-HT_2 receptors than the other classes. The 5-HT_4 receptors are most similar to the 5-HT_6/5-HT_2 group. Among the 5-HT_1 subclasses, the 5-HT_{1B} and 5-HT_{1D} receptors are most similar to each other, as are the 5-HT_{1E} and 5-HT_{1F} subclasses.

4. Sequence Conservation and Patterns of Variation Among Serotonin Receptors

For convenience, we will use the numbering convention of residues in TM helices devised by Ballesteros and Weinstein *(6)*, in which the most highly conserved residue in each helix is given an index number of 50. For example, the mostly highly conserved residue in helix 1 is referred to as residue 1.50, in helix 2 as 2.50, and so on. Other residues are numbered in relation to the index residue, so the residue in helix 1 that is one position closer to the amino-terminus of the protein is named residue 1.49 and so on. This system has the advantage of more easily being able to refer to homologous positions in different receptors by the same number.

Figure 5 shows an alignment of the known 5-HT receptors from *invertebrates*. Each of the TM helices except TM5 and TM6 contains at least one residue that is completely conserved. In TM1, the N1.50 residue is completely conserved. In TM2, D2.50 is completely conserved, as are L2.46, A2.47, and L2.51. In addition to the index residue R3.50, D3.32, S3.39, and I3.40 are conserved in TM3; 28 of the 35 receptors have a completely conserved "DRY" motif, and most of the remainder have very similar sequences (e.g., ERY, DRF, and GRY among others). Only the index residue W4.50 is completely conserved in TM4. In TM5, the index residue is P5.50, but in two of the receptors from the sea slug *Aplysia*, this residue is not a proline, but a serine in the *Aplysia* 5-HTB1 receptor (accession number Q16950) and a phenylalanine in the *Aplysia* 5-HTB2 receptor (accession number Q16951). The index residue in TM6 is P6.50, but in one of the 5-HT receptors from the nematode *C. elegans* (the Ser3 receptor, accession number NP–491954), this position is occupied by a glycine, which, interestingly, is also a helix-breaking residue. F6.44 and W6.48 are completely conserved among all of the invertebrate 5-HT receptors. ln TM7, the index residue P7.50 is completely conserved, as are W7.40, G7.42, S7.46, N7.49, and Y7.53. The residues N7.49, P7.50, and Y7.53 comprise the conserved NPxxY motif, which has several proposed roles in the function of other classes of GPCRs, including activation of small G proteins and internalization, first shown by Barak et al. *(7)* using the β_2 adrenergic receptor.

Figure 2 shows an alignment of 104 of the known 5-HT receptors from *vertebrates*; for clarity, sequences for which the databases contain incomplete or incorrect sequences (see above) have been omitted. In TM1, the index residue N1.50 is completely conserved among all 104 receptors; position 1.53 is a valine in all but the 5-HT6 receptors, in which it is a leucine. In helix 2, residue D2.50 is completely conserved among all 104 receptors, as are S2.45 and V2.57. In TM3, the DRY motif is conserved in all but one of the receptors, the exception being one of the two 5-HT7-like receptors from the pufferfish

5HTB1-APLYSIA
5HTB2-APLYSIA
5HT-Ae-AEDES
5HT7-ANO-ANOPHELES
5HT-DRO-DROSOPHILA
5HT-PLAN1-PLANARIAN
5HT-PLAN4-PLANARIAN
5HT-7He-SNAIL
5HT-Ser7-C-ELEGANS
5HT-DRO2-DROSOPHILA
5HT2A-ANO-ANOPHELES
5HT-Ser1-C-ELEGANS
5HT-ASC-ASCARIS
5HT-LYM2-SNAIL
5HT-LOB-PANULIRUS
5HT-DRO1A-DROSOPHILA
5HT1A-ANO-ANOPHELES
5HT-HEL-MOTH
5HT-BUT-PAPILIO
5HT-DRO1B-DROSOPHILA
5HT-TICK-BOOPHILUS
5HT-BEE-APIS
5HT-BOM-MOTH
5HT1B-ANO-ANOPHELES
5HT-LYM-SNAIL
5HT-1He1-SNAIL
5HT-AP1-APLYSIA
5HT-AP2-APLYSIA
5HT-Ser4-C-ELEGANS
5HT-HAEM-HAEMONCHUS
5HT-BARN-BARNACLE
5HT-SH-SHRIMP
5HT-Ser2-C-ELEGANS
5HT-Ser3-C-ELEGANS
5HT-C-ELEGANS
Conserved

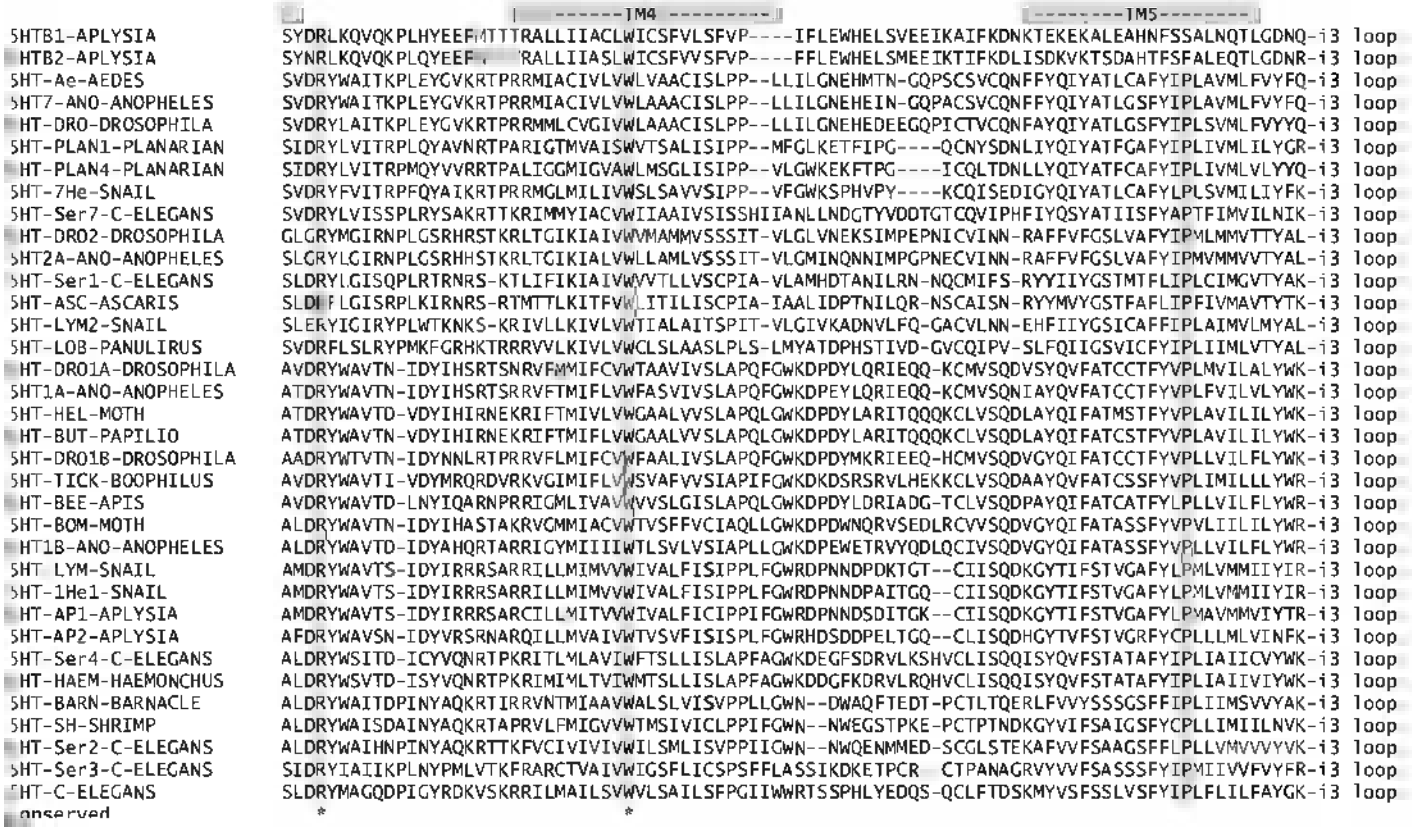


Fig. 5. Clustal W (1.82) multiple sequence alignment of 35 vertebrate serotonin receptors. Amino- and carboxy-terminus have been removed for clarity. Completely conserved residues are marked with an asterik (*) under the alignment.

(continued)

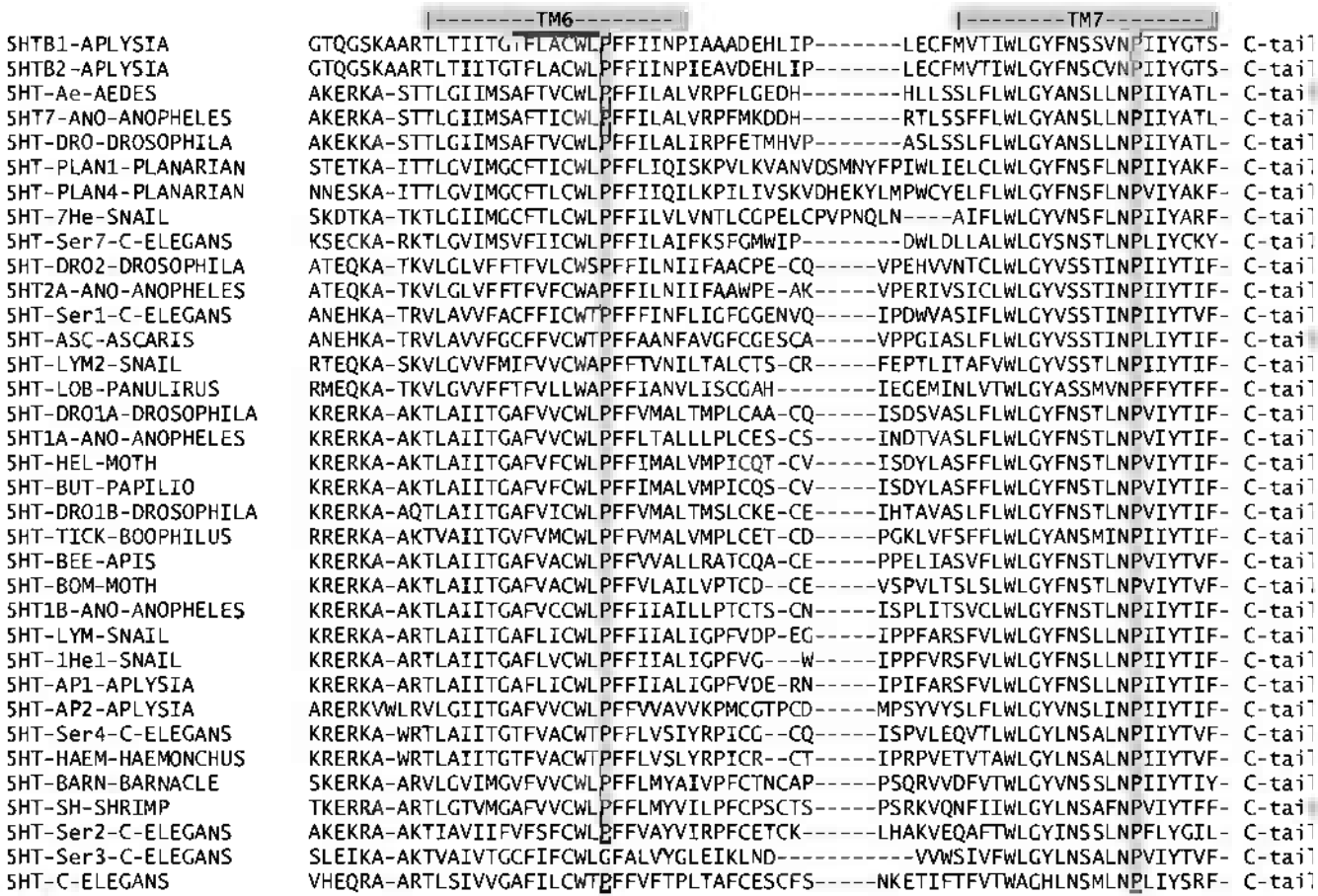


Fig. 5. *(continued)*

Tetraodon (accession number CAG09680). Additionally in TM3, residues D3.32, S3.39, I3.40, and I3.46 are completely conserved; the relatively high degree of sequence conservation in helix 3 is perhaps not surprising given its central role in ligand binding in these receptors. In helix 4, only the index residue W4.50 is completely conserved, and in TM5, residues P5.50 and Y5.58 are completely conserved. TM6 and TM7 are perhaps the most highly conserved helices, with four and seven completely conserved residues, respectively; this is probably also a reflection of the essential roles of many of these residues for binding of the natural ligand serotonin as well as serotonergic medications. Much information has been gleaned from mutagenesis studies on the functional roles of residues in the TM helices (*see* subsequent chapter, and refs. *2* and *3* for review).

5. Pseudogenes

Pseudogenes are sequences of genomic DNA that are very similar to "normal" genes but are nonfunctional because they lack functional promoters or have accumulated mutations that lead to premature stop codons in the coding sequence *(8)*. In the human genome, in addition to the 20,000 to 30,000 genes, there are at least 7800 pseudogenes *(8)*. Among the 5-HT receptors, pseudogenes have been described for the 5-HT_{1D} receptor *(9–11)*, the 5-HT_4 receptor *(12)*, and the 5-HT_7 receptor *(13–16)*. Interestingly, the human 5-HT_{5B} gene is known only as a pseudogene *(17)*, and a function for the human 5-HT_{5A} receptor is not known. Presumably, the functional role of the 5-HT5B receptor in other species has been taken over in humans by some other subclass of 5-HT receptor.

6. Chromosomal Location of Receptors

The chromosomal locations of the human 5-HT receptors are listed in Table 2. The 5-HT_{2C} receptor gene is on the X chromosome; the remainder are autosomal.

7. Introns and Exons

All of the 5-HT_1 receptor subclass genes have a single exon, whereas the remaining human 5-HT receptor genes have multiple exons. The 5-HT_2 receptor genes have a central exon of 201 bases (Fig. 6), suggesting a possible common origin for these genes. However, no clear pattern emerges from examination of the intron and exon sizes of the remaining human 5-HT receptor genes.

8. RNA Editing

An additional source of variation in 5-HT receptor sequences is provided by RNA editing, which was first shown for the rat 5-HT_{2C} receptor *(18)*.

Table 2
Chromosomal Location and Exon Numbers in Human 5-HT Receptor Genes

Receptor	Chromosomal location	No. of exons
5-HT_{1A}	5q11.2-q13	1
5-HT_{1B}	6q13	1
5-HT_{1D}	1p36.3-p34.3	1
5-HT_{1E}	6q14-15	1
5-HT_{1F}	3p12	1
5-HT_{2A}	13q14-q21	3
5-HT_{2B}	2q36.3-q37.1	3
5-HT_{2C}	Xq24	4
5-HT_{4}	5q31-q33	6
5-HT_{5A}	7q36.1	2
5-HT_{6}	1p36-p35	3
5-HT_{7}	10q21-q24	3

Burns et al. *(18)* showed that editing at various combinations of four sites in the mRNA for the 5-HT2C receptor could lead to the expression of six variant 5-HT_{2C} receptor proteins in addition to the wild-type unedited form. Further variants produced by editing at additional sites were subsequently described by others *(19–21)*. The process of RNA editing by adenosine deaminase leads to variation in the protein sequence of the second intracellular loop of the receptor, which, in turn, may lead to differences in G protein coupling and, therefore, constitutive activity, with the unedited form of the receptor showing the highest degree of constitutive activity *(18–20,22–29)*. The difference in G protein coupling has been attributed to differences in the structure of the i2 intracellular loop by molecular modeling *(30)*. Variants may differ in tissue distribution *(18)*, and the degree of editing varies among species *(22)*. Trafficking and desensitization of the receptor are also affected by editing, which may have effects on the interactions of the receptor with arrestin and G protein–coupled receptor kinase (GRK) *(31)*. In a recent study *(32)*, it has been shown that an intronic site of the 5-HT_{2C} receptor mRNA is also edited (site "F") and that editing at this intronic site affects both editing at site "D" and alternative splicing.

Whether differences in editing may have clinical implications is still an open question. One report suggests that suicide victims have significantly more editing at the "A" site *(33)*. Another report suggests that some sites (the "C" and "C′" sites) exhibit more editing in suicide victims, whereas others (the "D" site) have less editing, and the "A" site shows no significant difference between

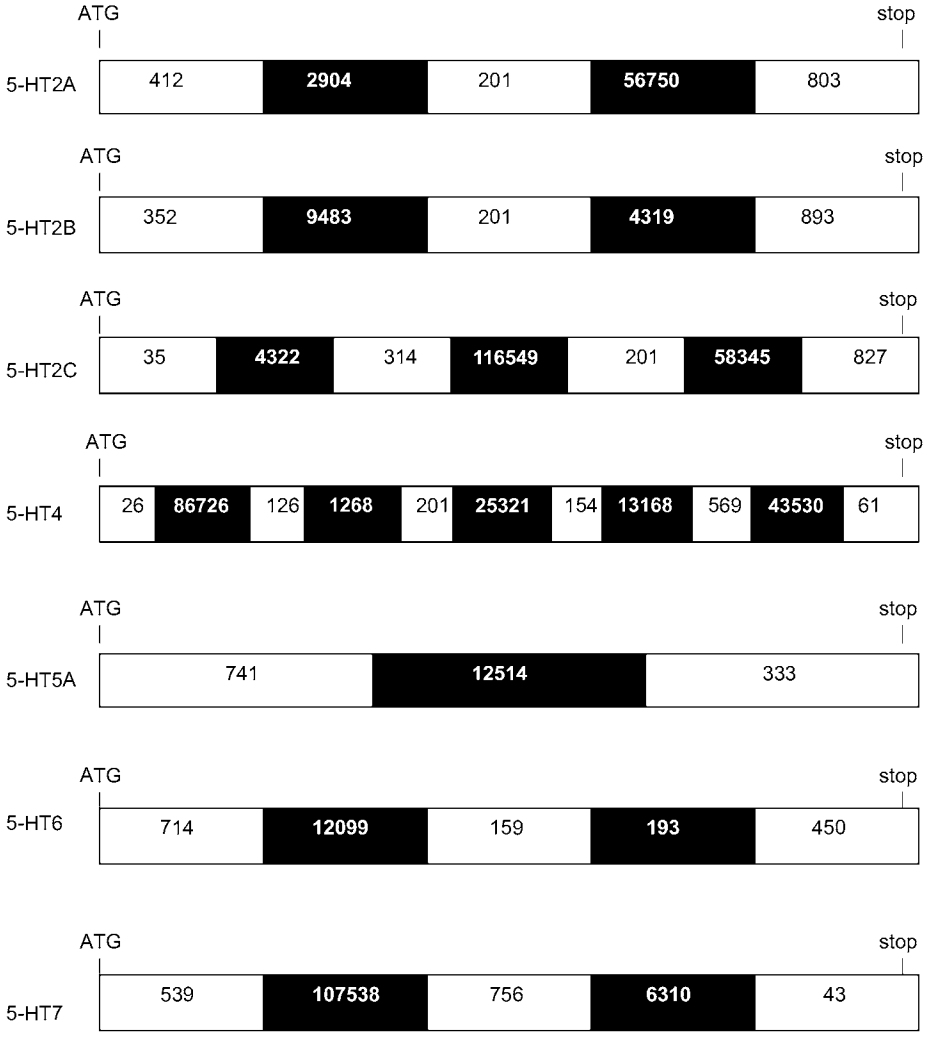


Fig. 6. Lengths of introns and exons within the coding region of human 5-HT receptors. Exons are shown in white background; introns are shown in black background. The 5-HT_1 class of receptors has a single exon, and is not shown.

suicide victims and controls *(34)*. The sample sizes of both these studies are relatively small, i.e., 18 *(33)* and 6 *(34)*, so the true nature of the relationship between 5-HT_{2C} receptor editing and suicidality awaits further work.

In another study *(21)*, it was suggested that editing may be reduced in schizophrenic subjects, and novel editing variants were described from these subjects. Thus, there may be a relationship between the extent of 5-HT_{2C} receptor editing

and various psychiatric disorders, but full appreciation of this relationship will depend on further data.

The degree of editing of the 5-HT_{2C} receptor may be modulated by various drugs. For example, administration of the tryptophan hydroxylase inhibitor *para*-chlorophenylalanine (pCPA) to mice, which depletes synaptic 5-HT, led to a significant decrease in editing at the "C" and "C′" sites *(35)*, whereas the 5-HT2A/2C partial agonist (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (DOI) caused a significant increase in editing at the "C′" site *(35)*. In a contrasting study, cocaine or reserpine, which increases 5-HT levels, had no apparent effect on 5-HT_{2C} receptor editing in rats *(36)*. What controls the level of editing in different tissues, in different individuals, and in different pharmacological circumstances is still largely unknown.

9. Alternative Splicing and Splice Variants

Alternative splicing as a means of providing additional sequence diversity among 5-HT receptors was first described for the rat 5-HT_4 receptor in 1995 *(37)*. Alternative splicing of 5-HT receptors presumably arose early in evolutionary history, because splice variants are known for 5-HT receptors from the nematodes *Ascaris (38)* and *C. elegans (39)*. Splice variants can be grouped into two main categories. The first group includes those in which the alternative splicing results in a premature stop codon before all seven TM regions are transcribed; this type of splice variant has been described for the human 5-HT_{2A} *(40)*, 5-HT_{2C} *(41,42)*, and 5-HT_6 receptors *(43)* and these receptors are likely to be nonfunctional. The second group of splice variants includes those that remain functional; for the most part, alternative splicing results in isoforms that differ in the carboxy tail or loops of the receptors, and these variants and their functions will be the subject of a subsequent chapter.

10. Single-Nucleotide Polymorphisms

Soon after the cloning of the first 5-HT receptors, it was recognized that the sequences of these receptors could be polymorphic *(44–46)*. After the development of higher-throughput methods of screening for such variation (*see* ref. *47* for an early example) and with the advent of the (post)-genomic era, a fuller appreciation of the extent and significance of these polymorphisms can be gained. These will also be the subject of a subsequent chapter.

11. Conclusion

The sequences of the known 5-HT receptors vary by species as well as by class and subclass of receptor. Additional variation arises from RNA editing, alternative splicing, and the existence of single-nucleotide polymorphisms. Why

all these receptors have evolved and been maintained remains enigmatic and likely to intrigue biologists in many fields for long into the future.

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2

Structure and Function Reveal Insights in the Pharmacology of 5-HT Receptor Subtypes

Richard B. Westkaemper and Bryan L. Roth

Summary

The purpose of this review is to examine experimental information concerning the structure and function of the G protein–coupled serotonin receptors in the three-dimensional context provided by the structure of rhodopsin. A critical examination of the suitability of rhodopsin as a template for serotonin receptor modeling from the level of sequence alignment to interpretation of biochemical experiments of relevance to the issues of structure–function relationships is presented.

Key Words: G Protein–coupled receptors; serotonin receptors; rhodopsin; sequence homology; molecular models; protein structure.

1. Introduction

The six G protein–coupled (GPCR) 5-HT receptor classes consist of the 5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7, which are further divided into a total of 13 subfamilies. GPCRs have long been known to consist of a single polypeptide chain having extracellular, intracellular, and membrane embedded domains. The membrane domain consists of seven α-helical segments connected by extracellular and intracellular loops with the N-terminus located extracellularly and the C-terminus located intracellularly. Signal transduction is initiated by ligands that bind in a site defined by the transmembrane helices and, perhaps, components of the extracellular loops. The intracellular domains are thought to interact with cytoplasmic proteins sensitive to agonist-induced conformational changes in receptor structure, ultimately giving rise to signaling that modulates cell function (*see* ref. *1* for a recent review).

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2. Structure–Function Relationships

Both direct and indirect methods can be used to explore structure–function relationships in GPCR systems. Often, indirect sequence-based observations can identify target residues, the role of which can be investigated by mutagenesis or other biochemical experiments. Multiple sequence alignments can reveal residues and sequence motifs that are conserved throughout a family of GPCRs. Such conserved features could be important in maintaining structure, thus determining function. Discrete differences in sequence between subtypes or species might point to residues that may be responsible for functional differences such as ligand selectivity. Sequence-based secondary structure prediction as well as location of and periodicity of amino-acid-residue physiochemical properties can assist in the identification of structural domains. For example, segments corresponding to the transmembrane domains of GPCRs have been identified as sequence segments with high hydrophobicity occurring in an amphiphilic pattern.

Site-directed mutagenesis is a widely used experimental method that can help directly establish the identity of residues particularly important for receptor structure and function. Mutagenesis studies have been used to identify residues lining the ligand-binding site, to determine the disposition of residues with respect to the aqueous pore and lipid layer, and to detect proximity of multiple residues to each other *(2,3)*. In such studies, single-point mutations that affect receptor properties (ligand affinity, activation, constitutive activity, etc.) are often presumed to be directly responsible for the effects observed; it is inferred that these residues line the ligand-binding site, making contact with bound ligand, or directly mediate conformational change reflecting the activation state. However, the possibility that mutated residues may indirectly affect receptor properties can seldom be conclusively ruled out. The demonstration that changes in properties induced by a single-point mutation that can be reversed or eliminated by complementary changes in a structurally associated feature, either ligand or second receptor residue (reciprocal mutations), provides strong evidence that the observed effects are the result of direct interaction between target residues rather than being an indirect consequence of long-range perturbation of the structure. Evaluation of the effects of native or engineered cysteine disulfide bond crosslinking can provide evidence for the proximity of the studied residues. Evaluation of the effects of metal ions (Zn^{2+}) on receptors with native or engineered ion-binding sites can also provide evidence for the proximity of the coordinating residues to a receptor histidine. Site-directed spin labeling can introduce nitroxide labels by disulfide bond formation with native cysteines or residues mutated to cysteine. The distance between labeled residues and changes in the distance between residues can be estimated by electron paramagnetic resonance spectroscopy. Amino acid

residues are individually mutated to cysteine in the substituted cysteine accessibility method (SCAM). The disposition of the targeted residue (solvent accessible, lipid accessible) is estimated by disulfide bond formation with water- and/or lipid-soluble labeling reagents. The location of a residue in the ligand-binding site can be further inferred from ligand protection studies. Several recent reviews provide compilations of the results of such studies with numerous GPCRs *(2–4)*.

3. Structure of the 5-HT Receptors

Data from structure-related experiments has little meaning in the absence of a structural hypothesis (i.e., a three-dimensional receptor model that allows the evaluation of experimental observations in a structural context). Most early 5-HT receptor model construction was based on the experimental structure of bacteriorhodopsin, although not a GPCR, and later on the low-resolution projection structures of rhodopsin (reviewed in ref. *5*). The solution of the crystal structure of the visual pigment bovine rhodopsin has opened new horizons in modeling GPCR structures *(6)*. There has been considerable discussion concerning the suitability of the rhodopsin structure as a template for the construction of models of other GPCRs *(2,3,7–9)*. The purpose of this review is to examine experimental information concerning the structure and function of the GPCR 5-HT receptors in the three-dimensional context provided by the structure of rhodopsin, a central issue being the suitability of rhodopsin as a template for 5-HT receptor modeling.

4. Rhodopsin as a Template for 5-HT Receptor Model Construction

Although several 5-HT receptors have been subjected to extensive structural studies (reviewed in ref. *10*), this review will focus on those of particular relevance to the validity of rhodopsin as a homology modeling template for hypothetical 5-HT receptor models. Establishing an alignment between the sequences of templates of known structure with sequences of the model protein is the first and most crucial step in any homology modeling exercise. Although sequence homology between the GPCRs and rhodopsin is very low, there are several highly conserved residues and motifs in the transmembrane helices that are common to both rhodopsin and nearly all other GPCRs (Fig. 1) *(11)*. Thus, if it is assumed that the number of residues in the corresponding helical segments of rhodopsin and the model target are identical, models of the transmembrane helices can be readily constructed. By far, most model building studies start with the assumption that there are no differences in the lengths of the transmembrane (TM) segments. This may not always be the case since irregularities

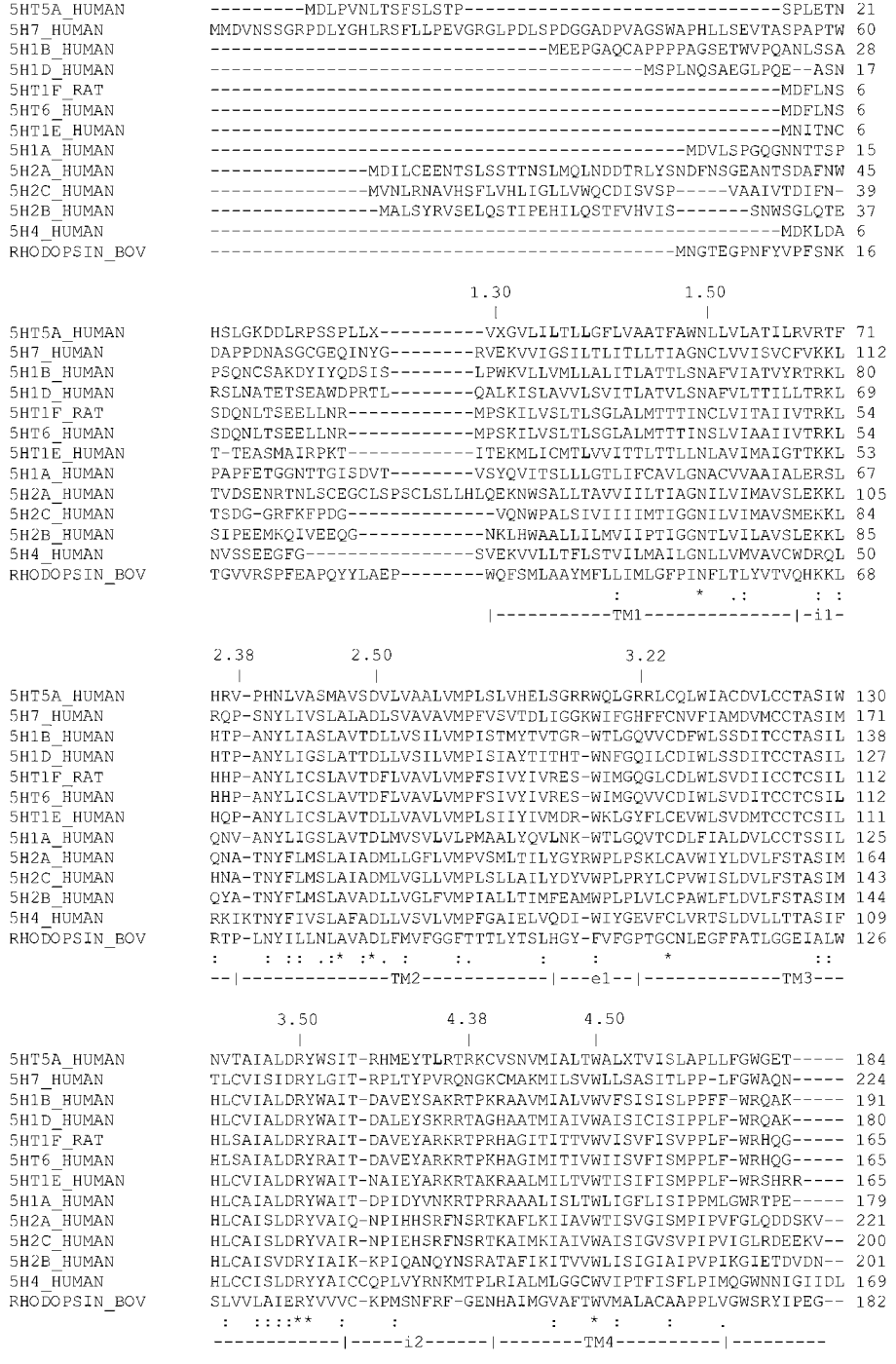

Fig. 1. CLUSTAL W (1.82) multiple sequence alignment for serotonin receptors and rhodopsin.

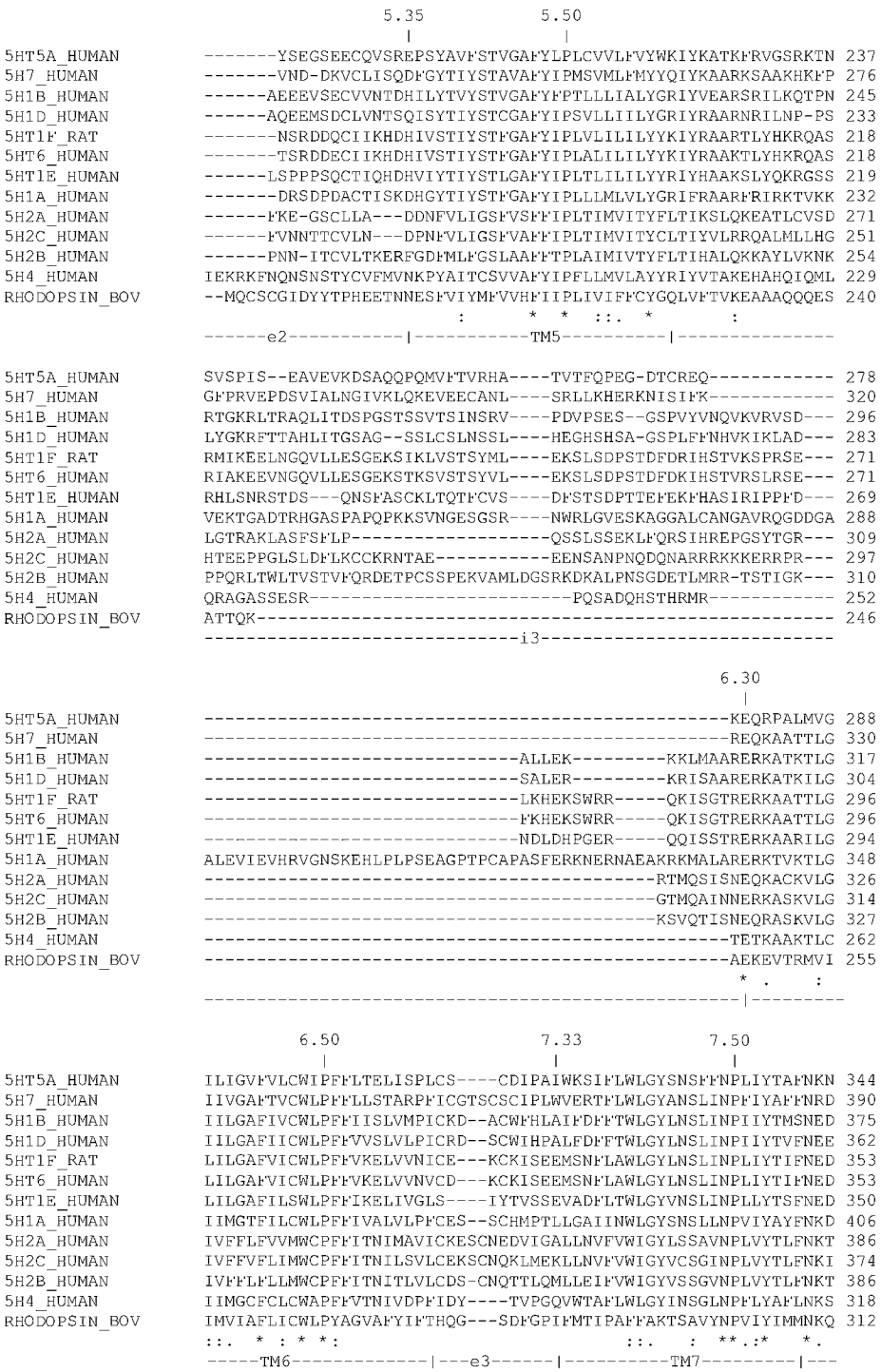

Fig. 1. *(continued)*

```
5HT5A_HUMAN       YNSAFKNFFSRQH------------------------------------------------ 357
5H7_HUMAN         LRTTYRSLLQCQYRNINRKLSAAGMHEALKLAERPERPEFVLRACTR---------RVLL 441
5H1B_HUMAN        FKQAFHKLIRFKCTS---------------------------------------------- 390
5H1D_HUMAN        FRQAFQKIVPFRKAS---------------------------------------------- 377
5HT1F_RAT         FKKAFQKLVRCRN------------------------------------------------ 366
5HT6_HUMAN        FKKAFQKLVRCRC------------------------------------------------ 366
5HT1E_HUMAN       FKLAFKKLIRCREHT---------------------------------------------- 365
5H1A_HUMAN        FQNAFKKIIKCKFCRQ--------------------------------------------- 422
5H2A_HUMAN        YRSAFSRYIQCQYKENKKP-LQLILVNTIPALAYKSSQLQMGQKK----------NSKQ 434
5H2C_HUMAN        YRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGRELNVNIYRHT----------NEPV 423
5H2B_HUMAN        FRDAFGRYITCNYRATKSVKTLRKRSSKIYFRNPMAENSKFFKKHGIRNGINPAMYQSPM 446
5H4_HUMAN         FRRAFLIILCCDDERYRRPSILGQTVPCS-------------------------TTTI 351
RHODOPSIN_BOV     FRNCMVTTLCCGKNPLGDDEASTTVSKTETSQVAPA------------------------ 348
                    .      .
                  --TM8---|------

5HT5A_HUMAN       ---------------------------------------
5H7_HUMAN         RPEKRPPVSVWVLQSPDHHNWLADKMLTTVEKKVMIHD 479
5H1B_HUMAN        ---------------------------------------
5H1D_HUMAN        ---------------------------------------
5HT1F_RAT         ---------------------------------------
5HT6_HUMAN        ---------------------------------------
5HT1E_HUMAN       ---------------------------------------
5H1A_HUMAN        ---------------------------------------
5H2A_HUMAN        DAKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV- 471
5H2C_HUMAN        IEKASDNEPGIEMQ--VENLELPVNPSSVVSERISSV- 458
5H2B_HUMAN        RLRSSTIQSSSIIL--LDTLLLTENEGDKTEEQVSYV- 481
5H4_HUMAN         NGSTHVLRDAVECGGQWESQCHPPATSPLVAAQPSDT- 388
RHODOPSIN_BOV     ---------------------------------------
```

Fig. 1. *(continued)*

in the three-dimensional structure of the α-helices of rhodopsin (π- and 3_{10}-helix segments in TM5 and TM7, respectively) could accommodate single amino acid insertions or deletions in modeled helices without perturbing the overall dimensions of the segment *(12,13)*. A major limitation to the use of rhodopsin as a template for modeling nonhelical domains of 5-HT receptors is the large differences in the lengths of corresponding structural elements. Fortunately, the largest discrepancies in sequence length occur in the N-terminus and C-terminus and intracellular loops, which are less likely to affect the structure of the ligand-binding site than the helices and extracellular loops. Where differences in segment length are too great to be modeled directly from the rhodopsin structure, investigators usually elect to generate a candidate loop structures, not from the rhodopsin structure but from the database of known protein structure using geometric (e.g., end-to-end distance) and sequence homology criteria. Unfortunately, there are usually no existing substructures with even modest homology to the 5-HT receptor sequences being modeled. Thus, loops created in this fashion are of questionable validity, particularly when the target sequences are significantly longer than those corresponding to rhodopsin. A final limitation in the existing structural data is that only the "dark" or inactive form of rhodopsin has been determined. The 5-HT receptor models derived from this structure are

thought to represent the inactive, resting, or antagonist occupied form of the receptor. The active form of rhodopsin would probably be the most appropriate template for the active or agonist occupied form of the GPCRs. Even though an experimental structure has not been determined directly, numerous biophysical approaches have provided insight into the nature of the conformational changes that rhodopsin undergoes on conversion to the light or activated form of the receptor *(14)*. To date, there has been at least one computational investigation directed toward simulating conformational changes on rhodopsin activation and β-adrenergic receptor activation based on distance constraints derived from biophysical experiments using constrained molecular dynamics simulations *(4)*. A similar approach might ultimately prove useful for generating models of the active form of 5-HT receptors.

The following is an examination of each structural element of rhodopsin and its suitability as 5-HT receptor model building templates. Figure 2 shows a graphic model of the 5-HT_{2A} receptor constructed from the rhodopsin template.

5. Extracellular Domains

5.1. N-Terminus

The N-terminal segment of GPCRs is highly variable in length (11–879 residues) *(15)*. The 5-HT receptor N-termini lengths range from 16 to 78 residues in length with very little conservation of sequence, with the possible exception of the first few residues in each sequence (Fig. 1). Given the total differences in length, it is difficult to envision a common N-terminus three-dimensional (3D) structure or function among the 5-HT receptors and rhodopsin. The rhodopsin N-terminus consists of a compact structure roughly parallel to the membrane that lies on top of the extracellular loops. If directly incorporated into 5-HT receptor models, this structure could limit the flexibility and disposition of the remaining extracellular loops. Deletion of the N-terminus from the 5-HT_{2A} receptor does not appear to affect receptor function *(16)*. Although no systematic mutagenesis studies have appeared, several single-nucleotide polymorphisms (SNPs) have been identified in the N-terminus of the 5-HT_{1A} *(17)* and 5-HT_{2A} *(18)* receptors, none of which affect receptor function. Together these observation suggest that explicit consideration of the N-terminal segment might not be necessary or desirable in the generation of 5-HT receptor models.

5.2. Extracellular Loop 1 (e1)

The first extracellular loop of rhodopsin directly connects TM2 and TM3 with a very short linker (six residues). With the exception of a single tryptophan, there is little sequence homology among the e1 loops of the 5-HT receptors. Fortunately, the lengths of the 5-HT receptor e1 segments are similar (six to seven residues) to the rhodopsin e1 loop. Thus, the rhodopsin e1 loop is

Fig. 2. A 5-HT_{2A}-receptor model constructed from the rhodopsin crystal structure. Serotonin is shown as a space-filled structure. Magenta colored backbone traces represent modeled structures of low reliability (N-terminus, C-terminus, and i3) as well as segments generated by insertion into or deletion of rhodopsin residues (e1, e2, e3, and i3 loops). Stick structures of the side chains of conserved residues (yellow), ligand-binding site residues (red), and residues involved in receptor activation are shown. (Illustration appears in color in insert that follows p. 240.)

likely to be a viable template for 5-HT receptor model structures. Even if the structure of the rhodopsin e1 loop is not considered explicitly, it is likely that protein database search-based generation of this segment will likely produce geometrically acceptable results for all of the 5-HT receptors.

5.3. Extracellular Loop 2 (e2)

One of the most striking features of the rhodopsin structure is the complexity and compactness of a helix bundle "cap" or "plug" formed from the extracellular interhelix loops and N-terminal segment *(19)*. Together, the N-terminal

sequence and the three loops e1 (TM2 to TM3), e2 (TM4 to TM5), and e3 (TM6 to TM7) form a layered, interlocking structure consisting partly of β-sheet loops. The bottommost of these is the e2 β-hairpin, which traverses the entire helix aggregate, extending from its origin at TM4 and its terminus at TM5 toward TM1 and TM7. The e2 loop is tethered to TM3 (Cys3.26) via a disulfide bond between the two highly conserved cysteines. The e2 loop of rhodopsin comes within the van der Waals distance of the covalently bound retinal chromophore forming the bottom of the plug, equivalent to the top of the retinal binding site. Thus, the relevant question becomes, is there an analogous structure that forms part of the ligand-binding site of neurotransmitter G protein–coupled receptors? It has been suggested that such a complete enclosure of the ligand-binding site is not likely for receptors that, unlike rhodopsin, must reversibly associate with the ligand *(7)*. With the exception of the 5-HT4 receptor (30 residues), most 5-HT receptors have relatively short e2 loops (16–21 residues) compared to the rhodopsin e2 loop (26 residues). The difference in lengths of the corresponding 5-HT and rhodopsin segments makes it difficult to model e2 structures directly from the rhodopsin template. However, the existence of the disulfide bond between TM3 and e2 does provide a geometric constraints that might help eliminate irrelevant structures generated by searching structural databases. It has been argued that the e2 loop of the 5-HT_{2A} receptor might not encroach as severely into the ligand-binding site as the corresponding structure for rhodopsin *(20)* because of the shorter length of the 5-HT_{2A} e2 loop. There have been no systematic mutagenesis studies conducted with the e2 loops of the 5-HT receptors. The P184L mutation did not affect 5-HT_{1A} receptor function in response to a wide range of agents *(17)*. Evaluation of chimers between 5-HT_{1B} and 5-HT_{1D} has led to the suggestion that the e2 loop might contribute to ligand selectivity *(21)* for some but not all investigated ligand *(10)* receptors. A recent SCAM study of the short e2 loop (approx 15 residues) supports the notion that the loop does in fact contribute to the ligand-binding site of the D2 receptor *(22)*. Thus, with caveats, the e2 loop of rhodopsin is a reasonable model of, if not a directly transferable template for, the general structural features of the e2 loops of 5-HT receptors.

5.4. Extracellular Loop 3 (e3)

The eight residue e3 loop of the rhodopsin receptor spans TM6 and TM7 on the periphery of the helical aggregate. The lengths of the e3 loop for the 5-HT receptors (7–11 residues) range from 1 fewer to 3 greater than the number of residues in the e2 loop of rhodopsin. Given the relatively remote disposition of the loop and the similarities in sequence length, it is expected that the rhodopsin e3 loop will be a reasonably good template for homology modeling. There have been no mutations of this segment reported for 5-HT receptors.

6. Intracellular Domains

6.1. Intracellular Loop 1 (i1)

The rhodopsin structure places the i1 loop adjacent to the short eighth membrane-embedded α-helix. With the exception of the 5-HT_{4A} receptor (six residues), the 5-HT receptors have the same length as the rhodopsin loop (seven residues). Interestingly a XKKLXXX motif is conserved between the rhodopsin sequence and the majority of the 5-HT sequences, suggesting that the i1 loops of rhodopsin and the 5-HT receptors could have a common structure. Systematic mutagenesis studies have not been conducted.

6.2. Intracellular Loop 2 (i2)

The second intracellular loop of rhodopsin consists of 11 residues that span TM3 and TM4 peripherally, extending into the membrane away from the helix aggregate. All 5-HT receptors are one residue longer, with the exception of 5-HT_4, which contains two additional residues compared to the rhodopsin sequence. Even in the absence of any sequence homology, the similarities in length suggest that the i2 loop of rhodopsin is at least a reasonable starting point for 5-HT receptor modeling.

6.3. Intracellular Loop 3 (i3)

The i3 loop has been shown to be a major site of receptor–G protein interaction and might have a potential role in mediating interhelical interactions. The large difference in sequence lengths in the i3 loop of 5-HT receptors and rhodopsin suggests that this feature represents the largest structural divergence between rhodopsin and the 5-HT receptors. The rhodopsin loop is relatively short (21 residues) and traverses the perimeter of the helix aggregate, extending slightly into the membrane near the membrane surface (away from the bundle). The 5-HT i3 loops are much longer, ranging from 57 to 121 residues. Because of the differences in sequence length, this feature cannot be modeled using rhodopsin as a template with any accuracy. Because there are no protein database structures with any degree of sequence homology to the GPCR loop, it is unlikely that this loop can be modeled with any accuracy. In one approach reported for 5-HT_{1A} and 5-HT_{2A} receptor models, the (113-residue) i3 loop was constructed in a stepwise fashion from segments with predicted α-helices (2 helices, 8 and 19 residues long) and β-conformations (two segments, 8 and 13 residues long) *(23)*. Backbone structures of segments predicted to be of random conformation were selected from sequences retrieved from a protein database search. It is not clear, however, how the tertiary structure of the loop was determined during this process. An alternative solution has been to omit the i3 loop from 5-HT (and other GPCR) models rather than explicitly including a structure that is most certainly wrong.

6.4. C-Terminus

The C-terminus of rhodopsin consists of 399 residues, only the first 18 of which are included in the crystal structure. A short eighth α-helical segment (approx 12 residues) initiates the C-terminus near the C-terminus of TM7. TM8 is approximately parallel to the membrane surface and perpendicular to the helix aggregate and is at the cytoplasm–lipid interface. The rhodopsin TM8 is preceded by the $NPXXY(X)_{5,6}F$ motif of TM7, a motif shared with 5-HT receptor sequences. Disruption of hydrophobic interaction between Y306/F313 alters H8 conformation and allows activation, suggesting that TM8 of rhodopsin acts as a membrane-dependent conformational switch-mediating activation *(24)*. The importance of an analogous interaction for 5-HT_{2C} receptors has been evaluated experimentally and results are consistent with models based on the rhodopsin structure *(25)*. It is likely that the N-terminus of rhodopsin including the eight helices is a reasonable 5-HT receptor model-building template.

6.5. Transmembrane Helix 1 (TM1)

The 3D structure of rhodopsin shows a kink in TM1 on the intracellular half of the helix. Rhodopsin has a proline at the 1.48 position (P53) that results in displacement of the extracellular portion of the helix toward and between TM2 and TM7 referenced from the extracellular side. There is no corresponding proline residue in any of the 5-HT receptors, suggesting that this helix irregularity might not be conserved. However, six subtypes (5-HT_7, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{2B}, and 5-HT_4) have either a glycine residue at 1.48, or 1.49, or both. Because glycine residues are often the site of irregularity in helices, the kink might very well be conserved in at least some these receptors. Because the cytoplasmic end of TM1 is tied to the cytoplasmic end of TM2 by the very short i2 loop in both the 5-HT and rhodopsin sequences, removal of the kink by replacement with an idealized helix in models of non-glycine-containing subtypes (5-HT_{1A}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_5, and 5-HT_6) would displace the intracelluar portion of TM1 away from TM2 and TM7. Interestingly, it has been pointed out with reference to other A-type GPCRs *(2)* that the region of helix irregularity might be a site of dynamic flexibility. None of the TM1 residues has been strongly implicated as being part of the ligand-binding site. Because TM1 is basically on the outside of the aggregate formed by the remaining helices, the precise nature of the geometry of TM1 might not be important in 5-HT receptor model generation, at least with respect to the ligand-binding domain. The role of 5-HT residues at 1.48 and 1.49 have not been investigated by site-directed mutagenesis to date.

6.6. Transmembrane Helix 2 (TM2)

The rhodopsin structure shows a helix irregularity in the extracellular half of TM2, placing it toward TM1, which is initiated by a pair of glycine residues

(2.56, 2.57) followed by three threonine residues (2.59–2.61). The threonine sequence appears to stabilize the helix bend with side chains hydrogen-bonding to the i-4 residue backbone carbonyl atoms *(2)*. Although none of the 5-HT receptor sequences have the threonine repeat, each has a conserved proline at 2.59, suggesting that this helix irregularity might be a structurally conserved feature of the 5-HT receptors. Several TM2 residues have been evaluated using site-directed mutagenesis. Mutation of the conserved aspartate (D2.50) to alanine abolished agonist affinity *(26)* for 5-HT_{1A} receptors. Mutation to asparagine in 5-HT_{1A} and 5-HT_{2A} receptors has been shown to selectively affect ligand affinity in an unpredictable fashion *(27)*. In a separate study *(28)*, the D2.50N mutation of the 5-HT_{2A} receptor did not affect agonist or antagonist affinity but reduced G protein coupling. The effect was reversed by a rescuing TM7 mutation N7.49D, which strongly suggest that residues at 2.50 and 7.49 are directly interacting with each other. The rhodopsin structure places both resides at positions within interacting distance, suggesting that the orientation of TM2 and TM7 represented by rhodopsin might also apply to 5-HT receptors.

6.7. Transmembrane Helix 3 (TM3)

The TM3 helix of rhodopsin deviates slightly from an ideal α-helix in that its axis has a slight precession creating a subtle S-shape. Backbone hydrogen-bonding residues at 3.33 and 3.43 (T118 and S127) might be responsible for the slight precession. All of the 5-HT receptors have multiple backbone hydrogen-bonding residues (Cys/Ser/Thr) at various positions (not conserved), suggesting that similar helix irregularities might be present in the 5-HT receptors. Whereas differences in structure of 5-HT TM3 segments might vary only slightly from rhodopsin, significant changes in function could result from changing the immediate environment by several structurally important residues, particularly TM3 D3.32 and TM3 D3.49. Numerous mutagenesis studies have supported the role of D3.32 and the ammonium ion counterion *(27,29–33)*. In addition, interaction of D3.32 with other receptor residues has been proposed for several GPCRs. In particular, interaction of D3.32 with a TM7 asparagine (N7.36) was proposed to maintain the receptors in the inactive state. Disruption of the interaction by ligand association (a "salt-bridge switch") was proposed to result in activation *(33)* for the adrenergic receptor. The observation that D3.32A, -E, -Q, and -N all result in receptors with decreased rather than increased constitutive activity is contradictory to the "salt-bridge" proposal but was consistent with an early 5-HT_{2A} receptor model *(30)*. Examination of the rhodopsin crystal structure indicates that residues at the 3.32 and 7.36 positions are in fact not within interacting distance.

The demonstration of potential interactions between the arginine (R3.50) of the highly conserved D(E)RY motif near the intracellular end of TM3 and a

glutamate (E6.30) near the intracellular end of TM6 is of particular importance to 5-HT_{2A} receptor structure and activation. The fact that this interaction stabilizes the inactive conformation of the receptor is suggested by the observation that interruption of the interaction by mutagenesis results in a high constitutive activity *(34)*. It has been suggested that activation of the 5-HT_{2A} receptor involves the relative displacement of the intracellular end of TM3 and TM6, as has been proposed on the basis of mutagenesis and biophysical studies for a variety of GPCRs. It was further suggested the displacement of TM6 is mediated at the TM6 kink located at the highly conserved P6.50 *(35)* for the adrenergic receptor. Residues and the 3.50 and 6.30 positions are in fact within interacting distance in rhodopsin, further suggesting that the rhodopsin TM3 is a viable template for 5-HT receptor models.

6.8. Transmembrane Helix (TM4)

The TM4 helix of rhodopsin is a short helix on the outer face of a helix "wall" around the ligand-binding site formed by the aggregate of TM2, TM3, and TM5. TM4 is a regular helix from the intracellular end up to a highly conserved proline at (P4.59) that results in the remaining few helix turns being displace away from the TM2 and TM3 helix ends toward lipid. Contrary to the assumption that highly conserved residue might be important for ligand binding, a highly conserved tryptophan residue (W4.50) faces the TM2/TM3 interface shielded from direct contact with the ligand-binding site in rhodopsin-based models. The relatively remote location of the rhodopsin TM4 is consistent with the observation that most 5-HT receptor mutations in TM4 have little effect on ligand affinity (reviewed in ref. *10*).

6.9. Transmembrane Helix 5 (TM5)

It has been suggested because sequence variability of all transmembrane helices among all GPCRs is greatest for TM5, much of the specificity of GPCRs could be attributable to TM5 properties *(13)*. All of the 5-HT receptors and rhodopsin share a highly conserved proline (P5.50) residue near mid-helix. The rhodopsin TM5 is relatively "straight" despite the proline residue, but it shows a significant deviation from ideal helical periodicity in that it has a single "underwound" π-helix turn (residues 5.44–5.49) near P5.50. Although relatively rare in general, it has been suggested that a π-helix might be a universal feature of all class A GPCRs because of the highly conserved nature of P5.50 *(13)*. In addition, there is some evidence that π-helices are frequently associated with ligand-binding domains *(13,36,37)*. Many experimental studies have suggested that portions of TM5 interact with the ligand. A hydrogen bond donating serine is present at either the 5.42 or 5.43 position in all 5-HT receptors. With the exception of the 5-HT_2 subtypes, a hydrogen bond donating residue is present at

both the 5.43 and 5.43 positions. The rhodopsin structure places these residues is a ligand-accessible position. Although not entirely self-consistent, mutation data for the 5-HT_{2A} suggest that a hydrogen bond donor might interact with a ligand having a hydrogen bond acceptor in a position analogous to that of the 5-OH group of 5-HT (reviewed in ref. *19*). If the helical "bulge" were replaced with a idealized helix, positions 5.42 and 5.43 would be rotated clockwise, placing both residues more toward the TM4–TM5 interface inaccessible to the ligand-binding site, suggesting that this helix irregularity might well be conserved in the 5-HT and other class A GPCRs. A phenylalanine at the 5.47 position is conserved in all 5-HT receptors. The neighboring 5.48 position is occupied by either a phenylalanine or tyrosine residue. The structure of rhodopsin places the 5.47 in a ligand-accessible orientation, but a 5.48 residue is clearly in lipid or at the TM5–TM6 interface. Mutation of each of these residues to alanine has been shown to selectively affect ligand affinity and both mutations reduce potency and efficacy of 5-HT-stimulated PI hydrolysis *(38)*, partially inconsistent with the lipid orientation of position 5.48. The partial discrepancy between the orientations of side chains dictated by the rhodopsin structure and mutagenesis data have led to the hypothesis that helix flexibility at the conserved proline 5.50 position and/or ligand-induced helix rotation might account for the inconsistencies *(2,10)*.

6.10. Transmembrane Helix 6 (TM6)

Rhodopsin TM6 has a pronounce kink at the uniformly conserved proline and position 6.50, tilting the extracellular half toward TM5. Evidence points to a major conformational change in this helix (particularly with respect to TM3) in the conversion of the inactive to the active state of rhodopsin. Spin-labeling and cysteine crosslinking studies in rhodopsin are consistent with a displacement of the cytoplasmic side of TM6 away from TM3 upon rhodopsin activation *(39,40)*. In addition, rhodopsin activation is prevented in a rhodopsin-containing engineered metal-ion-binding site *(41)*. It has been proposed that the conserved proline at this position represents a conformationally flexible hinge, the bending of which meditates TM6 conformational change on activation. An alanine scanning mutagenesis study of residues 6.28 through 6.40 on the intracellular half of TM6 of the 5-HT_{2A} receptor suggests that none has a direct effect on ligand binding *(34)* and all of these residues are distant from the ligand the ammonium ion-binding residue D3.32 of TM3. Single and reciprocal mutagenesis studies of E3.60 and R3.50 support the hypothesis that a salt bridge between these TM6 and TM3 residues might mediate receptor activation, as discussed earlier *(34)*. The rhodopsin structure in consistent with the potential presence of such an interaction in the inactive sate. Mutagenesis studies of conserved residues on the extracellular half (W6.48 and F6.52) of the receptor suggest that these

residues might be important for ligand binding for the 5-HT_{2A} receptor *(42)*. The aromatic side chains of both are in the proposed ligand-binding site in proximity to D3.32 of TM3 in a rhodopsin-based 5-HT_{2A} model *(20)*.

6.11. Transmembrane Helix 7 (TM7)

The 5-HT and rhodopsin receptors contain a highly conserved asparagine/proline pair at position 7.49 and 7.50. In addition to inducing a pronounce kink at this position, an unusual helix conformation occurs on the intracellular side of the kink consisting of a tightly wound 3_{10}-helix (7.43–7.46). This feature of the rhodopsin structure might be a consequence of retinal covalently bound to K296 (7.43), which is obviously not a property shared with the 5-HT receptors. Double-revertant studies of the 5-HT_{2A} receptor residues D2.50 on TM2 and N7.49 suggest that disruption of a hydrogen bond between these residues might be important for activation. Potential interaction between these two residues is evident in the rhodopsin structure and is consistent with a rhodopsinlike abnormal helix in this region *(28)*. In addition, a SCAM study of the D_2 receptor is more consistent with a 3_{10}-helix than a more regular, kinked α-helix *(43)*. Mutation of the highly conserved Y7.53 of NPXXY motif led to constitutively active 5-HT_{2C} receptors, suggesting that this locus has a role in receptor activation *(44)*. Further investigations of double-revertant Y/F pairs suggest that activation might, in part, be dependant on disruption of a direct interaction of Y7.53 and Y7.60 of the C-terminal helix 8 *(25)*, as has been proposed for rhodopsin. Such an interaction is readily apparent in the rhodopsin structure.

7. Conclusion

With a few notable exceptions, the rhodopsin structure appears to be a useful starting point for the generation of 3D models of the serotonin receptors that can be used to aid further receptor structure–function studies. Segments that almost certainly cannot be generated accurately from the rhodopsin structure include the N-terminus and i3 loop. Segments that almost certainly can be generated from the rhodopsin structure include the e1, e2, e3, and i1 loops, the TM2, TM3, TM6, and TM7 helices, and the C-terminus. Available data suggest that structural correspondence between rhodopsin and 5-HT receptor segments are not entirely consistent for TM1, TM4, and TM5.

Numerous 5-HT receptor models have been constructed from the experimental crystal rhodopsin structure with various levels of fidelity to the template. To date, models have been reported for 5-HT_{1A} *(23,45–47)*, 5-HT_{2A} *(20,23,30,34, 38,48–50)*, 5-HT_{2B} *(31,50)*, 5-HT_{2C} *(50)*, 5-HT_4 *(51)*, and 5-HT_6 *(52,53)* receptors. Not unexpectedly, each of the models presented has been useful in providing insight into the potential structural origins of the specific experimental feature being investigated (a limited set of mutations, SCAM of a

particular feature of the studied receptor). There have been very few systematic attempts to evaluate the usefulness of models for the generation of new ligands. Perhaps the most stringent and meaningful test of overall validity of GPCR homology models for this purpose is the application of virtual screening paradigms. Large databases of compounds with known activities (both active and inactive) are docked with receptor models in an automated fashion and the resulting complexes are scored. Overall model "fitness" can be determined statistically by comparing predicted affinity with the experimental results. Success would suggest that hypothetical models encode enough useful receptor structure information to allow prediction of ligand affinity for a large range of structural types of potential ligand; the models could be useful for structure-based ligand design. Encouraging results of this kind have been recently reported for the dopaminergic *(54)* and adrenergic *(55)* receptor models. A major limitation in the use of rhodopsin as a model template for screening purposes is that only the structure of the inactive form of the receptor is available. Thus, one would expect virtual screening using rhodopsin-based models to produce antagonists only. A second potential confounding factor in the use of rhodopsin as an explicit template for the generation of GPCR models has very seldom been mentioned explicitly. Ligand-binding sites of GPCR models based on the helix backbone of rhodopsin closely resemble the original retinal binding site (a "ghost site") even in the absence of sequence homology and even when the model side-chain geometries are constructed independent of the rhodopsin side-chain geometries *(20)*. Imprintation of a more realistic site has been accomplished by molecular mechanics minimization of receptor–ligand complexes or receptor–ligand ensemble complexes generated from manually docked orientations consistent with known site-directed mutagenesis studies *(20,54)*. The obvious liability of this approach is that the steric and electronic attributes of the imprinted or created site is highly dependent on the particular ligand(s) and ligand orientations selected. Simulations in which a ligand is randomly "solvated" with N- and C-blocked amino acids produces an artificial site quite effective in retrieving similar ligands from databases virtually screened in the automated docking algorithm. In fact, similar approaches have been used to generate explicit atomic models originally referred to as pseudoreceptors *(56,57)*. Although the use of GPCR models as virtual screening templates shows promise for both model validation and structure-based ligand design, results should be interpreted with appropriate skepticism.

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3

Polymorphic and Posttranscriptional Modifications of 5-HT Receptor Structure

Functional and Pathological Implications

Marilyn A. Davies, Chiao-ying Chang, and Bryan L. Roth

Summary

This chapter first describes the structural changes involved in genetic polymorphisms, mRNA editing, and alternative mRNA splicing of 5-hydroxytryptamine (5-HT) receptors. These structural changes lead to modifications in the production and characteristics of 5-HT receptors and affect protein expression. Functionally, they affect radioligand binding, signal transduction, and receptor sensitivity, thus affecting interindividual variation in responses to therapeutic agents, particularly antipsychotics and antidepressants. Studies indicate that genetic polymorphic and post-transcriptional modifications of 5-HT receptor structure contribute also to pathological processes related to irritable bowel syndrome, cardiopulmonary problems, psychiatric illness (i.e., schizophrenia and mood disorders), Alzheimer's disease, problems involving increased food and alcohol intake, and behavioral problems such as impulsivity, self-harm, and aggression. In the second part of this chapter, the $5HT_{2A}$, $5HT_{2C}$, and $5HT_4$ receptors are used to illustrate the structural changes involved in genetic polymorphisms, mRNA editing, and alternative mRNA splicing along with their functional consequences and pathological implications. Finally, this chapter describes the most salient posttranslational modifications of the 5HT receptors, which involve the chemical modification of the protein after its translation.

Key Words: Single-nucleotide polymorphisms; mRNA editing; alternative mRNA splicing; posttranslational modifications; $5HT_{2A}$, $5HT_{2C}$, and $5HT_4$ receptors.

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1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) exerts its physiological effects via its interaction with as many as 15 distinct and diverse G protein–coupled receptors (GPCRs), which are organized into seven distinct classes (5-HT_1 to 5-HT_7) based on their structural and operational characteristics. (*See* Chapter 1 for a thorough review.) Over the last decade, it has become clear that the molecular and functional diversity of the 5-HT receptor subtypes is even more heterogeneous when one considers the effects of genetic polymorphisms, posttranscriptional modifications (i.e., mRNA editing and alternative mRNA splicing), and posttranslational modifications (i.e., phosphorylation). These structural changes lead to modifications in the production and characteristics of 5-HT receptors and affect protein expression. Functionally, they affect radioligand binding, signal transduction, and receptor sensitivity, thus affecting interindividual variation in responses to therapeutic agents, particularly antipsychotics and antidepressants *(1–3)*. Given that the greatest concentration of 5-HT (90%) is found in the enterochromaffin cells of the gastrointestinal tract and that the effects of 5-HT are felt prominently in the cardiovascular and central nervous systems (CNS), it is not surprising to find that genetic polymorphic and posttranscriptional modifications of 5-HT receptor structure contribute also to pathological processes related to irritable bowel syndrome *(4–11)*, cardiopulmonary problems *(12–16)*, psychiatric illness (i.e., schizophrenia and mood disorders) *(17–21)*, Alzheimer's disease *(22–24)*, problems involving increased food and alcohol intake *(25–30)*, and behavioral problems (i.e., impulsivity, self-harm, and aggression) *(31–38)*.

This chapter first describes the structural changes involved in genetic polymorphisms, mRNA editing, and alternative mRNA splicing. The $5HT_{2A}$, $5HT_{2C}$, and $5HT_4$ receptors are used to illustrate these structural changes along with their functional consequences and pathological implications. The second part of this chapter describes the most salient posttranslational modifications, which involve the chemical modification of the protein after its translation.

2. Genetic Polymorphisms

Approximately 80% of sequence variations in genes encoding proteins involve an exchange of a single nucleotide (called single-nucleotide polymorphism or SNP) *(39,40)*. SNPs are present in both the coding and noncoding regions of the genome, they are usually biallelic, and two-thirds of them involve the replacement of cytosine (C) and thymine (T) (www.ncbi.nlm.gov). SNPs are of interest for several reasons *(41)*: First, linkage analysis has not yet identified loci for complex diseases *(42,43)*; second, SNPs are abundant (over 1 million SNPs are found across the human genome), thus providing many

markers near or in any locus-of-interest *(39)* assays in which wild-type, mutant, and heterozygous alleles are easily discriminated for association studies *(39,41,44,45)*.

Association studies examine whether SNPs located in genes are biological markers for disease-causing genes *(46)*. SNPs directly affect protein structure or expression levels, thus becoming candidates for disease development or risk factors affecting the course of disease *(47)*. Although individuals demonstrate similar physical or psychological disease and behavior phenotypes, pharmacogenetic studies indicate that distinct genetic profiles influence response to drug treatment *(48)*. Pharmacogenetic studies, which deal with the genetic basis underlying interindividual differences in drug response *(48)*, are designed to examine which SNPs affect the pharmacodynamics of drugs *(39)*.

3. Posttranscriptional Modifications

Genes can be altered posttranscriptionally by the processes of mRNA editing and alternative mRNA splicing.

3.1. mRNA Editing

The process of mRNA editing involves the alteration of codons (the sequence of nucleotides) in the RNA after it has been transcribed from DNA but before it is translated into protein *(49–51)*. RNA editing occurs by two mechanisms: substitution editing or insertion, and deletion or modification *(52)*. Substitution editing involves chemical alterations of nucleotides, which result in cytidine deamination (which converts cytidine to uracil) or in adenosine deamination (which converts adenosine to inosine) (5-$HT_{2C\text{-}VSV}$R) *(53)*. This change in codons of the mRNA leads to different protein isoforms, which are structurally truncated and/or contain amino acids not encoded by the original nonedited transcript *(49,51)*. The protein isoforms are cell- and tissue-specific, demonstrating distinct physiological properties *(2)*. Within the 5-HT receptor family, the 5-HT_{2C} receptor subtype is the only receptor known to be regulated by mRNA editing *(2,54)*. The specific mechanisms involved in 5-HT_{2C} receptor RNA editing are elucidated later in this chapter.

3.2. Alternative mRNA Splicing

In eukaryotes, pre-mRNA molecules undergo splicing, a process that removes introns (noncoding regions) from pre-RNA and leaves the exons to create the mature message. During alternative RNA splicing, exons are included or targeted for removal in different combinations, thereby providing an important source of 5-HT receptor heterogeneity *(55)*. Alternative splicing can occur in the 3′ or 5′ untranslated regions (UTRs) or in the protein-coding sequence, and it affects protein expression *(55)*.

Molecular biological studies indicate that isoforms or splice variants exist in the human brain for genes encoding receptor subtypes of 5-HT_{2A} *(56)*, 5-HT_{2C} *(3,57)*, 5-HT_3 *(58–60)*, 5-HT_4 *(61,62)*, 5-HT_6 *(63)*, and 5-HT_7 *(64)* receptors.

Alternative mRNA splicing can have both functional and pathophysiological *(64)* relevance. For example, the human 5-HT7 receptor gene contains at least two introns and encodes a 445-amino-acid 5-HT receptor; three splice variants of the 5-HT7 receptor (a, b, and d) are localized in various human tissues, including the heart, small intestine, colon, ovary, and brain *(65)*. The 5-HT_7 splice variants differ in the lengths of their intracellular carboxy-terminal tail *(66)* and several studies have examined their pharmacological profiles and ability to activate adenylyl cyclase (AC) *(66,67)*. Results indicated that any functionally important differences among the three isoforms are not likely to involve differences in ligand binding or differences in adenylate cyclase coupling. The 5-HT7 receptor splice variants in the brain are of interest because they are thought to be implicated in depression *(64)*.

3.2.1. 5HT_{2A} Receptor Heterogeneity

The human 5-HT_{2A} receptor gene, *HTR2A*, is located on human chromosome 13q14-21 (www.ncbi.nlm.nih.gov) and is composed of 471 amino acids *(68)*. It is widely distributed in peripheral and central tissues *(68,69)*. 5-HT_{2A} receptor heterogeneity is affected by genetic polymorphisms and alternative splicing.

3.2.1.1. Genetic Polymorphisms

Seven SNPs are located within the coding region of the 5-HTR_{2A} receptor. Two of the seven SNPs (T102C and C516T) are silent mutations and do not cause a change in the protein *(70,71)*. The T102C variant consists of a thymidine–cytidine substitution. Although the T102C polymorphism is silent, it has been the focus of many association studies examining the expression of psychosis and depression in individuals with schizophrenia *(72,73)*, mood disorders *(18,24,74–77)*, and Alzheimer's disease (AD) *(22,23,78,79)*. Results of associations in schizophrenia are varied with many case-control and family-based studies showing an association between the T102C SNP and the etiology of schizophrenia *(73,80–82)*. In their recent meta-analysis of 31 case-control studies, Abkolmaleky et al. *(73)* found a significant association of both the C allele and CC homozygosity with schizophrenia in European patients; this finding was not confirmed in samples from East Asian countries. Results of a metaanalysis of five family-based association studies found no significant association; however, the polled odds ratio (OR) was 1.3 ($p = 0.14$) *(73)*. In mood disorders, several studies *(19,74,77,83)* did not find an association between the 102C allele and the diagnoses; however, two studies *(76,84)* found a significant association between the 102C allele and suicidal ideation. For example, when

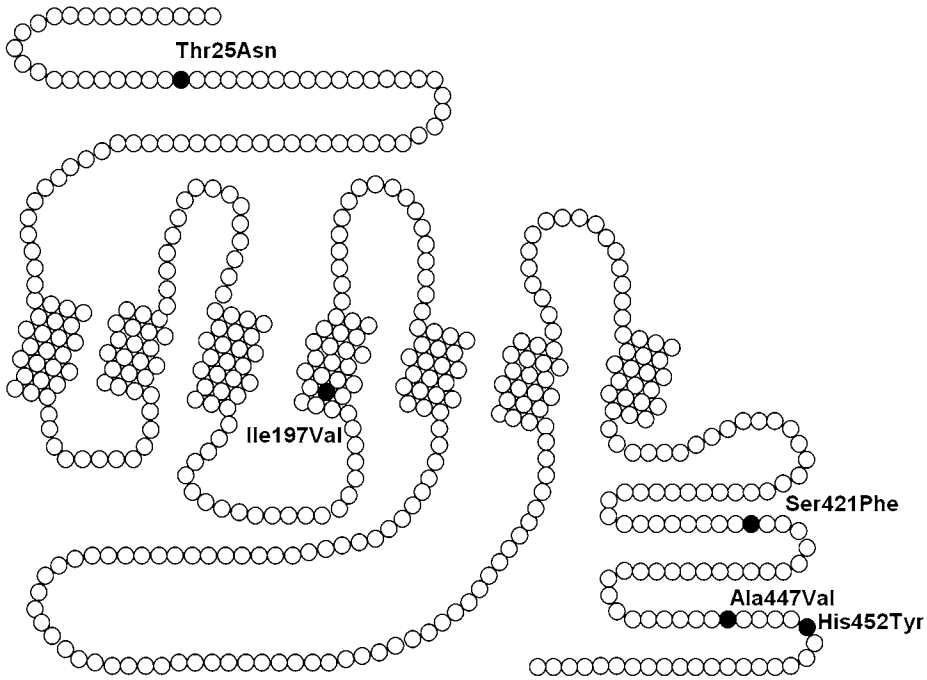

Fig. 1. SNPs on the 5-HT_{2A} receptor. Five SNPs (Thr25Asn, Ile197Val, Ser421Phe, Ala447Val, and His452Tyr) on the human 5-HT_{2A} receptor are shown by black circles.

Arias et al. *(84)* genotyped 159 Spanish patients with major depression and 164 unrelated and healthy controls, they found that those patients carrying the 102C allele had more than five times the risk for attempting suicide than non-carriers (OR = 5.50, $p = 0.01$). Finally, several association studies that focused on late-onset AD reported an association between the presence of the C102 allele and the presence of psychosis *(85,86)*, delusions *(79)*, hallucinations *(22)*, and comorbid depression *(23)*.

Five SNPs (Thr25Asn, Ile197Val, Ser421Phe, Ala447Val, and His452Tyr) result in a change in an amino acid (*see* Fig. 1). The Thr25Asn (T25N), variant involves a substitution of threonine by asparagine; it is located in the extracellular region on the N-terminus. The Ile197Val (I197V) variant involves a substitution of isoleucine by valine; it is located on the internal portion of the fourth intracellular loop. Finally, three variants (Ser421Phe, Ala447Val, and His452Tyr) are located in the C-terminal region of the receptor gene, which is thought to be a coupling domain *(87)*. The Ser421Phe (S421F) variant involves a serine to phenylalanine substitution; the Ala447Val (A447V) variant involves an alanine to valine substitution; and the His452Tyr (H452Y) variant involves a low-frequency histidine to tyrosine substitution. As indicated in Table 1, the rarer

Table 1
Allele Frequencies for Functional SNPs in the Coding Region of HTR2A and HTR2C

Receptor subtype	SNP	Average estimated heterozygosity	Minor allele frequency
5-HT_{2A}	Thr25Asn	0.041	0.021
	Ile197Val	0.015	0.008
	Ser421Phe	0.476	0.390
	Ala447Val	0.022	0.011
	His452Tyr	0.143	0.077
5-HT_{2C}	Leu4Val	0.027	0.014
	Cys23Ser	0.267	0.158

allele frequencies of these SNPs vary between 0.008 for I197V and 0.390 for S421F (www.ncbi.nlm.nih.gov).

The five functional SNPs might be important determinants of the pharmacodynamics of agonists and antagonists at the 5-HT_{2A} receptor. In a recently published in vitro study, Harvey and colleagues *(88)* assessed 5-HT binding, signal transduction, and receptor sensitivity for four SNPs (T25N, I197V, A447V, and H452Y) in a Sf9 insect cell line. Although they found no difference among the SNPs in sensitivity of the 5-HT_{2A} receptor to loxapine antagonism of serotonin stimulation, they did find a twofold reduction in sensitivity of the I197V polymorphism to clozapine antagonism of serotonin stimulation (IC_{50} value of wild type = 109.1 vs I197V = 223.4, $p = 0.03$). Additionally, the I197 allele did not have a significantly altered response from the wild-type allele to serotonin agonism, thus suggesting specificity for receptor antagonism. Two in vivo studies *(87,89)* examined intracellular 5-HT–induced Ca^{2+} release by platelets. In the first study of 16 patients diagnosed with seasonal affective disorder (SAD), Ozaki et al. *(87)* reported no significant difference in radioligand binding to platelet 5-HT_{2A} receptors; however, the rarer allele showed diminished signal transduction, as demonstrated by a blunting of the shape of the Ca^{2+} response mobilization peak for heterozygotes (452H/452T) compared with homozygotes (452H/452H). In their study of platelets of 27 male subjects (including 14 graduates of a residential alcohol unit and 13 nonpatient volunteers), Reist et al. *(89)* reported that subjects with increased measures of impulsivity showed decreased postreceptor 5-HT function and no significant effect of H452 genotype on 5-HT–induced Ca^{2+} release or fenfluramine-induced prolactin release by the pituitary. Notably, one available Y452/Y452 homozygote had diminished Ca^{2+} release and one of the highest levels of fenfluramine-stimulated prolactin release;

the low frequency (0.008) of this genotype in the population makes replication of this finding difficult *(38)*. Most recently, Hazelwood and Sanders-Bush *(90)* investigated agonist-stimulated signaling in NIH3T3 cells; their data indicated that the 452Tyr variant was associated with a loss of agonist-induced high-affinity binding, a decreased turnover of guanosine 5′-*O*-(3-[(35)*S*]thio)triphosphate after agonist stimulation, and a blunted signal downstream of receptor activation.

Several pharmacogenetic studies examined the relationship between H452Y and clinical response to clozapine in individuals with schizophrenia meeting criteria for treatment refractoriness or intolerance to typical antipsychotic therapy *(91)* (*see* Table 2). Although comparison across these studies is difficult because the studies varied in several parameters, including (1) sample size and ethnicity, (2) duration of drug treatment, (3) assessment methods, and (4) selected outcome measures, most studies supported an association between the frequency of the Y452 allele and nonresponse to clozapine. Duration of treatment is especially important because it has been observed that many patients need at least 3 mo to respond to clozapine *(92)*. In a meta-analysis of six studies conducted prior to 2000 (n = 373 responders and n = 360 nonresponders), Arranz et al. *(93)* showed a significant association between allele Y452 and poor response to clozapine (p = 0.008); homozygosity for Y452 was also related to poor clozapine response (p = 0.04). Only one study *(94)* examined the relationship between the T25N variant and clozapine response; no association was found.

Two pharmacogenetic studies examined the association between $5HT_{2A}$ SNPs and clinical responses to olanzapine and risperidone (Table 2). The first study *(95)* examined genotypic data from 41 patients and reported no correlation between T25N and H452Y SNPs and 6 wk olanzapine response. The second study *(96)* examined genotypic data from 73 Japanese patients at wk 8 of risperidone administration and found no association between the H452Y SNP and clinical symptoms. Because atypical antipsychotic drugs target multiple receptors, one research group investigated whether an atypical antipsychotic drug response could be predicted using a combination of polymorphisms in targeted receptors *(97,98)*. In a sample of 200 caucasians of British origin with schizophrenia, Arranz et al. *(97)* reported that the H452Y SNP, in combination with five other SNPs (from the $5\text{-}HT_{2C}$, the serotonin transporter [5-HTT] receptor genes, and Histamine2) predicted 76–86% clozapine response (p = 0.0001). The simpler combination of T102/– and H452/H452 in the $5\text{-}HT_{2A}$ receptor predicted good clozapine response in 80% of patients. In another sample of 92 patients from northern Spain, this same group reported that a combination of polymorphisms that included the H452Y SNP predicted a 3-mo olanzapine response (PPV = 76%, p = 0.07) and risperidone response (PPV = 88%, $p < 0.001$) as defined by the Positive and Negative Symptom Scale (PANSS) and Global

Table 2
Association Studies of SNPs of 5-HT_{2A} and Atypical Antipsychotic Drug Response

5HT2A variant	Drug (duration)	Participants (descent)	Response criteria	Results	Ref.
H452Y	Clozapine (over 6 wk)	102 Responders 58 Nonresponders 178 Normal controls (British)	GAS	Frequency of Try452 allele higher among nonresponders (11%) vs responders (6%)	*93*
H452Y T25N	Clozapine (at least 28 d)	73 Responders 73 Nonresponders (German)	GAS	Higher frequency of Try452 allele among nonresponders (16%) vs responders (6%) but not statistically significant	*94*
H452Y	Clozapine (wk 10)	SCZ or ScAD 21 Responders 49 Nonresponders	BPRS	Higher frequency of Try452 allele among nonresponders (16%) vs responders (6%) but not statistically significant	*169*

H452Y	Clozapine (6 mo)	97 Responders 88 Nonresponders	BPRS CGI	Frequency of Try452 allele higher among nonresponders (15%) vs responders (7%)	*170*
H452Y	Clozapine (over 6 wk)	79 Responders 35 Nonresponders	GAS	Frequency of Try452 allele higher among nonresponders (16%) vs responders (10%)	*93*
H452Y	Risperidone (8 wk)	73 Japanese patients with SCZ	PANSS	No correlation	*96*
H452Y T25N	Olanzapine 7.5–20 mg (up to 5 wk)	41 Patients with SCZ (21 First break; 20 Chronically ill)	BPRS SANS	No correlation	*95*

Abbrevations: CGI, Clinical Global Impressions scale; SCZ, schizophrenia; GAS, Global SczAD (schizoaffective disorder); PANNS, Positive and Negative Syndrome Scale; BPRS, Brief Psychiatric Rating Scale; SANS, Scale for the Assessment of Negative Symptoms.

Assessment Scale (GAS). These preliminary results suggest that a combination of polymorphisms in the *HTR2A* gene can be used to predict atypical antipsychotic drug response.

Because abnormalities in serotonergic function have been implicated in the etiology of bipolar affective disorder (BAD), several investigators have examined whether SNPs in the *HTR2A* gene confer genetic susceptibility to the disorder *(99–102)*; most studies reported no association. Arranz et al. *(99)* and Gutierrez et al. *(100)* reported no differences in frequency of T25N and H452Y genotypes for individuals with BAD vs controls for individuals of German, British, or Spanish origin. Etian et al. *(102)* reported no association between the H452Y polymorphisms and BAD in a large west European sample (356 BAD patients vs 208 healthy controls. Ranade et al. *(101)* reported a significant association between H452Y and bipolar I disorder (BDI) in a family-based sample of 93 BDI cases vs controls ($n = 92$).

3.2.1.2. Alternative Splicing

Guest et al. *(56)* reported an alternatively spliced 5-HT_{2A} receptor variant (a 118-bp insertion at the exon II/III boundary) that produces a frame shift in the coding sequence and a premature stop codon. They reported that the truncated receptor (5-$HT_{(2A\text{-}tr)}$) and native 5-$HT_{(2A)\text{-}R}$ were coexpressed in most brain tissues, with the highest levels being found in the hippocampus, corpus callosum, amygdala, and caudate nucleus. Western blot analysis of HEK-293 cells transfected transiently with a 5-$HT_{(2A\text{-}tr)}$ construct showed that a 30-kDa protein was expressed on cell membranes. Binding studies showed no effect of the 5-$HT_{(2A\text{-}tr)}$ variant on 3H-ketanserin binding to the native 5-$HT_{(2A)\text{-}R}$. Functionally, there was no effect on coupling of the 5-$HT_{(2A)\text{-}R}$ to 5-HT-stimulated Ca^{2+} mobilization.

3.2.2. 5-HT_{2C} Receptor Heterogeneity

The human $5HT_{2C}$ receptor gene, *HTR2C*, is located on human chromosome Xq24 (www.ncbi.nlm.nih.gov) and contains six introns and five exons *(103)*. It is expressed in the highest density in the choriod plexus and is widely distributed in the CNS (i.e., hippocampus, olfactory bulb, striatum, and cortex) *(68,104)*. Its heterogeneity is affected by genetic polymorphisms, RNA editing, and alternative splicing.

3.2.2.1. Genetic Polymorphisms

To date, investigators have located two SNPs within the coding regions of HTR2C: L4V and C23S (www.ncbi.nlm.nih.gov; *see* Fig. 2). The first SNP involves a leucine for valine substitution and is located in the extracellular region on the N-terminus. The frequencies of the L4 and V4 alleles are reported to be 0.984 and 0.0014, respectively (www.ncbi.nlm.nih.gov). In caucasians,

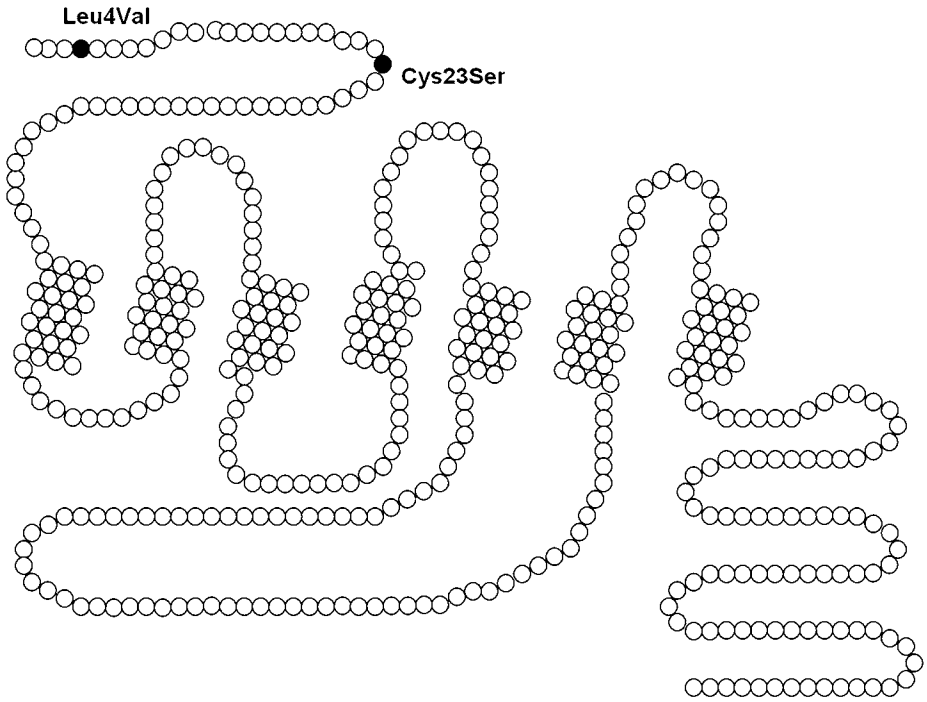

Fig. 2. SNPs on the 5-HT_{2C} receptor. Two SNPs (Leu4Val and Cys23Ser) of the human 5-HT_{2C} receptor are shown by black circles.

the frequencies of the Cys23 allele and Ser23 allele are reported to be 0.842 and 0.158, respectively *(105)* (www.ncbi.nlm.nih.gov). The second genetic polymorphism in the first exon, identified as the C23S polymorphism *(105)*, involves a cysteine for serine substitution and is located in the extracellular N-terminus region of the receptor gene (www.ncbi.nlm.nih.gov).

The C23S SNP has been studied and it appears to affect receptor function. Most recently, Okada et al. *(106)* examined receptor function in COS-7 cells. Ser23-expressed membranes showed reduced "high-affinity binding." Also, data from 5-HT–stimulated intracellular Ca^{2+} mobilization indicated that the Ser23 required higher 5-HT concentrations to elicit the same dose–response curve as Cys23; these researchers speculated that this difference could reflect increased desensitization of Ser23.

Regarding pathological processes, the C23S polymorphism has been examined for its association with psychotic symptoms in late-onset AD and both etiology and drug response in schizophrenia and mood disorders. In a late-onset AD sample, Holmes et al. *(22)* confirmed an earlier finding of an association between the presence of the Ser23 allele and visual hallucinations In addition,

there was an association between the C23S polymorphism and hyperphagia ($p = 0.03$). When Murad et al. *(107)* used the transmission disequilibrium test (TDT) to examine for linkage to schizophrenia in 207 nuclear families with a child with schizophrenia, they found no evidence linking C23S and schizophrenia. This finding supported an earlier study by Sodhi et al. *(167)* that reported no association between the presence of the C23S alleles and schizophrenia (102 patients vs 162 controls); however, this study did reveal a significant association between the Ser23 allele and clozapine response (in individuals with at least one Ser23 allele, 90.5% responded to clozapine vs in individuals without this allele, 59% responded). Another association study *(108)* indicated that the C23S variant is associated with an increased risk for hospitalization in individuals with schizophrenia. Meanwhile, Assal et al. *(79)* did not find an association of the C23S variant with psychosis in AD.

Using subjects from the European Collaborative Project on Affective Disorders, Lerer et al. *(109)* reported a significant excess of the Ser23 allele in 513 patients with recurrent major depression ($p = 0.006$) and 649 patients with bipolar disorder ($p = 0.02$) vs 901 normal controls. There was also considerable variability in the frequency of this allele across ethic groups.

The 5-HT_{2C} receptors are involved in the regulation of feeding behaviors; rodents lacking this gene become obese from increased food intake *(110,111)*. In humans, there is considerable evidence that a SNP in the promoter region of the *HTR2C* gene (–759C/T) is a risk factor for obesity *(112)*. Basile et al. *(113)* genotyped 80 patients with schizophrenia and reported a trend for patients carrying only the serine variant of the C23S polymorphism to have a higher mean weight gain following 6 wk of clozapine treatment. Westberg et al. *(114)* examined the association between the C23S SNP and weight loss in teenage girls. Data indicated that subjects in a weight-loss group ($n = 57$) differed significantly from normal-weight girls ($n = 91$) in the frequency of the serine allele (23.7% vs 7.7%, respectively).

3.2.2.2. mRNA Editing

A landmark paper by Burns and colleagues *(115)* first described RNA editing events in rat brain. RNA editing involved an adenosine to inosine substitution, required the action of two separable double-stranded RNA (dsRNA) and adensosine deanimase(s) (ADARs), and occurred at four sites (A, B, C, and D) located within the second intracellular loop of the receptor. RNA editing in the r5-HT_{2C} receptor resulted in seven major isoforms (VNV, VSV, VNI, VSI, INV, ISV, and INI) encoded by 11 RNA species *(115)*. Also, patterns of RNA editing were tissue-specific; the most common variants (VNV, VSV, VNI, and VSI) were expressed in the whole brain and hippocampus, whereas the least common variants (INV, ISV, and INI) were expressed in the choroid plexus. Functionally,

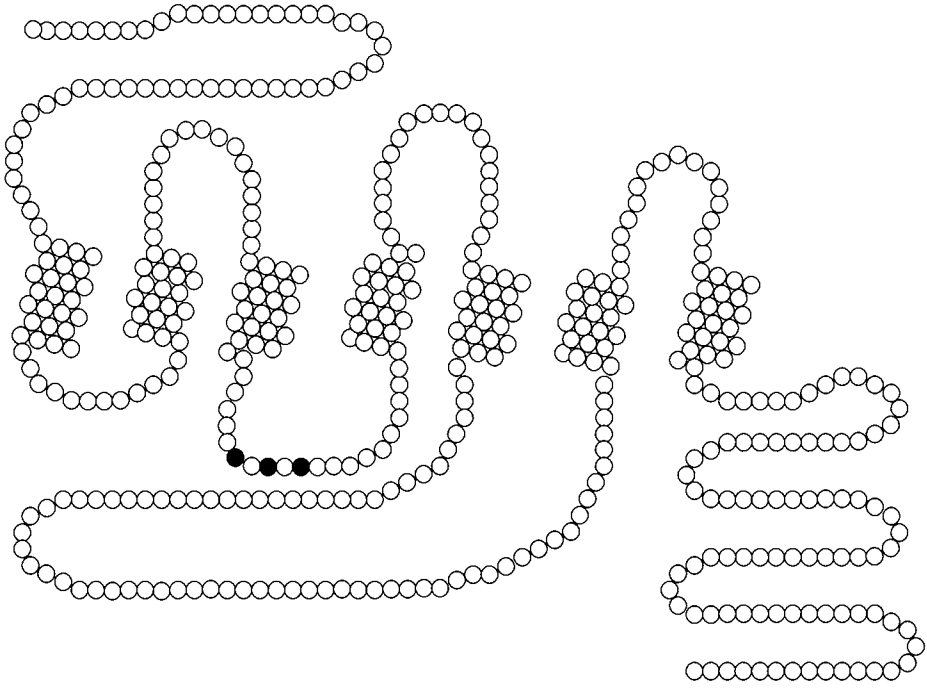

Fig. 3. RNA editing of the 5-HT_{2C} receptor. RNA editing sites of the human 5-HT_{2C} receptor are shown by black circles.

the VSV receptor isoforms was associated with decreased potency of 5-HT for active phosphoinositide hydrolysis *(115)*.

Extending studies to human brains, Fitzgerald et al. *(52)* generated stable cell lines by transfecting human embryonic kidney 293E (HEK293E) cells with cDNAs for the INI, VNV, VSV, or VGV edited isoforms of human 5-HT_{2C} receptor mRNA. Results indicated that RNA editing occurred at five sites (A, B, C, D, and E); the additional site (E) contributed to the diversity of the receptor by resulting in 14 protein isoforms. The editing is catalyzed by three adenosine deaminases (ADAR1, ADAR2, and ADAR3) and their isoforms *(57,116)*. The posttranscriptional modification that involves adenosine-to-inosine RNA editing occurs on exon 5 in a region that encodes the second intracellular loop of the 5-HT_{2C} receptor, a region that is important for G protein coupling *(115,117,118; see* Fig. 3).

Functionally, in mammalian cells, edited 5-HT_{2C} receptor mRNA isoforms appear to be tissue-specific and function less efficiently than the original non-edited transcript *(52,119)*. Marion et al. *(120)* reported that RNA edited forms of the 5-HT_{2C} receptor showed lesser degrees of constitutive activity than the

nonedited forms of the receptor in HEK293 cells. In addition, their internalization is mediated by a G protein–coupled receptor kinase (GRK)/β-arrestin 2-dependent mechanism. Wang et al. *(116)* described a 13-fold reduction in agonist potency and reduced basal level activity for a thalamus-specific isoform (5-$HT_{2C\text{-}IGV}$R) and a fourfold to fivefold reduction in agonist potency for two other isorforms (5-$HT_{2C\text{-}MSV}$R and 5-$HT_{2C\text{-}IGV}$R) in the amgdala and choriod plexus. Likewise, another extensively edited isoform (5-$HT_{2C\text{-}VSV}$R) in the human brain exhibited lower constitutive activity as compared to the nonedited version (5-$HT_{2C\text{-}INI}$ receptor) *(121,122)*. Niswender et al. *(121)* demonstrated that the hallucinogenic drugs DOI and DMT exhibited lower affinity and potency when interacting with the edited h5-$HT_{2C\text{-}VSV}$ receptor as compared to h5-$HT_{2C\text{-}INI}$ receptor. However, the agonist LSD and three antipsychotic medications (clozapine, loxapine, and risperidone) were unable to elicit a phosphoinositide hydrolysis response expressing the h5-$HT_{2C\text{-}VSV}$ receptor.

To test the hypothesis of a possible role for 5-HT_{2C}R editing in the etiology and pharmacotherapy of schizophrenia, several studies *(3)* have used total RNA obtained from postmortem human brain samples to investigate the efficiency of 5-HT_{2C}R editing in the dorsolateral prefrontal cortex (DLPFC) of individuals diagnosed with schizophrenia (SCZ); results are contradictory. One study *(167)* examined samples from Brodman area 46 of five SCZ patients and five normal controls (NCs) and reported reduced RNA editing, increased expression of the unedited 5-$HT_{2C\text{-}INI}$ isoform ($p = 0.001$) and decreased expression of two novel mRNA edited variants [5-$HT_{2C\text{-}VSV}$ and 5-$HT_{2C\text{-}VNV}$] isoforms for the schizophrenia groups. In contrast, two studies *(3)* suggested that there were no alterations in editing of the h5-HT_{2C} receptor that are associated with schizophrenia. One study *(3)* examined Brodman area 46 and found that editing efficiencies at all five editing sites were not significantly different between 15 elderly individuals diagnosed with SCZ and 15 NCs ($ts < 1.3$, $df = 28$, $p > 0.24$). Although the second study by Niswender et al. *(20)* found no differences in mRNA levels between 13 subjects with SCZ or depression and 13 NCs, they did report that individuals who had committed suicide exhibited a small but significant elevation of editing at the A-site, regardless of diagnosis. These findings were further supported by a recent study *(123)* of prefrontal cortex samples of patients with bipolar disorder, schizophrenia, and major depression. Compared with controls, there were nonsignficant trends for increased site-D RNA editing in depression patients ($p = 0.08$) and for increased site A RNA editing in suicide victims ($p = 0.07$). Most recently, Gurevich et al. *(124)* reported a significant increase in E-site editing and a significant decrease in D site editing (Brodman area 9) among suicide victims with a history of major depression. Noting that, in mice, fluoxetine resulted in editing changes that were opposite those seen

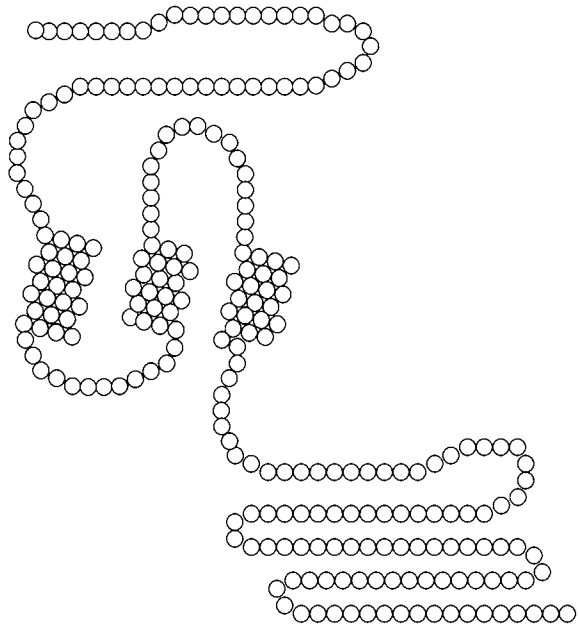

Fig. 4. Alternative splicing of the 5-HT_{2C} receptor. Shown is the truncated form of the human 5-HT_{2C} receptor with a premature termination that results in a truncated receptor.

in suicide victims, Gurevich et al. *(124)* suggested that this antipdepressant drug might reverse RNA editing abnormalities in depressed suicide victims.

3.2.2.3. Alternative Splicing

In the 5-HT_{2C} receptor, alternative splicing results in the generation of two variants: a truncated nonfunctional receptor (5-HTR-tr) and 5-HT_{2C}R-COOH *(116,125)*; both encode nonfunctional receptor proteins (*see* Fig. 4). In addition, an alternatively spliced 5-HT_{2C} receptor RNA that contains a 95-nt deletion in the region coding for the second intracellular loop and the fourth transmembrane domain of the receptor has been identified *(103)*. This deletion appears to lead to a frame shift and premature termination so that the short isoform RNA encodes a putative protein of 248 amino acids. Xie et al. *(103)* reported that the ratio for the short isoform over the 5-HT_{2C} receptor RNA was higher in a choroid plexus tumor than in normal brain tissue, suggesting the possibility of differential regulation of the 5-HT_{2C} receptor transcription in different neural tissues or during tumorigenesis. In another study *(3)*, which examined whether the splicing of 5-HT_{2C} receptor mRNA was in the DLPFC of 15 elderly individuals diagnosed with SCZ vs 15 NCs, they found no differences between the groups. However, it was suggested that alterations in the expression of the

truncated receptor relative to the functional protein could be associated with subject-to-subject variances in the course of the treatment of schizophrenia.

3.2.3. 5-HT$_4$ Receptor Heterogeneity

The human 5-HT$_4$ receptor is located on human chromosome 5q31-5q33 *(126)* (www.ncbi.nlm.nih.gov) and is composed of 387 amino acids *(127,128)*. It is widely distributed in both central and peripheral tissues. Its heterogeneity is affected by genetic polymorphisms and alternative splicing.

3.2.3.1. Genetic Polymorphisms

Suzuki et al. *(126)* used denaturing high-performance liquid chromatography (dHPLC) followed by direct sequencing to detect one silent mutation in the HTR4 coding region and six SNPs in the intron regions of the HTR4 gene. Ohtsuki et al. *(129)* identified eight polymorphisms of the HTR4 gene; four were at or very near the four splice variant regions encoding the C-terminal tail of the receptor and showed significant association with bipolar disorder (OR > 1.5). To date, there are no reported functional SNPs in the coding region of the HTR4 gene (www.ncbi.nlm.nih.gov).

3.2.3.2. Alternative Splicing

The HTR4 gene produces nine splice variants: 5-HT$_{4(a)}$, 5-HT$_{4(b)}$, 5-HT$_{4(c)}$, 5-HT$_{4(d)}$, 5-HT$_{4(h)}$, 5-HT$_{4(gef)}$, 5-HT$_{4(n)}$ *(61,130)*. Although the stimulation of cAMP is triggered by the activation of 5-HT$_4$, there appear to be fine functional differences in the pharmacological profiles of spliced variants. When Bender et al. *(61)* compared the pharmacological profile of variant h to isoforms a and b, they found no differences in receptor binding in COS-7 cells; however, they did find that the 5-HT$_4$ antagonist GR113808 functioned as a partial agonist at the 5-HT$_{(4hb)}$ variant compared to its antagonist action at the other two variants. In contrast, Pindon et al. *(131)* reported that both ligand binding and signal transduction varied between the 5-HT$_{4(a)}$ and h5-HT$_{4(b)}$ receptor splice variants stably expressed in human embryonic kidney (HEK) 293 cells. The fraction of the [3H]5-HT high-affinity site relative to the whole receptor population measured with [3H]GR113808 was higher for the h5-HT$_{4(a)}$ isoform than for the 5-HT$_{4(b)}$ isoform. Also, the potency and efficacy of several compounds (5-methoxytryptamine, prucalopride, SDZ-HTF 919, and SB204070) varied at each isoform.

Because the 5-HT$_4$ receptors mediate physiological effects in the heart, gut, and CNS *(132)*, splice variants of this receptor are thought to be involved in atrial arrhythmia, irritable bowel syndrome, and neurodegenerative diseases. Medhurst et al. *(132)* used TaqMan real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) to investigate the mRNA distribution of 5-HT$_4$ receptor C-terminal splice variants in multiple human CNS and peripheral tissues. They

reported that the highest CNS expression of 5-HT_4 receptor mRNA was observed in the basal ganglia, amygdala, and hippocampus; however, there were differences in expression levels and distribution patterns of each variant. The 5-$HT_{4(b)}$ splice variant was more highly expressed in all areas than other variants. Highest levels of the 5-$HT_{4(a)}$ variant were found in the amygdala; 5-$HT_{4(c)}$ expression was highest in the pituitary gland; and the highest levels of 5-$HT_{4(g)}$ were observed in the hypothalamus and cortex. Very low levels of 5-$HT_{4(d)}$ mRNA were detected in the small intestine. In a recent study, Yusuf et al. *(133)* indicated that left atrial dilatation favored the expression of the b isoform (5-$HT_{4(b)}$) after coronary artery bypass grafting (CABG) as compared to the a isoform (5-$HT_{4(a)}$), which might predispose to atrial fibrillation or help prolong arrhythmias post-CABG.

4. Posttranslational Modifications of Serotonin Receptors

Various covalent modifications can occur after a polypeptide has been translated. The modifications include phosphorylation, glycosylation, methylation, attachment of lipids, ubiquitination, and so on. These modifications have been implicated in the involvement of many biological pathways such as protein folding, signal transduction, proteolysis, membrane anchoring/association, and protein targeting. Nevertheless, among the various modifications, only palmitoylation, glycosylation, and phosphorylation have been reported to occur in serotonin receptors thus far.

4.1. Palmitoylation

Palmitoylation is a post-translational covalent attachment of fatty acids (mainly palmitic acid) to proteins via labile thioester bonds to cysteine residues that occurs posttranscriptionally *(134)*. It is a lipid modification that is usually reversible and it is known that proteins involved in signal transduction such as GPCRs, α-subunits of G proteins, phospholipase C, adenylyl cyclase, and nonreceptor tyrosine kinase can be targets for palmitoylation *(135–137)*. In GPCRs, dynamic palmitoylation and depalmitoylation have been demonstrated to play roles in the regulation of receptor function. For example, substitution of the palmitoylated cysteine in β_2-adrenergic receptors leads to the receptor being highly phosphorylated and largely uncoupled from G's in response to agonist stimulation *(138)*. In addition, the rate of palmitoylation of muscarinic acetylcholine receptor M2 subtypes was markedly accelerated by the addition of the agonist and it was found that palmitoylation enhances the ability of the receptors to interact with G proteins *(139)*. These findings show that palmitoylation had different effects in different GPCRs.

Not much has been addressed regarding palmitoylation of serotonin receptors. However, it was found that the mouse 5-HT_{1A} receptor is modified by

palmitic acid and palmitoylation efficiency was not modulated by agonist stimulation *(140)*. Blocking protein synthesis with cycloheximide resulted in a significant reduction of receptor palmitoylation, which suggests that palmitoylation occurs early after synthesis of the 5-HT_{1A} receptor. Pulse-chase experiments also showed that fatty acids were stably attached to the receptor and this means that it is probably a relatively irreversible modification. By site-directed mutagenesis studies, two conserved cysteines (residue 417 and 420) at the proximal C-terminal domain were identified as palmitoylation sites. Mutation of individual cysteine residues reduced the ability of the receptor to couple with Gi and impaired inhibition of adenylyl cyclase activity. Moreover, when the two mutations were combined, the 5-HT_{1A} receptor no longer coupled with Gαi subunits, which demonstrate the importance of palmitoylaion in activation of the Gαi protein. In addition, in nonpalmitoylated mutants, inhibition of forskolin-stimulated cAMP formation as well as receptor-dependent activation of extracellular signal-regulated kinase were also affected indicating the important role of palmitoylation in receptor signaling via both Gαi and Gβγ-mediated pathways. Two ways in which palmitoylation could affect 5-HT_{1A} receptor function have been proposed: (1) It might be required for the receptor to adapt a comformation, which facilitates receptor–G protein recognition/interaction, and (2) it might be involved in receptor trafficking and/or localization to the membrane subdomains, like lipid rafts.

Analysis of the primary protein structure of the human 5-HT_{1B} receptor reveals a putative site for palmitoylation, i.e., a cysteine residue located in the short carboxyl tail of the receptor *(141)*. A recombinant c-myc epitope-tagged 5-HT_{1B} receptor was expressed in Sf9 insect cells and palmitoylation of the receptor was demonstrated by metabolic labeling of the cells with [^{3}H]palmitic acid.

In the mouse, four 5-HT_4 receptor isoforms have been cloned *(142)*. It has been shown that among them, the 5-$HT_{4(a)}$ isoform undergoes reversible palmitoylation *(143)*. Upon agonist stimulation, the turnover rate for receptor-bound palmitate increases. In a later study, the functional roles of palmitoylation have been addressed in a series of site-directed mutagenesis experiments that mutated the potential palmitoylation sites in the 5-$HT_{4(a)}$ receptor *(143)*. It has been found that in addition to Cys^{328} and Cys^{329}, a cysteine residue Cys^{386}, which is located in the very distal part of the COOH-terminus of the receptor, was also demonstrated to be another palmitoylation site. It was demonstrated that mutation of the proximal palmitoylation site (Cys^{328}/Cys^{329}) significantly increases the capacity of receptors to convert from the inactive (R) to the active (R*) form in the absence of agonist, whereas no such effect had been detected in $Cys^{386} \rightarrow$ Ser as well as the triple, nonpalmitoylated mutant. It was therefore suggested that the palmitoylation state of the receptor is probably able to

modulate the agonist-independent constitutive 5-$HT_{4(a)}$ receptor activity. On the other hand, palmitoylation does not seem to play roles in 5-HT_4's ability to interact with Gs, to stimulate adenylyl cyclase activity, or to activate cyclic nucleotide-sensitive cation channels upon agonist treatment. Palmitoylation also had no effect on the intracellular distribution of nonpalmitoylated receptors. Based on the results, it was proposed that complete palmitoylation of the 5-$HT_{4(a)}$ receptor might result in the formation of two additional intracellular loops (i4 and i5), which might be important in determining the basal level of receptor activity in the absence of agonist. Inhibition of palmitoylation at the receptor might change its conformation and affect its activation state. However, the details underlying the mechanism are still unclear.

4.2. Glycosylation

Glycosylation is the addition of oligosaccharide side chains to a protein. Glycosylation has been suggested to play roles in protein folding and targeting, cell–cell adhesion, or regulatory roles in receptor signaling (e.g., Notch) as well as many unknown functions *(168)*. Glycosylation can be roughly divided into two types: N-linked glycosylation and O-linked glycosylation. N-linked glycosylation transfers oligosaccharide to the side-chain NH_2 group of an asparagine amino acid in the protein, whereas O-linked glycosylation adds oligosaccharide to the side-chain OH group of a serine or threonine residue.

So far, not much is known regarding the glycosylation of serotonin receptors. However, when the rat 5-HT_{2C} receptor was first identified by antibodies from both cell line (3T3/2C) and rat brain, it was demonstrated that 5-HT_{2C} receptors are N-glycosylated *(144)*. In order to inhibit the *de novo* addition of sugar to polypeptides, cultures of the 5-HT_{2C} receptor cell line were grown in the presence of tunicamycin, which inhibits the biosynthetic addition of N-linked sugars. Upon tunicamycin treatment, 5-HT_{2C} receptor proteins exhibited masses of 40 and 41 kDa, significantly less than the 51- to 52- and 58- to 68-kDa receptors from control cells. In addition, applying *N*-glycosidase F (PNGase F) cleaves the bond between asparagines and *N*-acetylglucosamine and results in 5-HT_{2C} receptors with masses of 41 and 42 kDa. On the other hand, *O*-glycosidase did not significantly alter the mass of the deglycosylated (N-linked) 5-HT_{2C} receptor. However, the possibility of existence of O-linked sugars still could not be ruled out.

Identification of the 5-HT_{2C} receptor as a 60-kDa N-glycosylated protein in the choroid plexus and hippocampus has also been carried out in another study *(145)*. When rat choroid plexus and hippocampal membrane lysates were treated with PNGase F, 5-HT_{2C} receptor polypeptides from both tissues were converted to a sharp 38-kDa band. In a time-course experiment performed in the choroid plexus,

it was demonstrated that PNGase F converted the broad 5-HT_{2C} 60-kDa band to a 5-HT_{2C} 48-kDa band before the final 5-HT_{2C} 38-kDa band appeared *(146)*. Interestingly, it seems that glycosylation was heterogeneous and irregular after transient or stable transfection of cultured cell lines. The different results obtained from either in vivo or in vitro sources suggest that observations made in cell lines might not reflect the in vivo situations.

The 5-HT_{3A} receptor is a ligand-gated ion channel that exists as a pentameric oligomer. This receptor was predicted to have three potential N-linked glycosylation sites with consensus amino acid sequence N-X-S/T *(147)*. Quirk et al. *(146)* used tunicamycin treatment and site-directed mutagenesis to inhibit selectively N-linked glycosylation at each glycosylation site in the murine 5-HT_{3A} receptor and examined the resulting effects on receptor function and expression in transiently transfected heterologous cells. Their findings suggested that N109 is necessary for receptor assembly, whereas N174 and N190 are important for membrane targeting and ligand binding. Moreover, each glycosylation site has been shown to be involved in 5-HT_{3A} receptor-mediated Ca^{2+} influx. Recently, Monk et al. *(148)* investigated the presence and potential role of N-glycosylation of the human 5-HT_{3A} receptor subunit expressed in COS-7 cells. They demonstrated that by substituting four identified N-terminal asparagines (N5, N81, N147, N163) using site-directed mutagenesis, each expressed 5-HT_3 mutant displayed a reduced molecular weight indicating that each of the residues was subject to N-glycosylation. Further experiments showed that inhibition of N-glycosylation by mutating the glycosylation sites either prevented (N81, N147, N163) or greatly reduced (N5) the production of a 5-HT_3 receptor binding site. In addition, immunocytochemical studies demonstrated that each asparagine substitution either prevented (N81, N147, N163) or reduced considerably (N5) mutant protein expression on the cell membrane. In these studies, the importance of each glycosylation site in receptor function was clearly demonstrated.

4.3. Phosphorylation

The majority of the studies examining the phosphorylation of serotonin receptors have emphasized its role on receptor desensitization. A general mechanism of G protein–coupled receptor desensitization involves phosphorylation of the intracellular domains of the receptor by second–messenger kinases such as protein kinase A (PKA) or C (PKC) and specific G protein–coupled receptor kinases, that lead to the binding of arrestins to the receptor and G protein uncoupling *(149,150)*. The mechanisms have been best elucidated for the β-adrenergic receptors, where desensitization is mediated by both βARK and PKA *(151)*. However, mechanisms underlying desensitization of serotonin

receptors are still under investigation and current findings are summarized as follows.

4.3.1. 5-HT$_{1A}$ Receptor

Protein kinase C has been shown to induce phosphorylation and desensitization of the human 5-HT$_{1A}$ receptor in Chinese hamster ovary (CHO) cells in an early study *(152)*. It was found that there was a time- and dose-dependent increase in phosphorylation of the 5-HT$_{1A}$ receptor by PMA, a PKC activator. Raymond and Olsen *(152)* later examined the effects of short-term treatment with PKA activators on coupling to the inhibition of adenylyl cyclase and phosphorylation of a human 5-HT$_{1A}$ receptor in CHO cells and the data suggested a potential subtle role for PKA in receptor phosphorylation and desensitization. However, activation of PKA was found to be able to augment the actions of submaximally but not maximally activated PKC on a 5-HT$_{1A}$ receptor. It therefore suggested a cooperativity between PKA and PKC in 5-HT$_{1A}$ receptor phosphorylation and desensitization existed. On the other hand, a study performed in Sf9 insect cells *(153)* showed that brief pretreatment with 5-HT resulted in 5-HT$_{1A}$ receptor desensitization and increased phosphorylation on serine and threonine residues. Nevertheless, the 5-HT$_{1A}$ receptor appeared to be a substrate for phosphorylation by an endogenous, agonist-activated, heparin-sensitive kinase but not PKA or PKC, as demonstrated by treating the cells with various kinase inhibitors.

Lembo and Albert *(154)*, on the other hand, identified multiple phosphorylation sites that are required for pathway-selective uncoupling of the rat 5-HT$_{1A}$ receptor by PKC in Ltk$^-$ fibroblasts. In Ltk$^-$ cells, acute pretreatment with TPA (which activates several isoforms of PKC) selectively reduces 5-HT-induced increases in PI turnover and intracellular calcium but not inhibition of the cAMP level. Mutant 5-HT$_{1A}$ receptor lacking one, two, or three potential PKC phosphorylation sites in the third intracellular loop had been constructed by site-directed mutagenesis and tested. Upon treatment with TPA, there was a progressive recovery to 74% of the control 5-HT–induced increase in calcium mobilization as PKC phosphorylation sites were moved from the receptor, suggesting the role of these sites in receptor desensitization mediated by PKC. The residual inhibitory effect of PKC activation on the triple mutant might be due to phosphorylation at additional sites on the receptor, G protein, or downstream effectors. Later, in a study performed in the excitable neuronal cell line F11 (a dorsal root ganglion neuron crossed with a neuroblastoma cell), the role of specific PKC phosphorylation sites for modulation of the inhibitory coupling of a 5-HT$_{1A}$ receptor to N-type calcium channels was investigated using patch-clamp techniques *(155)*. The results suggested that among the four phosphorylation sites within the 5-HT$_{1A}$ receptor (one in the i2 loop and three in the i3 loop),

uncoupling of the 5-HT_{1A} receptor from inhibition of N-type calcium channels mediated by PKC is partly dependent on a PKC site (T149) located in the second intracellular loop. However, it was proposed that downstream action of PKC on G proteins or channel subunits might also be involved.

4.3.2. 5-HT_{1B} Receptor

Amino acid sequence analysis of the human 5-HT_{1B} receptor has revealed consensus phosphorylation sites in all intracellular loops for PKA and PKC. Phosphorylation of the 5-HT_{1B} receptor was demonstrated by metabolic labeling of the 5-HT_{1B} receptor-expressing Sf9 cells with [^{32}Pi]phosphate *(141)*. This posttranslational modification was proposed to be involved in receptor regulation such as desensitization.

4.3.3. 5-HT_{2A} Receptor

In order to identify residues essential for a agonist-mediated desensitization rat 5-HT_{2A} receptor, Gray et al. *(156)* mutated, individually or in groups, all of the 37 serines and threonines (which are potential phosphorylation sites) in the cytoplasmic domains of the receptor and assessed effects of these mutations. It was found that mutation of two nonconserved serine residues (S421 in the carboxyl-terminal tail and S188 in the second intracellular loop [to alanine]) significantly blocked agonist-induced desensitization of the receptor. Interestingly, a SNP at the S421 locus (S421F) has been reported, and this mutation was also demonstrated to have a significant effect on receptor desensitization. However, no direct evidence of phosphorylation of these residues has been reported and the role of phosphorylation in 5-HT_{2A} receptor desensitization needs to be further investigated.

4.3.4. 5-HT_{2C} Receptor

The 5-HT_{2C} receptor exhibits agonist-independent constitutive receptor activation. In the NIH/3T3 cell, it was demonstrated that the 5-HT_{2C} receptor is phosphorylated under basal conditions and phosphorylation is increased by agonist treatment conditions that result in desensitization of receptor signaling *(157)*. In a later study, Backstrom et al. *(144)* generated phophorylation-deficient rat 5-HT_{2C} receptors to determine if phosphorylation promotes receptor desensitization. It was found that deletion of the PDZ recognition motif prevents receptor phosphorylation. Moreover, the findings showed that phosphorylation-deficient 5-HT_{2C} receptors displayed identical initial responses as the wild-type receptor in PI hydrolysis and calcium release assays, but they exhibited diminished secondary responses and a delayed recovery relative to wild-type receptors. NIH/3T3 cells stably expressing 5-HT_{2C} receptors with a S459A mutation also showed reduced receptor phosphorylation and secondary calcium responses. It

was therefore proposed that 5-HT_{2C} receptor phosphorylation promotes resensitization of receptor-mediated responses.

4.3.5. 5-HT_3 Receptor

To examine the phosphorylation of the myc-tagged wild-type 5-HT_{3A} receptor subunits from the guinea pig, stably transfected HEK 293 cells were metabolically labeled with [^{32}P]phosphoric acid *(158)*. It was demonstrated that both splice variants of the 5-HT_{3A} receptor subunit were phosphorylated. Moreover, site-specific mutagenesis revealed that phosphorylation occurs at Ser409, a potential target of PKA.

4.3.6. 5-HT_4 Receptor

In a study characterizing human 5-$HT_{4(D)}$ receptor desensitization in CHO cells, four putative PKA phosphorylation sites located in the third intracellular loop or the C-terminal tail were mutated by site-directed mutagenesis, given the prominent role of PKA in agonist-mediated desensitization *(159)*. However, none of the 5-$HT_{4(D)}$ mutants showed impaired receptor desensitization, which suggests that PKA might act on certain nonconsensus sites. Alternatively, the 5-$HT_{4(D)}$ receptor might not necessarily be directly phosphorylated by PKA upon receptor activation. Other proteins involved in the signaling pathway might be the substrates for PKA instead.

4.3.7. 5-HT_5, 5-HT_6, and 5-HT_7 Receptors

To date, no published data have been shown to indicate the role of phosphorylation in receptor regulation.

5. Conclusions

Evidence from in vitro and in vivo studies indicate that SNPs in the coding regions, mRNA editing (in the 5-HT_{2C} receptor), and alternate mRNA splicing events occur in several human 5-HT receptors. These modification are associated with the symptoms of some physical diseases and psychiatric disturbances; they can alter the pharmacodynamics of antidepressants and antipsychotic drugs. Further insights into these cellular mechanisms are important from a public health perspective, as many antidepressants and antipsychotic medications are being used in the treatment of psychiatric disorders, either as a monotherapy or augmentation strategy *(160–166)*.

Regarding posttranslational modifications, palmitoylation, glycosylation, and phosphorylation have been found in 5-HT receptors; each of these modifications has distinct roles in regulation of receptor functions. It is well known that phosphorylation, the modification of the serotonin receptors that has been studied most widely, plays an important role in receptor desensitization.

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4

Strategies for the Development of Selective Serotonergic Agents

Richard A. Glennon

Summary

Many of the ligands in use today to investigate serotonin receptors and serotonin receptor pharmacology were serendipitous discoveries; this includes ligands that are commonly regarded as being "selective" for a given population of serotonin receptors. Nevertheless, there still remains a number of serotonin receptor types that lack a truly selective agonist and/or antagonist. Over the past 20 yr, there have been various attempts to rationally develop ligands with greater selectivity, or selective ligands for serotonin receptor types for which such agents were lacking. To this end, we describe some of our efforts to develop selective serotonergic agents by presenting a series of case studies. Several different strategies have been employed to achieve this goal. In particular, the "deconstruction–reconstruction–elaboration" approach is shown to be useful for aiding the development of selective ligands where a lead structure is already known, and the utility of the "standard series" approach is illustrated where a lead structure is not known. The discussion is focused on these and other methods that could have general applicability for the development of other selective serotonergic and nonserotonergic agents.

Key Words: Serotonin agonists; serotonin antagonists; selectivity; drug design; standard series; deconstruction–reconstruction–elaboration; serendipity.

1. Introduction

Chemical entities might be viewed as a primordial soup of nonselective agents from which selective agents can evolve. The primordial pool might

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already contain some selective agents, but appropriate pharmacological techniques and methodologies must be available (or developed) so that they can be "fished out." At the heart of medicinal chemistry is the design and synthesis of agents with a given pharmacological action. Although this no longer remains the problem it once was, one of the most difficult tasks in medicinal chemistry remains the design of *selective* agents; that is, once an agent with a given biological function has been identified (a relatively straightforward goal), a subsequent and more difficult task is the introduction of selectivity to, for example, reduce undesirable side effects or develop tools that might be useful as selective agonists, antagonists, or radioligands for pharmacological studies. Difficulties encountered in the development of selective ligands are epitomized in the field of serotonin. In addition to interacting with the serotonin transporter (SERT), serotonin binds at seven major families or populations of receptors (5-hydroxytryptamine; 5-HT_1 to 5-HT_7) *(1,2)*. Multiple subpopulations (and species homologs and splice variants) exist for many of these. With this number of targets, development of an agonist or antagonist ligand with selectivity for one serotonin receptor (sub)population over another represents a rather daunting challenge. Several populations of 5-HT receptors are still without selective agonists and/or antagonists. Indeed, even identification of a novel nonselective structure type can sometimes present challenges.

The purpose of this chapter is not to review those agents that are currently thought to be selective for one population of serotonin receptors over another, or even to describe the various agents that have been investigated for serotonergic activity. Rather, the intent is to discuss some of the strategies that we have employed in our attempts to develop "selective" serotonergic agents. What is presented are selected case studies that have emanated from our laboratory; the chapter documents some of our efforts (and problems encountered along the way). The studies should be instructive in that they might eventually lead to serotonergic agents with even greater selectivity. In most instances, the approaches are quite general and also should be applicable to nonserotonergic fields of study.

The field of serotonin research exploded over the past two decades with thousands of articles now appearing each year (nearly 3000 articles were published in the first three quarters of 2004 alone). Certainly, our work has not been conducted in a vacuum. Without question, our work has been enormously dependent on, and impacted by, the results of others. Given limitations of space, we focus primarily on our studies, giving just due to certain other investigators whose key results highly influenced the direction of our work. We apologize in advance for not having the space to present a more thorough review of the contributions others have made to this field or even to provide a more comprehensive account of those investigations that directly or indirectly bear on the

studies that are presented here. Early work (covering the literature to 1985) on the medicinal chemistry of serotonergic agents has been reviewed *(3)*. For detailed descriptions of the different populations of 5-HT receptors and their ligands, a number of recent reviews are available *(2,4–6)*.

What does selectivity mean? Also, how selective must an agent be to be termed "selective"? Selectivity refers to the ability of a compound to recognize its target without interacting with other, related targets *(7)*. The selectivity that an agent shows for one target over another is sometimes referred to as the *selectivity index*. A selectivity index of 1000 to 10,000 or more is considered ideal, but in some instances 50-fold to 100-fold can provide sufficient selectivity to derive a good therapeutic index *(7)*. Thus, selectivity is relative. A drug need not be selective to be useful or therapeutically effective—indeed, most drugs are not; some drugs are even quite nonselective (to wit the antipsychotic agent clozapine). Nevertheless, selective agents make good pharmacological tools, and much is learned along the way to achieving selectivity that can be applied to the formulation of structure–affinity relationships (SAFIR) and/or structure–activity relationships (SAR). The latter information can often be applied to drug design. At this point, it might also be noted that an agent can display *binding selectivity* and/or *functional selectivity*. The two concepts are not strictly related. Binding selectivity is usually related to *affinity*. Functional selectivity, which can be achieved without binding selectivity, is the more complex of the two and is complicated by issues of efficacy; it can sometimes be related to the pharmacological assay employed. For example, an agonist ligand might show some selectivity for one receptor population over another (i.e., it binds with higher affinity at one receptor than another); however, if its efficacy is significantly lower at the first than at the second, it might appear functionally selective for the latter. An extreme case is where an agent binds equally well at two populations of receptors but is an agonist at one and an antagonist at the other; such an agent would appear to be a functionally selective agonist in an in vivo assay even though it possesses no binding selectivity between the two receptor populations. Antagonists can also demonstrate functional selectivity. For example, an antagonist binds equally well at two receptor populations but one population is inaccessible; this might be encountered if one population is located only in the periphery and the other only in the brain, and the antagonist is unable to penetrate the blood–brain barrier. The studies described in this chapter deal almost exclusively with binding selectivity. Our work has been predicated on the need to identify an agent that binds in a selective fashion at one serotonin receptor population over the others prior to any investigation of functional selectivity.

In its most general terms, the overall question to be addressed is *How does one go about developing a selective agent in the absence of information*

necessary to achieve selectivity? With specific application to serotonin, how can selectivity be achieved for one 5-HT receptor population over another? With the discovery of some of the newer 5-HT receptors, little more might be known other than that 5-HT binds at the receptor. Certain other agents might have been examined at the receptor, but these are generally standard, nonselective, structurally unrelated agents used to authenticate the novelty of the receptor. Often, the structures of these latter agents are such that they cannot be related to that of 5-HT or to one another, and they are generally not very useful for subsequent design purposes. Some of the agents examined might even be selective for another 5-HT receptor population and are included in an investigation to show that they do not bind to a newly identified receptor. Because it is known that 5-HT binds at a receptor (i.e., 5-HT represents a unique and naturally occurring template structure, as do other neurotransmitters), there is a temptation to explore derivatives of 5-HT to develop selective serotonergic agents. Although this approach is sometimes successful (and examples will be provided later), it should be realized that the closer a structure approaches that of 5-HT, the greater the likelihood that it will not be selective for one 5-HT receptor population over another; after all, 5-HT itself is a nonselective agent. In contrast, if a close structural analog of 5-HT can be made selective for a specific population of 5-HT receptors, there is reasonable likelihood that it will not bind to nonserotonergic receptors because serotonin itself does not display appreciable affinity for such receptors.

Today, several different approaches exist for developing or identifying novel agents. Perhaps the most useful of these is *high-throughput screening* (HTS), for which hundreds or thousands of compounds can be relatively quickly screened in an applicable pharmacological assay. However, the method is predicated on the *a priori* availability of a selective agent(s) or radioligand(s) (or a pharmacological preparation that possesses only one specific population of receptors that is responsive to a nonselective ligand). In other words, useful though the method might be, previously generated information is required for this approach to be most successful. Furthermore, once a "lead" structure has been identified, structural modification is very frequently required to optimize its actions and/or selectivity. Other methods include application of *pharmacophore models* and utilization of *quantitative SAR (QSAR) techniques*. Here, too, these approaches presuppose the availability of ligands, or assay data, to which these techniques can be applied. A more recent approach—*receptor structure-based drug design*, or simply *structure-based drug design*—is the utilization of three-dimensional graphics models of the various serotonin receptors to design agents that will interact with specifically identified amino acid residues thought to be present in a binding pocket. This approach, coupled with site-directed mutagenesis, offers a powerful means for the development of selective agents. To date, however,

not every 5-HT receptor type has been modeled. Furthermore, because of the lack of receptor crystal structures and because of the different modeling techniques being utilized, multiple homology (or *de novo*) models can be described for a given receptor. So, at the moment, this approach is one of trial and error; models are constructed and ligands are designed, synthesized, and evaluated to determine whether they bind. The resulting information is then used to further modify the model; that is, for the most part, this approach is currently focused as much on verification of various receptor models as it is for drug design purposes. Nevertheless, this technique is still quite young and holds great promise for the future.

In the absence of a "lead" structure or "structural template," it seems that the above methods have not brought us any closer to the desired goal: development of selective agents. When developing a selective ligand, it is first necessary to identify a structural template—typically a nonselective structural template—and to then modify its structure in such a manner so as to achieve enhanced selectivity. Thus, this is actually a two-part problem. If it is specifically desired to develop a selective agonist (or partial agonist, or antagonist), this then becomes a more complicated three-part problem, with efficacy forming the third leg of the triad (*vide supra*). This process becomes yet more complex when attempting to develop a new clinical entity because pharmacokinetic, metabolic, and other properties need to be considered as well; this is usually less of a problem or concern when developing pharmacological tools. We have employed several different means for template identification and selectivity enhancement, and two of the most productive have been the "deconstruction–reconstruction–elaboration" approach and application of the "standard series" concept. The former relies on the availability of a known nonselective agent that shows some affinity for a given receptor type. The latter approach is applied when there is no known lead structure. Both will be described below.

It should be noted that the selectivity of serotonergic agents has been a temporal phenomenon *(8)*; that is, as the number of 5-HT receptor types has continued to grow, agents once thought selective for a particular population might eventually be shown to bind at one or more of the newer 5-HT receptor subpopulations. This has required the constant re-examination of "selective" ligands at new receptor types once they are identified. Of course, earlier pharmacological findings also need to be re-evaluated or reinterpreted once a so-called "selective" agent is found to no longer possess the originally claimed selectivity. Sometimes, this can present enormous problems. For example, if a particular pharmacological effect (e.g., contraction of a particular isolated tissue preparation) has been classified as being mediated via a specific population of receptors on the basis that the effect was produced (or antagonized) by a so-called selective agent and then this pharmacological effect is used to classify

newer agents, considerable effort might be required to correct the literature if the agent originally used to classify the effect is subsequently shown to be nonselective or more selective for a different population of receptors. Indeed, the field of serotonin is booby-trapped with pharmacological data that have eventually required re-evaluation as new receptor populations were identified and as newer, more selective agents have been developed. The interested reader is cautioned to rely on the most recent findings to avoid some of the confusion surrounding the older literature.

2. Selected Case Studies

2.1. 5-HT$_2$ Agonists

It should be fairly evident from the foregoing discussion that each of the above general approaches has as its basis the requirement that a certain amount of information be already in hand. What is often required to achieve selectivity is some sort of structural template that can be exploited. Frequently, other than for the structure of 5-HT itself, such a lead structure might be unavailable; in such cases, a structural template must be identified. Our work with serotonin dates back more than 30 yr to the early 1970s; put in proper perspective, these might be considered the doldrum-days of serotonin research. Although serotonin had been seemingly implicated as being involved in various psychiatric and cardiovascular disorders, proper tools (assay methods, chemical entities) were not readily available. In fact, some investigators still considered serotonin a "putative neurotransmitter." As a consequence, little work was being conducted with serotonin. At that time, it was recognized that there existed at least two types of peripheral serotonin receptor (the so-called D- and M-type 5-HT receptors) (reviewed in ref. *9*), but far less was known about central 5-HT receptors. What follows is a discussion of the identification of certain phenylalkylamine derivatives as 5-HT$_2$ ligands (some of this work has been previously reviewed in refs. *10* and *11*); this is an example of where selective ligands pre-existed in the primordial pool but were unrecognized as such because of the lack of appropriate pharmacological methodologies.

Our earliest foray into the world of serotonin was associated with studies aimed at elucidation of the mechanism of action of hallucinogenic agents. Certain classical tryptaminergic hallucinogens, such as *N*,*N*-dimethyltryptamine (DMT; **2** in Fig. 1), its 4-hydroxy and 5-methoxy analogs (psilocin and 5-OMe DMT [**3** and **4** in Fig. 1], respectively), and (+)lysergic acid diethylamide (LSD; **5**), because of their obvious structural similarity to serotonin **(1)** were thought to act via a serotonergic mechanism *(12)*. Despite this supposition, whether hallucinogenic agents behaved as serotonin agonists or antagonists was to remain controversial for the next several decades *(13)*.

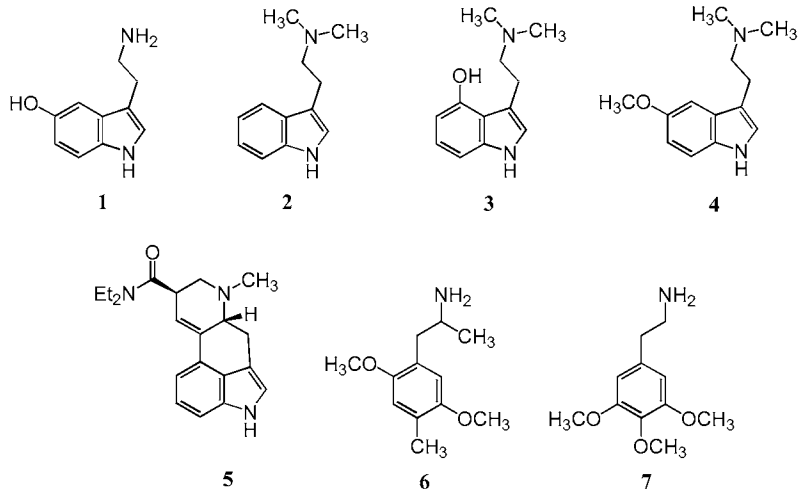
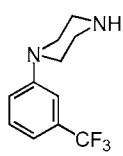


Fig. 1. Chemical structures of serotonin (5-HT, **1**), and several classical hallucinogens including DMT (**2**), psilocin (**3**), 5-OMe DMT (**4**), LSD (**5**), DOM (**6**), and mescaline (**7**).

Other hallucinogenic agents, such as the phenylalkylamines 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM; **6**) and mescaline **(7)**, reportedly produced somewhat similar effects in humans, but were structurally distinct in that they did not possess a tryptaminergic nucleus. Actually, because of their structural similarity to the catecholamine neurotransmitters, they were speculated by some investigators to act via either an adrenergic or dopaminergic mechanism (*see* discussion in ref. *13*). Two of the first questions we set out to answer were as follows: (1) Do phenylalkylamine hallucinogens bind at serotonin receptors? and (2) Can examples of tryptamine and phenylalkylamine hallucinogens produce similar behavioral effects? Because of its popularity at the time and because radioligand-binding assays for serotonin receptors had not yet been developed, we initially utilized an isolated peripheral tissue preparation—rat fundus tissue—that was thought to be activated by serotonin via a single type of serotonin receptor (i.e., a D-type serotonin receptor). Serotonin, even at very low concentrations, contracts fundus tissue in a robust manner that can be reliably and accurately measured. Using this technique, it was demonstrated that examples of both classes of hallucinogen (tryptamine and phenylalkylamine hallucinogens) bind at serotonin receptors (reviewed in ref. *10*). Using a drug discrimination paradigm with rats trained to discriminate either 5-OMe DMT, LSD, DOM or mescaline from the vehicle, we showed that stimulus generalization occurred among this group of agents independent of which was used as the training drug. This was the first indication that these structurally different agents were capable of producing

similar stimulus effects in rats. (The *drug discrimination* paradigm is a behavioral procedure where animals, typically rats, are trained to make one of several possible responses when administered a *training drug*; in tests of *stimulus generalization*, *test agents* are administered to the same animals to determine if they produce an effect similar to that of the training drug [i.e., to determine if substitution occurs]. Where stimulus generalization or substitution occurs, the test drug is considered to be producing stimulus effects similar to those of the training drug.) Next, using rats trained to discriminate DOM from vehicle, we examined a large number of arylalkylamines (i.e., tryptamines and phenylalkylamines), formulated structure–activity relationships, and found that the stimulus potencies of these agents in the rat behavioral paradigm paralleled their human hallucinogenic potencies (in those cases where human data were available in the literature). With evidence from the isolated tissue assays that these compounds might be acting as serotonergic agents, we reasoned that there might exist a relationship between their stimulus or hallucinogenic potency and their affinity for serotonin receptors. Indeed, this was demonstrated to be the case *(10)*. Both the hallucinogenic potency and stimulus potency of various hallucinogens were significantly correlated with their affinity for the serotonin receptors of the rat fundus preparation. It was tentatively concluded that the tryptamine and phenylalkylamine hallucinogens were acting as serotonin agonists. Well and good, but how can the action of centrally acting hallucinogenic substances be adequately, and satisfyingly, explained by their affinity for rat gut serotonin receptors? Apart from coincidence, the only logical conclusion was that the serotonin receptors present in the fundus preparation must be similar to those found in the brain. However, brain serotonin receptors had yet to be investigated in detail.

At about this same time, two novel serotonergic agonists were reported: 1-(3-triflurormethyl-phenyl)piperazine (TFMPP; **8**) by Fuller and colleagues at Eli Lilly *(14)* and 8-hydroxy-2-(*N*,*N*-di-*n*-propylaminotetralin) (8-OH DPAT; **9**) by Hacksell and co-investigators in Sweden *(15)*. We obtained samples of these two agents and administered them to the DOM-trained animals in tests of stimulus generalization. If hallucinogens are acting as serotonin agonists, animals trained to discriminate a hallucinogen should recognize these new serotonin agonists. This did not happen. Substitution (stimulus generalization) normally occurs when two agents produce similar stimulus effects in animals. What the results told us was that even though each of these agents might be serotonin agonists, they produce dissimilar stimulus effects in animals. We eventually trained separate groups of rats to discriminate TFMPP and 8-OH DPAT from the vehicle. Not only did substitution fail to occur when hallucinogens were administered to these animals, but the TFMPP-trained animals failed to recognize 8-OH DPAT, and 8-OH DPAT-trained animals failed to recognize TFMPP. Evidently, the hallucinogens, TFMPP, and 8-OH



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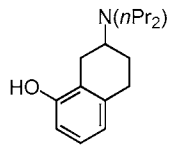

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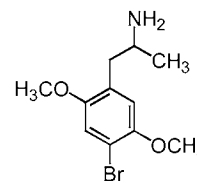

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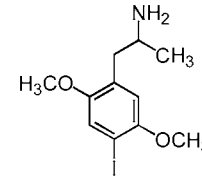

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DPAT—although all being purported serotonin agonists—produced different stimulus effects in animals (reviewed in ref. *16*).

We examined more carefully the stimulus effects of DOM to determine if serotonin receptors were actually involved in mediating its stimulus actions; stimulus antagonism studies were conducted with various neurotransmitter antagonists, including serotonin antagonists. In tests of stimulus antagonism, pretreatment of the animals with an antagonist that specifically blocks the neurotransmitter mechanism underlying the actions of an agonist will cause the animals to make a different response than that typically seen following administration of an agonist. The only antagonists that consistently attenuated the DOM stimulus were serotonin antagonists. However, not all serotonin antagonists were equally effective. Certain serotonin antagonists had little to no effect on the DOM stimulus. Similar studies were conducted with the TFMPP- and 8-OH DPAT-trained animals. Clearly, there were notable differences. It was apparent that these serotonin agonists were producing distinct stimulus effects in animals; because stimulus effects are centrally mediated, the only conclusion that could be reached, other than that these agents activated yet to be identified receptor types, was that there must exist more than one type of serotonin receptor in the brain.

Using radioligand-binding methodology, two populations of brain 5-HT receptors were proposed by Peroutka and Snyder in the fall of 1979: 5-HT_1 receptors and 5-HT_2 receptors *(17)*. A third type of peripheral receptor, the M-type serotonin receptor, was eventually found in the brain and named 5-HT_3 receptors (reviewed in refs. *9* and *18*). The possibility existed, then, that the three types of agents we had been examining (i.e., DOM, TFMPP, 8-OH DPAT) might be representative ligands for these three receptor types. Although this was not to be the case, we had no way of knowing it at the time. We attempted to sort out the actions of these agents.

Several landmark studies were reported in the early 1980s that help to clarify the situation. Pedigo et al. *(19)* found that 5-HT_1 receptors could be further subcategorized as 5-HT_{1A} and 5-HT_{1B} receptors, ketanserin and pirenpirone were identified as the first useful 5-HT_2-selective antagonists *(20)*, and it was proposed that 8-OH DPAT was a 5-HT_{1A} agonist (reviewed in ref. *21*). Tritiated ketanserin

and tritiated 8-OH DPAT were subsequently introduced as radioligands to label brain 5-HT_2 and 5-HT_{1A} receptors, respectively, and it became possible to measure the affinity of hallucinogens at 5-HT_2 and 5-HT_{1A} receptors. Although certain of the tryptaminergic hallucinogens displayed affinity both for 5-HT_{1A} and 5-HT_2 receptors, the phenylalkylamine hallucinogens such as DOM displayed high affinity for 5-HT_2 receptors and lacked affinity for 5-HT_{1A} receptors. In addition, we found that the stimulus actions of DOM were potently antagonized by the then novel 5-HT_2 antagonists ketanserin and pirenpirone, indicating that DOM was acting as a 5-HT_2 agonist *(22)*. We further demonstrated that the rat brain 5-HT_2 receptor affinity of hallucinogens was significantly correlated with their stimulus potency using rats trained to discriminate DOM from the vehicle *(23)*, and we later found a similar correlation between human 5-HT_2 receptor affinity and human hallucinogenic potency *(24)*. It seemed that we had identified the first 5-HT_2-selective agonist: DOM **(6)**.

In the same series of studies, we had provided mechanistic insight to the actions of hallucinogens and had identified what appeared to be a novel 5-HT_2 receptor structural template. The phenylalkylamine template was used to investigate structure–activity and structure–affinity relationships. The availability of these compounds also permitted pharmacophoric investigations and application of QSAR methods *(25,26)*. The bromo and iodo counterparts of DOM (i.e., DOB and DOI [**10** and **11**], respectively) were identified as possessing structural features deemed optimal for 5-HT_2 action. Interestingly, these latter two agents were the most potent phenylalkylamine agonists in contracting rat fundus tissue. The low nanomolar affinities of these latter two ligands in radioligand-binding studies using brain homogenates led to the introduction of [^{3}H]DOB and [^{125}I]DOI as 5-HT_2 receptor agonist radioligands for use in binding and autoradiographic investigations *(27,28)*. Subsequently, [^{123}I]DOI and R(−)[^{123}I]DOI were developed for SPECT imaging studies and R(−)[^{76}Br]DOB was examined for autoradiographic studies *(29,30)*.

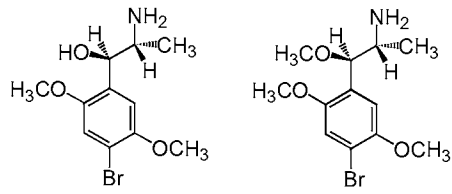
Our enthusiasm was seriously dampened following several very disconcerting literature reports. A third population of 5-HT_1 receptors (termed 5-HT_{1C} receptors at the time) was reported *(31)*, and shortly thereafter we found that DOM and related phenylalkylamine hallucinogens bind with high affinity at this new receptor population. It was also reported that brain 5-HT_2 receptors were not identical with rat fundus receptors *(20)*. This latter finding questioned the significance of our earlier correlation between stimulus potency of hallucinogens and receptor affinity for rat fundus serotonin receptors. Subsequently (and fortunately for us), on the basis of additional pharmacological investigation, 5-HT_{1C} receptors were identified as being a member of the 5-HT_2 family; the original 5-HT_2 receptors were renamed 5-HT_{2A} receptors, and 5-HT_{1C} receptors were renamed 5-HT_{2C} receptors *(1)*. Rat fundus receptors, also later found to

belong to the 5-HT_2 family, were initially termed 5-HT_{2F} receptors *(32)* and have been since renamed 5-HT_{2B} receptors *(1)*. So, nearly 20 yr after our initial studies, the circle had been closed. Agents such as DOM, DOB, and DOI are now recognized as being rather selective 5-HT_2 agonists, but they show almost no selectivity for the three 5-HT_2 subpopulations; that is, these agents bind with nearly comparable affinity at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors *(33)*. Our initial correlation between fundus (now 5-HT_{2B}) serotonin receptor affinity and hallucinogenic potency might have been fortuitous, but now could be satisfactorily explained; for the series of agents we had examined, 5-HT_{2B} receptor affinity correlated significantly with brain 5-HT_{2A} receptor affinity *(33)*. Phenylalkylamines are widely used today as 5-HT_2 agonists; actually, depending on the assay system being used, the agents are high-efficacy partial agonists with the R(−) isomers being more effective than their S(+) enantiomers. These compounds have also served as the basis for further ligand development; for example, with the proper aryl substituents appended, DOM-related structures have been shown to display 5-HT_2 antagonist action (*see* Section 2.2).

In effect, a structural template, the aryl-substituted phenylalkylamines, was identified where no structural template had previously existed. Some of the agents, such as DOM, were known but it remained for them to be identified as being selective until the necessary pharmacological techniques were available—and, indeed, for several specific receptor populations to be identified and classified. However, normally, this is not the manner in which selective agents are developed.

DOM **(6)**, DOB **(10)**, DOI **(11)**, and certain related agents are hallucinogenic in humans. Our investigations led to the "5-HT_{2A} hypothesis of hallucinogenic drug action" for the classical hallucinogens *(11,23)*. Could this information be put to use for the development of agents with possible therapeutic benefit? Recently, it was demonstrated that activation of 5-HT_{2A} serotonin receptors represents a novel approach to lowering intraocular pressure—an approach that might be useful in the treatment of glaucoma *(34)*. A local ocular site of action seems to be sufficient for achieving decreased intraocular pressure in a primate model of ocular hypertension. Because 5-HT_{2A} agonists might also produce undesirable central effects should sufficient quantities enter the brain, attempts were made to identify 5-HT_2 agonists with reduced propensity to penetrate the blood–brain barrier. In this manner, a 5-HT_{2A} agonist that does not readily penetrate the blood–brain barrier should be effective following local ocular application, and central side effects might be minimized.

Two general strategies are typically employed to reduce the ability of a compound to enter the brain: quaternization of an amine and introduction of polar substituents. Because structure–affinity studies had already shown that quaternization essentially abolishes the affinity of phenylalkylamines for



5-HT_2 receptors *(35)*, quaternization was not a viable approach. Limited to the introduction of a polar substituent, it was again necessary to consult the structure–affinity results to determine what and where a polar substituent might be tolerated. 1-(4-Bromo-2,5-dimethoxyphenyl)-2-aminopropan-1-ol **(12)**, an analog of the 5-HT_2 agonist DOB **(10)** bearing a benzylic hydroxyl group, was identified as a candidate structure; the hydroxyl group might sufficiently lower lipophilicity to impede penetration of the blood–brain barrier relative to DOB *(36)*. However, the effect on 5-HT_{2A} affinity of such a structural modification was unknown. Because of the presence of two chiral centers, four optical isomers are possible; all four were prepared and evaluated. Of the four optical isomers, the 1*R*,2*R*-isomer **12** (5-HT_{2A}, K_i = 1.3 n*M*) was found to bind at 5-HT_{2A} receptors with an affinity similar to that of *R*(−)DOB (K_i = 0.5 n*M*). Like *R*(−)DOB, **12** behaved as a partial agonist. In an in vivo test of central action (i.e., stimulus generalization with rats as subjects), **12** was >15 times less potent than *R*(−)DOB, suggesting that it might not as readily enter the brain *(36)*. In collaboration with Alcon Laboratories, intraocular administration of **12** to ocular hypertensive monkeys was shown to effectively reduce intraocular pressure *(36)*. Given the route of administration (i.e., topical) and concentrations necessary to reduce intraocular pressure, compounds such as **12** should demonstrate minimal central effects at potentially useful therapeutic doses and offer useful leads for further development. Interestingly, O-methylation of **12** (i.e., **13** in Fig. 2; 5-HT_{2A}, K_i = 0.7 n*M*) resulted in an agent that behaved as a full 5-HT_{2A} agonist, indicating that certain oxygen functions at the benzylic position of phenylalkylamines are not only tolerated, but that they can also influence efficacy *(36)*.

2.2. 5-HT_2 Antagonists

The dopaminergic agent spiperone (**14** in Fig. 3) was used to initially identify and distinguish 5-HT_1 from 5-HT_2 receptors *(17)*. It was the first recognized 5-HT_2 antagonist, and [^{3}H]spiperone was used for several years to label 5-HT_2 receptors in radioligand-binding studies. Later, it was demonstrated that spiperone binds at a subpopulation of 5-HT_1 receptors (i.e., 5-HT_{1A} receptors) *(19)*. One of the most significant early advances in 5-HT_2 research

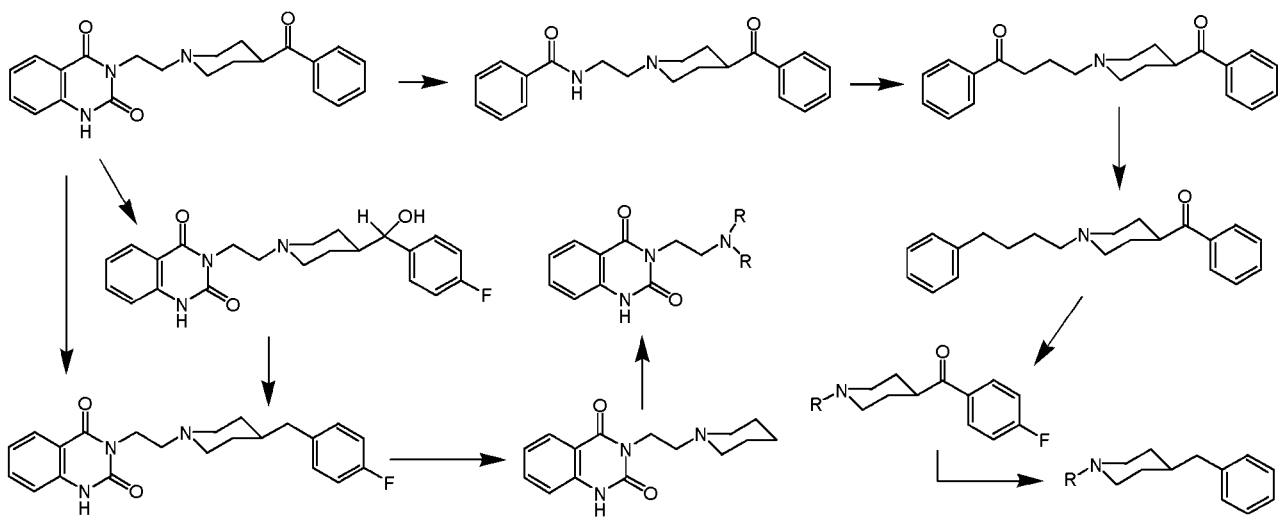


Fig. 2. Examples of some partial structures of ketanserin (**15**) examined to determine the minimal structural requirements for binding.

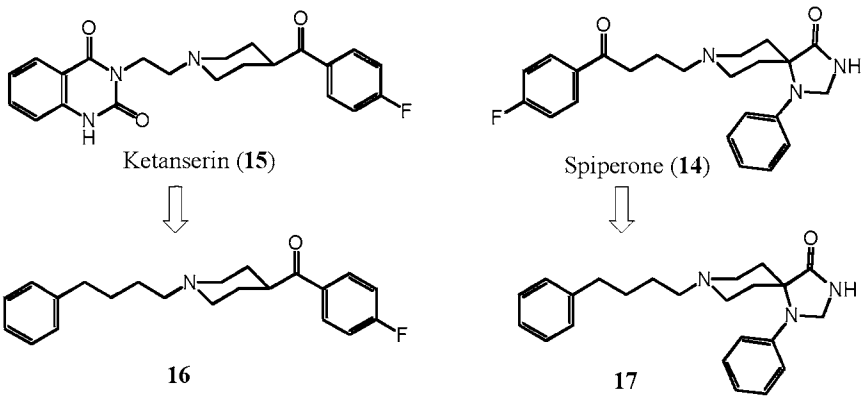


Fig. 3. Ketanserin (**15**), which binds at 5-HT_{2A} receptors with high affinity (K_i = 3.5 n*M*) and only 14-fold selectivity over 5-HT_{2C} receptors, can be abbreviated to **16**, which binds with similar 5-HT_{2A} affinity but enhanced (120-fold) selectivity. Spiperone (**14**), which binds with similar 5-HT_{2A} affinity (K_i = 2 n*M*) can be abbreviated to **17** (5-HT_{2A}; K_i = 4 n*M*); both of these compounds display 1000-fold 5-HT_{2A} vs 5-HT_{2C} selectivity. Although there is no assurance that **16** and **17** bind in a similar fashion, if they do it would appear that the spiro ring portion of **17** contributes to its greater 5-HT_{2A} selectivity.

was the identification of ketanserin **(15)** as a 5-HT_2 antagonist by investigators at Janssen Pharmaceutica *(20)*. Unlike spiperone, ketanserin displayed low affinity for 5-HT_{1A} and dopamine D_2 receptors. However, unlike ketanserin, spiperone enjoyed the advantage of reduced affinity for 5-HT_{2C} vs 5-HT_{2A} receptors; that is, spiperone binds with high affinity at 5-HT_{2A}, 5-HT_{1A}, and dopamine D_2 receptors and with 500- to 1000-fold selectivity for 5-HT_{2A} vs 5-HT_{2C} receptors. In contrast, ketanserin displays reduced affinity for 5-HT_{1A} and dopamine D_2 receptors, but displays little selectivity for 5-HT_{2A} vs 5-HT_{2C} receptors. Because of the diverse and complicated pharmacology associated with 5-HT_2 receptors, interest has focused over the past decade or so on the development of antagonists with selectivity for 5-HT_{2A} over 5-HT_{2C} receptors, and vice versa.

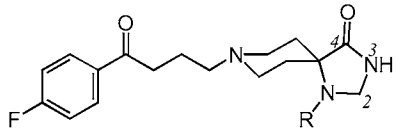
We were intrigued by spiperone because it was the first agent to show substantial 5-HT_{2A} vs 5-HT_{2C} selectivity, and at the time our studies were initiated, spiperone was the only agent to show such selectivity. Might it be possible to modify the structure of spiperone so as to retain 5-HT_{2A} affinity while reducing or eliminating 5-HT_{1A} and dopamine D_2 affinity? We tackled this problem by employing what we have termed a "deconstruction–reconstruction–elaboration" approach; that is, we "deconstructed" the structures of ketanserin **(15)** and spiperone **(14)** to determine their minimal structural requirements for binding. Next,

we reintroduced various structural features (i.e., the "reconstruction" step) in a stepwise manner to determine how various substituents influenced affinity and selectivity (some sample "partial structures" for ketanserin are shown in Fig. 2). In so doing, we found that the cyclic nature of ketanserin actually detracted from selectivity, that the azaspiro portion of spiperone was important to selectivity, and that a related structure-type could be derived from each (i.e., compounds **16** and **17**; Fig. 3) *(37,38)*.

Comparing the abbreviated structures **16** and **17** derived from ketanserin and spiperone, respectively, it was found that **16** binds with similar 5-HT_{2A} affinity but greater 5-HT_{2A} vs 5-HT_{2C} selectivity than ketanserin **(15)** *(37)*. Furthermore, comparing **16** with **17**, it was apparent that the presence of the spiro ring system makes a substantial contribution to 5-HT_{2A} selectivity.

In the "elaboration" step, we utilized the information gained from the above studies and further modified structure **17** by introducing substituents not found in spiperone *(38)* and by examining conformationally constrained analogs *(39)*. It was found that a small alkyl substituent at the N_3-position (*see* structure **18**) was tolerated, but that as its size increased, 5-HT_{2A} receptor affinity and, in particular, 5-HT_{2A} selectivity decreased. Removal of the C_4 carbonyl group decreased 5-HT_{2A} affinity by 100-fold; replacement of the N_3 nitrogen atom by a methylene group and other structural changes to the five-membered ring decreased either 5-HT_{2A} receptor affinity and/or selectivity *(38)*. Replacement of the N_1-phenyl group of **18** (where R = Ph) by a hydrogen atom resulted in a compound that lacked affinity for any of the receptors being examined. This was a major clue; apparently, the phenyl group is a key determinant of 5-HT_{2A}, 5-HT_{1A}, and dopamine D_2 receptor affinity. Modification of the N_1-substituent could conceivably result in compounds that display different affinities for the various receptors, unless each receptor had identical binding requirements associated with this position. Continued examination of N_1-substituents culminated in the identification of *KML-010* **(19)** as a novel 5-HT_{2A} antagonist. *KML-010* binds with high affinity at 5-HT_{2A} receptors (K_i = 20 n*M*), lacks affinity for 5-HT_{1A} and 5-HT_{2C} receptors (i.e., K_i > 10,000 n*M*), and binds at dopamine D_2 receptors with >400-fold lower affinity than spiperone *(38)*.

The deconstruction–reconstruction–elaboration approach was successful in identifying those structural features that contribute to the 5-HT_{2A} vs 5-HT_{2C} receptor affinity and selectivity of spiperone and to the lack of 5-HT_{2A} selectivity of ketanserin. In addition, during the elaboration process, *KML-010* was identified as a selective 5-HT_{2A} antagonist. In summary, the concepts behind this approach are as follows: (1) identify the minimal structural requirements for the binding of a nonselective agent, (2) reintroduce substituents in a stepwise fashion to determine their influence on affinity and selectivity, and



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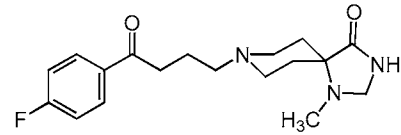

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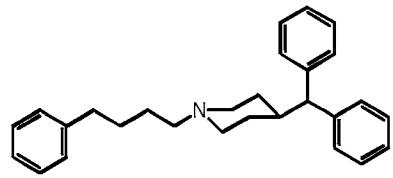

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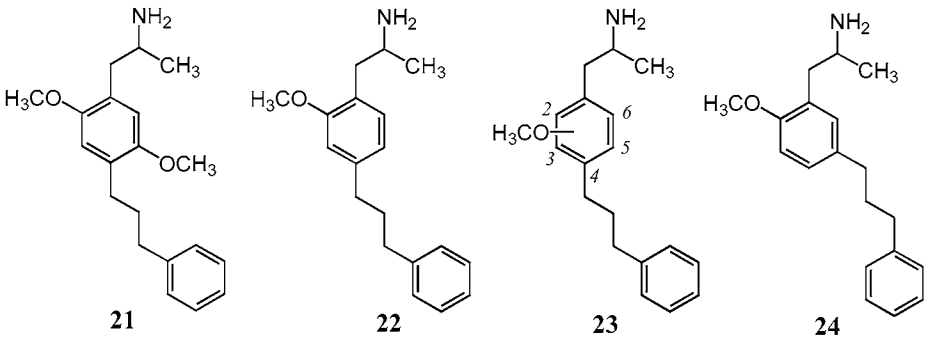
(3) use this information to synthesize an optimized structure and then utilize this structure for subsequent structural modification.

Elaboration of certain of the ketanserin partial structures (*see* Fig. 2) also provided some useful insight regarding the three-dimensional structure of 5-HT_{2A} receptors. On the basis of molecular modeling studies, a proposed mode of docking of ketanserin to 5-HT_{2A} receptors had indicated that a portion of the molecule was in the vicinity of a TM6 phenylalanine (F340) moiety, even though the amino acid might not directly participate in binding. While probing a potential region of bulk tolerance, several benzylic-substituted ketanserin partial structures were prepared, including **20** (K_i = 213 n*M*). Even with their reduced affinity, these compounds retained 5-HT_{2A} antagonist character. It was reasoned that steric hindrance between the phenyl group of **20** and F340 might contribute to its low affinity. It was further reasoned that replacing this amino acid with a less bulky leucine might allow the receptor to better accommodate the benzylic substituents. Indeed, **20** displayed nearly 70-fold enhanced affinity (K_i = 3.4 n*M*) at the F340L mutant receptor than at the wild-type receptor *(40)*. Similar results were obtained with other partial structures bearing bulky groups on the benzylic carbon atom. This is probably the first instance where bulk tolerance was introduced in a G protein-coupled receptor by site-directed mutagenesis and provides some evidence that F340 is located near the ketanserin-binding pocket *(40)*.

In the course of our investigations with the phenylalkylamine 5-HT_2 agonists (Section 2.1), we investigated the structure–affinity relationship (SAFIR) for binding. Although the 2,5-dimethoxy pattern was not essential for the binding of these compounds at 5-HT_2 receptors, it appeared to be optimal. It was also found that lipophilic substituents at the ring 4-position played a major role in affinity modulation and that affinity increased as lipophilicity increased. For



example, increasing the alkyl chain length from one to eight carbon atoms produced a consistent increase in affinity *(26)*. Interestingly, as determined using functional assays, once the alkyl chain was longer than an *n*-propyl substituent, there was a decline, and eventually a loss, of agonist action. Indeed, some of the longer-chain analogs, or those bearing a bulky substituent (e.g., 4-*tertiary*-butyl), actually showed the 5-HT_2 antagonist character. One compound that captured our attention was **21**; **21** (5-HT_{2A} K_i = 30 n*M*) displayed good affinity and 5-HT_2 antagonist properties in several assay systems. What made these types of compound interesting is that (1) we had previously proposed that agonists and antagonists bind differently at 5-HT_2 receptors, (2) phenylalkylamines (e.g., DOM, DOB, DOI) generally behave as 5-HT_2 agonists, and (3) compounds such as **21** are phenylalkylamines, but are also antagonists. In other words, do the phenylalkylamine antagonists bind more like the phenylalkylamine agonists or like nonphenylalkylamine antagonists? If the phenylalkylamine antagonists bind differently than phenylalkylamine agonists, there is a good possibility that their structure–activity relationships will differ from that of the agonists; if this is true, the affinity/activity of the antagonist phenylalkylamines would probably not yet have been optimized. For phenylalkylamines with agonist action, the 2,5-dimethoxy substitution pattern is optimal for affinity, and removal of one of the methoxy groups decreases affinity substantially. Removal of the 5-methoxy group of **21** had little effect on 5-HT_{2A} receptor affinity (i.e., **22**, K_i = 8 n*M*) *(40)*. In fact, the 2,3-dimethoxy, 2,6-dimethoxy, and 3,5-dimethoxy analogs **(23)** (K_i = 3–4 n*M*) all displayed 10-fold higher affinity than **21** *(41)*. Various other 4-position substituents were examined, as was translocation of the 4-position substituents to the 5-position *(42)*. Compound **24** (K_i = 13 n*M*) was identified as another type of novel 5-HT_{2A} antagonist (PI hydrolysis) *(42)*. It would appear, then, depending on what ring substituents are present, that phenylalkylamines can behave as 5-HT_2 agonists, partial agonists, or antagonists and that phenylalkylamine agonists and antagonists probably bind somewhat differently because of apparent differences in their SAFIRs. Although none of the phenylalkylamine

antagonists displayed >10-fold selectivity for 5-HT_{2A} vs 5-HT_{2C} receptors, as a class these ligands remain to be fully exploited.

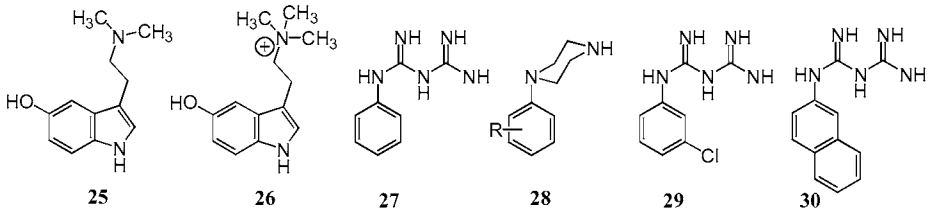
2.3. 5-HT_3 Agonists

The 5-HT_3 receptors, in the form of M-type serotonin receptors, represent one of the earliest investigated populations of 5-HT receptors. However, they were not identified in the mammalian brain until the late 1980s. Nevertheless, a plethora of very high-affinity and selective 5-HT_3 antagonists quickly became available (e.g., ref. *43*). What was lacking for the longest time was a useful 5-HT_3 agonist.

How might one go about designing a 5-HT_3 agonist? Initially, the only available agonist was 5-HT itself. Obviously, 5-HT lacks selectivity for 5-HT_3 receptors; furthermore, its affinity (K_i ~ 1000 n*M*) for 5-HT_3 receptors is not particularly high. Other than for 5-HT_3 receptors, all other populations of 5-HT receptors belong to the G protein–coupled receptor superfamily *(1,2)*. Studies that we had conducted at the time suggested that 5-HT receptors typically did not tolerate quaternary amines. 5-HT_3 receptors, being ion channel receptors rather than G protein-coupled receptors, might be able to accommodate quaternary amines much in the same way that certain other ion channel receptors accommodate quaternary amines. Our first investigation included a comparison of serotonin **(1)**, *N*,*N*-dimethylserotonin or bufotenine **(25)**, and its simplest quaternary amine counterpart, 5-HTQ **(26)**. N,N-Dimethylation of 5-HT (K_i = 750 n*M*) to bufotenine (**25**; K_i = 280 n*M*) was tolerated and resulted in several-fold increased affinity; further methylation to the quaternary amine 5-HTQ (**26**; K_i = 75 n*M*) not only resulted in a more selective compound, but the affinity of 5-HTQ was about 10-fold higher than that of 5-HT itself *(44,45)*. Before we could publish our findings, others independently showed that **26** is a 5-HT_3 agonist in a peripheral (i.e., M-receptor) preparation *(46)*. Here is one instance, then, where a close structural relative of serotonin does indeed show selectivity for one 5-HT receptor population over the others.

5-HTQ might be considered as one of the first serotonergic agents specifically designed to interact in a selective manner with a given 5-HT receptor population. 5-HTQ continues to be used as a 5-HT_3 agonist. However, its utility is restricted by the very feature that makes it selective for 5-HT_3 receptors; that is, being a quaternary amine, 5-HTQ would not be expected to readily penetrate the blood–brain barrier and, as a consequence, it is only of limited utility as a centrally acting 5-HT_3 agonist when administered via systemic routes. On the other hand, this property makes it a useful tool for examining the peripheral actions of a 5-HT_3 agonist following systemic administration.

At about the same time that 5-HTQ was identified, it was reported that phenylbiguanide **(27)** represents a novel type of 5-HT_3 agonist *(47)*. Arylpiperazines **(28)** were also found to bind at 5-HT_3 receptors *(48)*, and some were

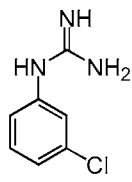


25 **26** **27** **28** **29** **30**

subsequently shown to behave as 5-HT$_3$ agonists (reviewed in ref. *49*). In general, phenylbiguanide was a selective but low-affinity (K_i ~ 1000 n*M*) compound, whereas the arylpiperazines were much higher-affinity but less selective 5-HT$_3$ ligands. We undertook an investigation to determine what accounted for the affinity and selectivity, or lack there of, of compounds such as **27** and **28**. Because 3-chlorophenylpiperazine and the benz-fused naphthylpiperazine displayed higher 5-HT$_3$ receptor affinity than phenylpiperazine itself, we prepared and examined the 3-chloro and benz-fused analogs of phenylbiguanide (i.e., **29** and **30**, respectively). Although arylpiperazines and arylbiguanides might bind in a comparable manner at 5-HT$_3$ receptors (i.e., this remains to be determined), the notion that they might provided a working hypothesis for design purposes. Both compounds (i.e., **29** and **30**; K_i ~ 20–30 n*M*) were found to bind with significantly higher affinity than phenylbiguanide (K_i = 1200 n*M*) *(50)*. The 3-chloro compound **29** (subsequently referred to as *m*CPBG) was also independently reported by Kilpatrick and co-workers *(51)*.

Although the naphthylbiguanide **(30)** has not seen much pharmacological application, the 3-chloro derivative *m*CPBG **(29)** has become a popular 5-HT$_3$ agonist despite the fact that it is not very brain penetrant. What accounts for the 5-HT$_3$ receptor affinity of *m*CPBG? Can the structure be modified to increase its lipophilicity and, thus, its ability to penetrate the blood–brain barrier? This is another instance where application of the deconstruction–reconstruction–elaboration approach should be useful.

Without going into detail, suffice it to say that examination of various "partial structures" of *m*CPBG revealed that optimal 5-HT$_3$ receptor affinity was associated with the intact and unaltered molecule *(50)*. Interestingly, however, the entire biguanide moiety was found unnecessary for binding or agonist action. For example, *m*CPG (MD-354, **31**; K_i = 32 n*M*), an example of a novel class of 5-HT$_3$ ligands—the arylguanidines—binds with nearly the same affinity as *m*CPBG (K_i = 18 n*M*) and displayed agonist action in several different functional assays *(50,52)*. During the "elaboration" step, analogs of **31** and **32** were prepared, QSAR studies were conducted, and conformationally constrained analogs were investigated *(54,55)*. Introduction of additional chloro substitutents resulted in enhanced affinity. The highest-affinity derivative identified was



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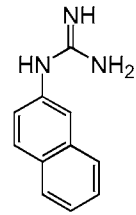

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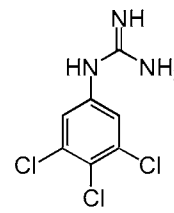

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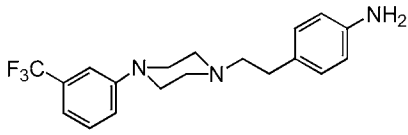
3,4,5-trichlorophenylguanidine (YC-30, **33**; $K_i = 0.7$ n*M*) *(53,54)*, which displayed nearly 50 times the affinity of **31** and >3000 times the affinity of the parent structure, phenylguanidine.

Recently, Dukat and co-workers *(56)* have demonstrated that *m*CPG **(31)** binds at α_{2B}-adrenergic receptors ($K_i = 20$ n*M*) with an affinity comparable to its affinity for 5-HT_3 receptors. It would seem that the deconstruction–reconstruction–elaboration approach could once again be applied to divorce 5-HT_3 from α-adrenergic properties.

2.4. 5-HT_{1A}-Selective Agents

de Stevens *(57)* categorizes drug discovery approaches as being of two general types: *structured research* and *serendipity*. Indeed, one of the most fruitful, if unreliable, factors in drug design is serendipity. During the early 1980s, four populations of 5-HT receptors were recognized: 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, and 5-HT_3. It was assumed that if an agent displayed affinity for one of these receptor types and did not bind at the others, it must be selective. A few arylpiperazines had been examined and found to display affinity for 5-HT_{1B} receptors, but lower affinity for 5-HT_{1A} and 5-HT_2 receptors (their affinities for 5-HT_3 receptors had not yet been investigated); thus, it nearly became dogma at the time that arylpiperazines were 5-HT_{1B}-selective agents. This concept was to cause a temporary setback in 5-HT receptor research. However, in the early 1980s, Shih and co-workers reported at a symposium that arylpiperazine **34** (PAPP), an N_4-substituted analog of TFMPP **(8)**, binds at 5-HT_{1A} receptors; the details of their findings were later described more completely *(58)*. The finding, although unrecognized and perhaps unappreciated at the time, would eventually alter the course of 5-HT_{1A} research. Arylpiperazines could no longer be considered 5-HT_{1B}-selective agents!

We were intrigued with this observation and began an investigation of the binding of various arylpiperazines at 5-HT_{1A} receptors. Simple arylpiperazines (i.e., those either not bearing an N_4-substituent or those that possessed only a small N_4-substituent) displayed low to modest affinity for multiple populations of 5-HT receptors, whereas certain other arylpiperazines, depending on the nature



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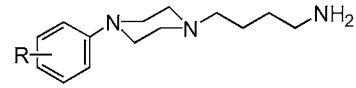

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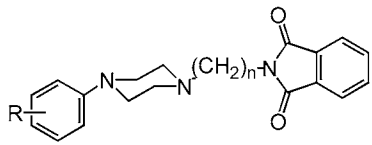

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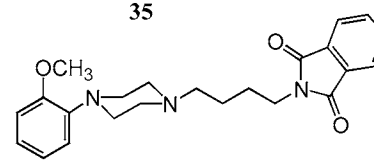

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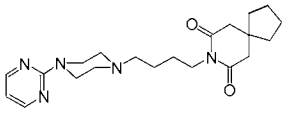
of their N_4-substituents, displayed enhanced 5-HT_{1A} receptor affinity *(59)*. The latter, which we designated "long-chain arylpiperazines" (i.e., LCAPs), depending on the nature of their N_4-substituents, also displayed enhanced selectivity for 5-HT_{1A} receptors. We reasoned that if a basic amine (as found in **34**) was a contributor to 5-HT_{1A} affinity, compounds such as **35** with a more basic amine (and with perhaps a slightly shorter or longer chain length) might represent 5-HT_{1A} ligands. We prepared derivatives of compound **35** and found them to lack affinity for 5-HT_{1A} receptors. In the course of varying the alkyl chain length of **35**, synthetic intermediates—phthaloyl-protected intermediates **36**—were prepared. One of these intermediates (NAN-190, **37**; K_i = 0.6 n*M*) was submitted for binding and, unexpectedly, displayed high affinity and appreciable selectivity for 5-HT_{1A} receptors *(59–61)*. Hence, NAN-190 represented a serendipitous discovery and opened a door for additional studies.

At about the same time, several arylpiperazines were being independently developed by the pharmaceutical industry as antianxiety agents and/or antidepressants. In fact, some had been originally synthesized years earlier as possible antipsychotic agents. Subsequent studies showed that some of these agents displayed affinity for 5-HT_{1A} receptors and it was eventually established that they likely produced their anxiolytic actions via a 5-HT_{1A} agonist or partial agonist mechanism. The first of these agents to be used clinically was buspirone **(38)**. The agent 8-OH DPAT **(9)** had already been described as a selective 5-HT_{1A} agonist and certain of the LCAPs were also recognized now as being either 5-HT_{1A} agonists or partial agonists. A problem that plagued 5-HT_{1A} research for the longest time was lack of a 5-HT_{1A} antagonist. During the course of our studies, the LCAP NAN-190 **(37)** was found to act as a 5-HT_{1A} antagonist *(60,61)*. Thus, not only was the binding of NAN-190 a serendipitous discovery, its action as a 5-HT_{1A} antagonist was also fortuitous. Bristol-Meyers independently developed BMY 7378 **(39)**, which also displayed 5-HT_{1A} antagonist actions. These two compounds, NAN-190 and BMY 7378, represented the first useful 5-HT_{1A} antagonists; however, depending on the assay system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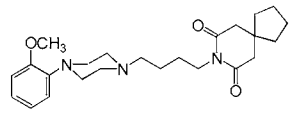
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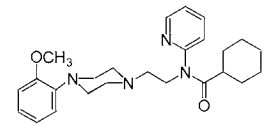
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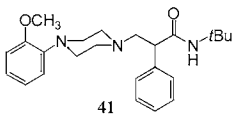
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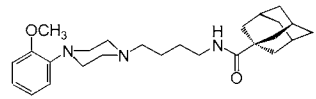
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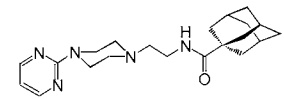
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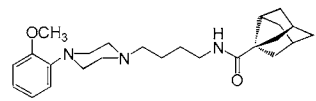
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employed, both compounds were eventually found to be low-efficacy (intrinsic activity = 0.1–0.3) partial agonists or postsynaptic antagonists with some presynaptic agonist action (reviewed in ref. *62*). Studies conducted with [^{3}H]NAN-190 also indicated that NAN-190 might be a very low-efficacy partial agonist but that in various functional studies, its efficacy was simply too low to be routinely observed *(63)*. Later, Wyeth-Ayerst, by variation of the linker moiety of the LCAPs, reported the first 5-HT$_{1A}$ "silent antagonists" WAY-100635 **(40)** and WAY-100135 **(41)** (reviewed in refs. *64* and *65*).

We continued our work on the SAFIRs of arylpiperazine analogs as 5-HT$_{1A}$ ligands and found that many displayed low nanomolar to subnanomolar affinity. Structure–affinity investigations led us to describe structural features important for 5-HT$_{1A}$ binding; these are summarized in Fig. 4 *(62,64)*. This paved the way for the development of a large number of 5-HT$_{1A}$ ligands. Certain derivatives, however, depending primarily on the nature of their N$_4$-substitiuent (Terminus group and Spacer length), also displayed affinity for 5-HT$_2$, dopamine D$_2$ and/or α_1-adrenergic receptors. NAN-190, for example, binds nearly equally well at 5-HT$_{1A}$ and α_1-adrenergic receptors. Taking advantage of the structure–affinity findings shown in Fig. 4 and structural requirements for adrenergic receptor binding, we identified RK-153 (**42**; K_i = 0.4 n*M*) *(66)*, which displayed >160-fold selectivity for 5-HT$_{1A}$ vs α_1-adrenergic receptors and retained 5-HT$_{1A}$ antagonist character; unfortunately, RK-153 displayed enhanced affinity for dopamine D$_2$ receptors. This same compound was independently reported by Wyeth-Ayerst in the same year. Subsequently, Wyeth-Ayerst *(67)* reported adatanserin **(43)**, a 5-HT$_{1A}$ partial agonist/5-HT$_2$ antagonist with reduced affinity for α-adrenergic and dopamine D$_2$ receptors, as a potential antidepressant. Continued work with

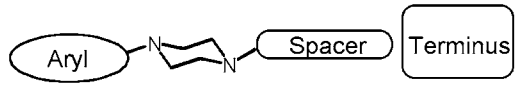
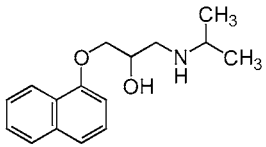


Fig. 4. Structural features important for the binding of arylpiperazines at 5-HT_{1A} receptors *(62,64)*. The Aryl group is a phenyl, substituted phenyl, heteroaryl, or substituted heteroaryl group, whereas the Spacer is usually a branched or unbranched carbon chain of two to five atoms in length (and might contain a heteroatom in place of one of the methylene groups). The Terminus is typically an amide, reverse amide, imide, or aryl group; the terminus function can be quite large, as this area is probably associated with a region of bulk tolerance.

the Terminus portion of RK-153 ultimately resulted in NBUMP (**44**; 5-HT_{1A} $K_i = 0.1$ n*M*) *(68)*—one of the highest affinity ligands ever reported for 5-HT_{1A} receptors. Although NBUMP **(44)** displayed 460-fold selectivity for 5-HT_{1A} vs α_1-adrenergic receptors and 260-fold selectivity over dopamine D_2 receptors, it was found to be a partial agonist with an intrinsic activity of approx 0.4; that is, it behaved both as an agonist and as an antagonist in an adenylate cyclase assay. Evidently, modification of the Terminus moiety influences both selectivity and efficacy *(62)*.

We terminated our work with 5-HT_{1A} ligands in the very early 1990s, but not before identifying what is probably a region of bulk tolerance associated with the Terminus group. It was determined that this portion of the molecule could be quite bulky. Considerable variation is also allowed in the Spacer and Aryl portions of the LCAPs. The LCAPs represent a versatile structural template *(62,69)* that can lead not only to 5-HT_{1A} ligands but also, by variation of the structural features shown in Fig. 4, to ligands for other receptor types (e.g., 5-HT_{2A}, α-adrenergic, dopamine D_2, σ receptors). Over the ensuing years, thousands of LCAPs were prepared and examined by various investigators at 5-HT_{1A} (and other) receptors, and more continue to be reported.

Another example of the deconstruction–reconstruction–elaboration approach involves the use of propranolol **(45)** as a 5-HT_{1A} antagonist. In the early 1980s, several groups of investigators found that the β-adrenergic antagonists propranolol and pindolol bind at 5-HT_{1A} receptors and behave as 5-HT_{1A} antagonists; *see* Pierson et al. *(70)* for further discussion. These agents were only of limited utility for in vivo studies because they displayed higher affinity for β-adrenergic receptors than for 5-HT_{1A} receptors. For example, propranolol **(45)** binds with nearly 50-fold lower affinity at 5-HT_{1A} receptors ($K_i = 90$ n*M*) than at β-adrenergic ($K_i = 2$ n*M*) receptors; in addition, propranolol binds at 5-HT_{1B} receptors ($K_i = 50$ n*M*) *(70)*. Nevertheless, propranolol provided a novel lead structure that could be exploited. Prior investigations had shown that the alkyl hydroxyl group and *N-iso*propyl moiety were optimal features for the



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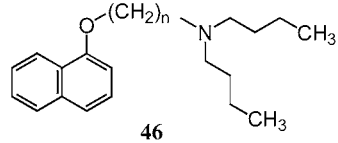

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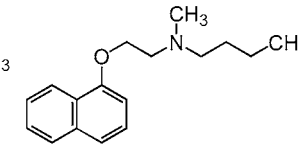

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β-adrenergic actions of propranolol. We *deconstructed* the structure of propranolol and were not surprised to find that removal of these two features decreased β-adrenergic affinity; interestingly, these functional groups were not required for binding at 5-HT_{1A} receptors. The simple elimination of the hydroxyl and isopropyl groups already enhanced 5-HT_{1A} selectivity somewhat. During the *elaboration* step, we examined the role of amine substitution. The *N*,*N*-di-*n*-butyl analog of the primary amine of des-hydroxypropranolol (i.e., **46**, where $n = 3$; 5-HT_{1A} $K_i = 225$ n*M*) retained much of the affinity of propranolol for 5-HT_{1A} receptors but showed dramatically lower affinity for β-adrenergic ($K_i = 8300$ n*M*) and 5-HT_{1B} ($K_i = 5600$ n*M*) receptors *(70)*. Shortening the alkyl chain from three to two methylene groups resulted in a further increase in selectivity (i.e., **46**, where $n = 2$; 5-HT_{1A} $K_i = 150$ n*M*, β-adrenergic; and 5-HT_{1B} $K_i \geq 10{,}000$ n*M*). The investigation culminated with the identification of several compounds that displayed the 5-HT_{1A} antagonist character; one of these, compound **47** (5-HT_{1A} $K_i = 39$ n*M*), showed a higher affinity than propranolol for 5-HT_{1A} receptors and displayed a low affinity for β-adrenergic ($K_i = 5000$ n*M*) and 5-HT_{1B} receptors ($K_i = 1100$ n*M*) *(62,70)*. These studies were being conducted at the same time as those described earlier with the arylpiperazines; because some of the LCAPs displayed antagonist actions and subnanomolar affinity for 5-HT_{1A} receptors, further work on propranolol-derived aryloxyalkylamines as 5-HT_{1A} antagonists was curtailed. Nevertheless, these studies showed that it was possible to alter the selectivity of aryloxyalkylamines for the different receptor subtypes by making relatively simple structural changes.

In one of our final forays in the 5-HT_{1A} area, it was desired to develop fluorescent probes for cellular mapping of 5-HT_{1A} receptors using fluorescence microscopy. Several fluorescent moieties are available including nitrobenzo-2-oxa-1,3-diazole (NBD), fluorescein (FLU), and 7-amino-4-methylcoumarin-3-acetic acid (AMCA) derivatives. Incorporation of these moieties into a structure generally renders the structure fluorescent under appropriate conditions. However, the moieties are rather large or bulky; so, the question became one of where can such substituents be appended on a 5-HT_{1A} ligand without significant detriment to 5-HT_{1A} receptor affinity? As discussed above, we had identified the LCAP Terminus region (Fig. 4) as one that could tolerate significant bulk.

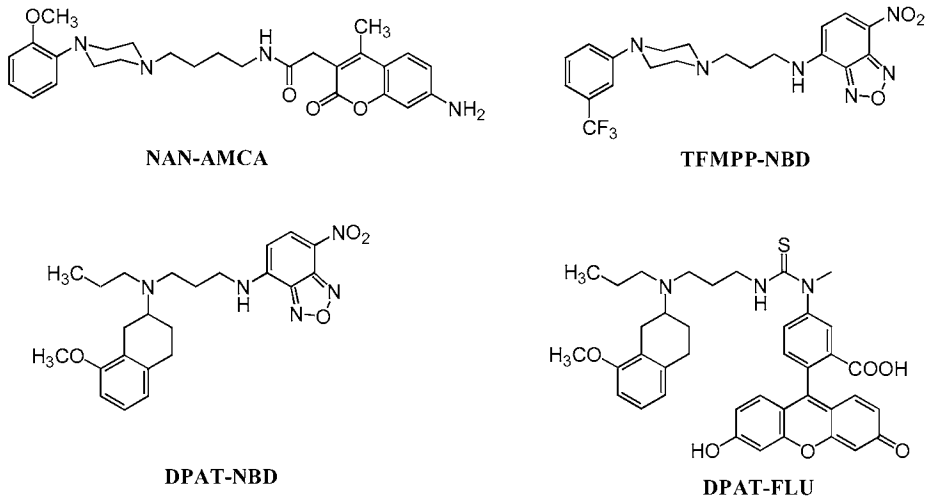


Fig. 5. Structures of 5-HT_{1A} serotonin receptor fluorescent probes. (From ref. *71*.)

In a collaborative effort with Research Biochemicals Inc., the first compounds prepared were derivatives of NAN-190 (more specifically, derivatives of **35)** and TFMPP **(8)** *(71)*. Compounds NAN–AMCA (5-HT_{1A} $K_i = 0.4$ n*M*) and TFMPP–NBD (5-HT_{1A} $K_i = 11$ n*M*) displayed good affinity and selectivity for 5-HT_{1A} receptors (structures are shown in Fig. 5); TFMPP–NBD, in particular, showed little affinity ($K_i > 10{,}000$ n*M*) for 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_3 receptors (i.e., the only 5-HT receptors known at that time). Because we already had shown that there is also a region of bulk tolerance associated with the amine substituent of 8-OH DPAT **(9)** *(72)*, we surmised that similar functionalities should be accommodated. Indeed, DPAT–NBD (5-HT_{1A} $K_i = 0.4$ n*M*) (Fig. 5) displayed high affinity and ≥100-fold 5-HT_{1A} selectivity. Curious as to how far we might push this region of bulk tolerance, we prepared the fluorescein derivative DPAT–FLU (Fig. 5); although DPAT–FLU retained 5-HT_{1A} affinity ($K_i = 22$ n*M*), its affinity was not as great as that of DPAT–NBD *(71)*. Apart from obtaining the desired fluorescent ligands, we also showed that the amine regions of 5-HT_{1A} ligands could withstand substantial steric bulk.

2.5. 5-HT_{1D} Ligands

Initial attention in the serotonin field was focused on the first three receptor populations to be identified: 5-HT_{1A}, 5-HT_{1B}, and 5-HT_2 receptors. However, once the presence of 5-HT_{1B} receptors in the human brain was questioned, interest in this population temporarily subsided. With the discovery of a new population of 5-HT_1 receptors, 5-HT_{1D} receptors, came the realization of a structural and functional similarity between 5-HT_{1B} and 5-HT_{1D} receptors. On the basis of

acquired pharmacological findings, it was hypothesized that 5-HT_{1D} receptors might represent the human counterpart of rodent 5-HT_{1B} receptors. The antimigraine agent sumatriptan (displaying affinity for 5-HT_{1D} receptors with 2- to 10-fold selectivity over rodent 5-HT_{1B} receptors and about 10-fold selectivity over 5-HT_{1A} receptors) was introduced and its migraine-abortive mechanism of action was hypothesized to involve an agonist interaction at 5-HT_{1D} receptors. Given the clinical success of sumatriptan, there was considerable interest in developing additional 5-HT_{1D} agonists. Interest in the relationship between 5-HT_{1B} and 5-HT_{1D} receptors, and their associated pharmacology, also increased at this time because it was thought that what was known about the former (e.g., about structure–activity relationships) might be applied to the design of novel 5-HT_{1D} ligands. However, it quickly became apparent that some compounds displayed affinity for one population but not the other. There might be similarities between these two receptor types, but there were also some obvious differences *(73)*. One of the enigmatic agents, (−)propranolol, binds with high affinity (K_i ~ 15–60 n*M*) at 5-HT_{1B} receptors, but with much lower affinity (K_i ~ 2000 to >10,000 n*M*) at 5-HT_{1D} receptors. Shortly after the cloning of these receptors, receptor modeling studies were undertaken. In fact, the first investigation of its kind in the serotonin field to apply a combination of graphics modeling, site-directed mutagenesis, synthesis, and structure–activity concepts involved comparisons of 5-HT_{1B} and 5-HT_{1D} receptors with propranolol as its primary focus *(74)*. Specific amino acid residues were identified that might account for differences in binding at the receptor types.

Even with the success of sumatriptan, there was a need for newer agents. One of the shortcomings of sumatriptan is its propensity to cause undesirable cardiovascular side effects (coronary artery constriction) in certain patient populations. Initially, it was hypothesized that these cardiovascular effects might be attributable to the affinity of sumatriptan for 5-HT_{1A} receptors. We were approached by Allelix Bioharmaceuticals to develop a novel 5-HT_{1D} agonist ligand that, unlike sumatriptan, would display reduced affinity for 5-HT_{1A} receptors. To initiate these studies, we used what we have termed the "standard series" approach.

Confronted with an ever-growing population of 5-HT receptors, it was of interest to determine what structural features of 5-HT were important for binding at each of the receptor types. We devised a scheme whereby approx 15 serotonin-related tryptamine analogs (the "standard series") was selected for examination in an initial screen to obtain binding data. Over time, this series grew to include about two dozen compounds. Thus, whenever we initiated a study with a new serotonin receptor type, we would begin by examining the standard series. This approach offered several advantages. First, the series was already in-hand and did not require the synthesis of any new compounds each

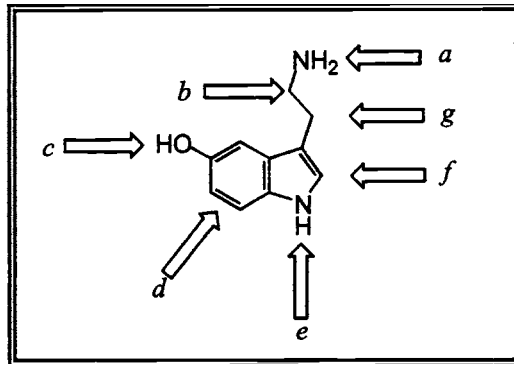
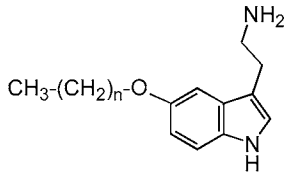


Fig. 6. In the "standard series" approach, various tryptamine analogs are examined at a receptor population to identify initial SAFIRs and to obtain information about selectivity. Compounds in the series are specifically selected to answer the following questions: (*a*) Can the primary amine be replaced with a secondary, tertiary, or quaternary amine? Is bulk tolerated? (*b*) Is α-substitution tolerated? Does stereochemistry play a role in binding? (*c*) Is the hydroxyl group required for binding? Is it a hydrogen-bond donor or acceptor (i.e., can it be replaced by a methoxy group)? Is bulk tolerated? (*d*) Are substituents tolerated at other ring positions? (*e*) Is the indolic nitrogen atom required for binding? Is the NH required? Is bulk tolerated? (*f*) Is substitution at the 2-position tolerated? Is the intact "pyrrole" portion of the indolic nucleus required for binding? (*g*) Can the alkyl chain be lengthened or shortened by one methylene unit? What chain conformation is optimal for binding?

time a new project was begun. Second, each of the compounds was, structurally, only once removed from some other member of the series (i.e., data for any given compound could be related back to data on a close structural relative); typically, structures were such that there was frequently some redundancy in a structural modification to assure reliable structure–affinity conclusions to be formulated. Third, sufficient binding information would be obtained on a closely related series of compounds so that some preliminary QSAR studies could be performed, if desired. Fourth, in addition to providing structure–affinity information for a given receptor type, a comparison of data from the same compounds across several receptor types afforded clues to what structural features might impact selectivity. The compounds in the series were selected to answer a number of basic questions; some of these are shown in Fig. 6. Answers to a large number of questions could be obtained by examining a relatively small number of compounds. Once preliminary information was in hand that a particular substituent(s) was or was not important, a second phase of the study was begun by synthesizing novel structures to address specific questions that were raised by the standard series. In using the *standard series*



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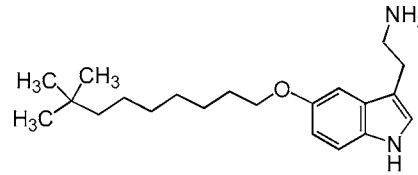

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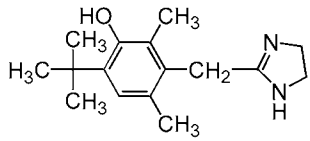
approach, sometimes a lead structure could be identified where no lead had previously existed (*see also* Section 2.6).

One of our first applications of the standard series approach was to develop a 5-HT_{1D}-selective ligand. Comparison of 5-HT_{1A} and 5-HT_{1D} receptor-binding data for the series offered few clues other than that both receptor types possess a region of bulk tolerance associated with the tryptamine 5-position *(75)*. Given the similarity in structural requirements for the binding of tryptamines at the two populations of receptors, but because of the low degree of homology between the two receptor types, it was assumed that greater structural differences might lie outside the tryptamine-binding pocket than within the pocket. Might it be possible to take advantage of this? That is, if receptor differences are greater in regions remote from where the tryptamine portion of serotonin binds, it is possible that the regions of bulk tolerance associated with the indolic 5-position are also somewhat different with respect to substituents they might accommodate. To test this hypothesis, we prepared a series of linear *O*-alkyl analogs of serotonin, **48** ($n = 0$–10), where the alkyl group ranged from *O*-methyl to *O*-undecyl *(76,77)*. *O*-Alkyl analogs bearing three to eight carbon atoms displayed little selectivity for 5-HT_{1D} over 5-HT_{1A} receptors. However, the nonyloxy analog (NOT; i.e., **48**, where $n = 8$) showed decreased affinity for 5-HT_{1A} receptors, whereas it retained affinity ($K_i = 1$ n*M*) for 5-HT_{1D} receptors. 5-HT_{1D} receptor affinity decreased where the *O*-alkyl group was greater than nine carbon atoms in length. Hence, whereas 5-methoxytryptamine showed no selectivity for 5-HT_{1D} vs 5-HT_{1A} receptors, nonyloxytryptamine (NOT) displayed 315-fold selectivity for the former population. NOT (**48**, where $n = 8$) was found to be a 5-HT_{1D} agonist and lacked 5-HT_{1A} agonist action. Subsequent investigations examined compounds bearing branched alkoxy groups (e.g., **49**; 400-fold 5-HT_{1D} vs 5-HT_{1A} selectivity) *(77)*, alkoxy groups bearing aryl substituents, and other structural modifications at the indolic 5-position *(77,78)*. During the course of these later investigations, two individual populations of 5-HT_{1D} receptors were identified (now termed human 5-HT_{1D} [*h*5-HT_{1D}] receptors and *h*5-HT_{1B} receptors). Furthermore, it was subsequently proposed that *h*5-HT_{1B} (or human 5-HT_{1B}-like) receptors, not 5-HT_{1A} receptors, might be responsible for the cardiovascular side effects associated with sumatriptan. The various 5-alkoxytryptamine analogs that we had prepared were examined at *h*5-HT_{1D}

and *h*5-HT_{1B} receptors and, like sumatriptan (and other triptans known at the time), they failed to show >10-fold selectivity for one population over the other. Examination of standard series data for *h*5-HT_{1D} and *h*5-HT_{1B} receptors provided no obvious clues of how selectivity might be achieved using a tryptamine nucleus as the basis for development of novel agents with greater selectivity. This required a change in strategy; it was now necessary to develop agents with selectivity for *h*5-HT_{1D} vs *h*5-HT_{1B} receptors. Here, we used a different tactic. We shifted our focus to "nontryptaminergic" 5-HT_{1D} ligands; what was required, however, was identification of a new lead structure.

It had been reported that the (α-adrenergic agent oxymetazoline **(50)** binds at calf caudate 5-HT_{1D} receptors and displays action as a 5-HT_{1D} agonist *(79)*. Obviously, oxymetazoline was a nonselective agent, but it provided a new structural template for examination. We examined oxymetazoline at 5-HT_{1D} receptors (human brain homogenates) and found it to bind with high affinity. Shortly after *h*5-HT_{1D} and *h*5-HT_{1B} clones became available, we examined oxymetazoline and found that it binds with high affinity at both populations of receptors (K_i < 1 n*M*) but displays no selectivity. Could the structure of oxymetazoline be modified to afford a more *h*5-HT_{1D}-selective agent? This seemed an appropriate situation in which to apply the deconstruction–reconstruction–elaboration approach. Beginning with oxymetazoline **(50)**, we stripped the structure of most pendent substituents, and this included examples of imidazoline analogs where the ring was opened to an amidine, and then reintroduced the substituents in a stepwise fashion to determine how they contributed to affinity and *h*5-HT_{1D}/*h*5-HT_{1B} selectivity *(80)*. This led to the identification of a novel lead structure; it was found that the phenolic group of oxymetazoline played a major role in its lack of selectivity. The *des*-hydroxy compound **51** (K_i: *h*5-HT_{1D} = 0.7 n*M*, *h*5-HT_{1B} = 14 n*M*) displayed similar *h*5-HT_{1D} affinity, but 20-fold *h*5-HT_{1D} selectivity, relative to oxymetazoline (K_i: *h*5-HT_{1D} = 0.4 n*M*, *h*5-HT_{1B} = 0.3 n*M*). Certain other analogs (e.g., **52**, 100-fold *h*5-HT_{1D} selective) displayed even greater selectivity. In the "elaboration" step, structure–affinity and QSAR studies were conducted. Both *h*5-HT_{1D} and *h*5-HT_{1B} receptors appeared to possess a limited hydrophobic binding region associated with the aryl 4-position; relatively small lipophilic substituents (e.g., methyl, ispropyl, bromo) at this position resulted in enhanced affinity, with a *tertiary* butyl group (as found in **51**) seemingly optimal. Lipophilic substituents with greater bulk/size were not as well tolerated *(80)*. These findings suggested that some other part of the molecule should be examined if greater selectivity was desired *(81)*. Attention was turned to the imidazoline portion of **51**. The ring-expanded compound **53** (K_i: *h*5-HT_{1D} = 27 n*M*, *h*5-HT_{1B} = 4370 n*M*) *(81,82)*, although binding with lower affinity than oxymetazoline, was identified as the most (i.e., 160-fold) *h*5-HT_{1D}-selective agent at that time.



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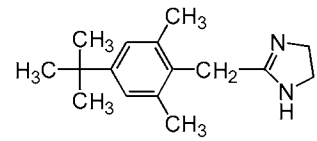

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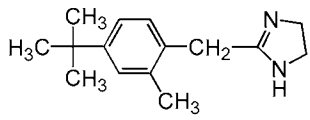

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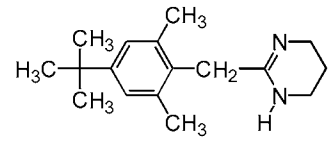

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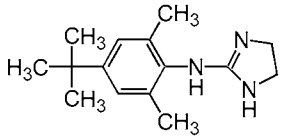
The deconstruction–reconstruction–elaboration approach identified a number of novel nontryptaminergic *h*5-HT$_{1D}$ agonists and many of them displayed enhanced selectivity for this receptor population over *h*5-HT$_{1B}$ receptors. Several demonstrated good selectivity for *h*5-HT$_{1D}$ over other serotonin and nonserotonin receptors and acceptable pharmacokinetic properties *(83)*. In the course of these studies, related analogs, such as the anilinoimidazolines and the amidines, also were identified and have yet to be fully exploited. For example, the anilinoimidazoline counterpart of **51** (**54**; *h*5-HT$_{1D}$ K_i = 0.3 n*M*) binds with 40-fold *h*5-HT$_{1D}$ selectivity *(83)*, whereas amidine **55** (*h*5-HT$_{1D}$ K_i = 13 n*M*) displays 45-fold selectivity *(80)*; that is, both of these compounds are at least as *h*5-HT$_{1D}$-selective as **51**. Neither of these series has been examined in detail; nor has their activity/selectivity been fully optimized.

Before leaving the topic of 5-HT$_{1D}$ receptors, one other study is worth mentioning—not so much because it resulted in a selective agent (because it did not), but because a compound with little measurable affinity (K_i > 10,000 n*M*) was converted to one with significantly enhanced affinity. As already mentioned, propranolol **(45)** binds at rat 5-HT$_{1B}$ receptors with high affinity, but it lacks affinity for *h*5-HT$_{1D}$ and *h*5-HT$_{1B}$ receptors. Results of graphics modeling studies suggested that the ether and hydroxyl oxygen atoms of propranolol contributed to their high affinity for rat 5-HT$_{1B}$ receptors and interact with amino acid residues that are not found in either the *h*5-HT$_{1D}$ or *h*5-HT$_{1B}$ receptors *(74)*. It was also found that structural modifications similar to those described earlier (*see* Section 2.4) could result in derivatives with enhanced *h*5-HT$_{1D}$ affinity. Aryloxyalkylamines bearing a two-atom chain and a secondary amine showed good affinity for *h*5-HT$_{1D}$ receptors; optimal affinity appeared associated with amines bearing an *N*-monomethyl group *(77)*. Hence, propranolol (*h*5-HT$_{1D}$ K_i > 10,000 n*M*) was ultimately converted to **56** (K_i = 34 n*M*), which showed a significant improvement in affinity. Compound **56** is a fairly conformationally flexible agent. Occasionally,



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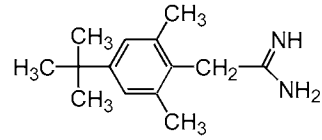

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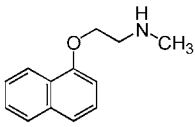

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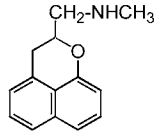

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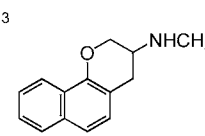

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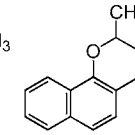

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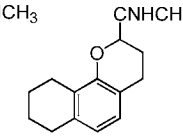

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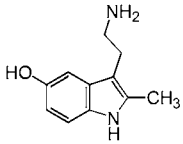
the affinity and/or selectivity of a compound can be enhanced if it is constrained in a conformation favorable for binding at a particular receptor population. Several different conformationally constrained analogs of **56** can be envisioned: **57**–**59**. We prepared all three compounds and found that whereas **57** and **58** bind with reduced affinity, **59** (h5-HT$_{1D}$ K_i = 10 nM) binds with an affinity at least comparable to that of **56** (K_i = 34 nM) *(84)*. Furthermore, **60** (K_i = 4 nM), a tetrahydro version of **59**, was found to bind with even higher affinity. Although **59** and **60** possessed only 10- to 15-fold selectivity for h5-HT$_{1D}$ vs h5-HT$_{1B}$ receptors, this exercise demonstrated that even compounds with little affinity for a receptor (e.g., propranolol h5-HT$_{1D}$ K_i >10,000 nM) can be converted to analogs with substantially enhanced affinity (e.g., **60**; h5-HT$_{1D}$ K_i = 4 nM) *(84)*. As with the 5-HT$_{1A}$ studies on aryloxyalkylamines, h5-HT$_{1D}$ ligands of greater interest were identified (i.e., the oxymetazoline analogs), and work on this project has not yet been completed. Efforts are still required to further enhance the h5-HT$_{1D}$ selectivity of compounds such as **59** and **60**.

2.6. 5-HT$_6$ Serotonergic Ligands

Another example of the "standard series" approach is illustrated by our investigations in the 5-HT$_6$ area. We initiated our studies in the mid to late 1990s when no selective agent was yet known for these receptors. We began by examining the "standard series" *(85)*. One of the compounds in the series is 2-methyl 5-HT **(61)**. Interestingly, 2-methyl 5-HT showed high affinity for this receptor population (h5-HT$_6$ K_i = 46 nM) *(85)*. Typically, 5-HT receptors do not readily accommodate a methyl group at the 2-position of serotonin; 5-HT$_3$ receptors are an exception, Despite its relatively low affinity, 2-methyl 5-HT (**61**; 5-HT$_3$ K_i ~ 1200 nM) has long been used as a 5-HT$_3$ agonist. Now, 2-methyl 5-HT could no longer be considered a 5-HT$_3$-selective agent. Although the affinity of 2-methyl 5-HT was higher at 5-HT$_6$ than at 5-HT$_3$ receptors and although



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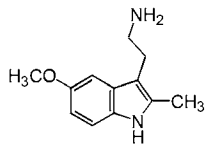

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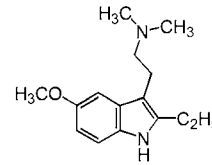

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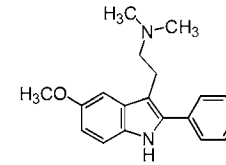

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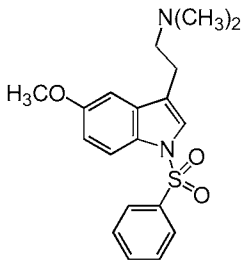
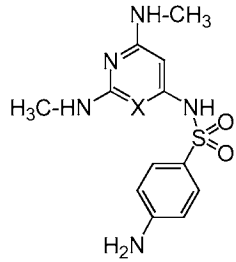
it showed agonist action (adenylate cyclase assay), it could not be called a 5-HT_6-selective agent either. Years earlier we had examined the standard series at 5-HT_3 receptors and we had shown, as had several other groups of investigators, that 5-HT_3 receptors do not readily accommodate O-methylation of serotonin. In theory, 2-methyl-5-*meth*oxytryptamine **(62)** should bind at 5-HT_6 receptors (we already knew from the standard series that 5-HT_6 receptors accommodate a tryptaminergic 5-methoxy group), but should bind with reduced affinity at 5-HT_3 receptors. The agent, on hand from previous studies, was quickly demonstrated to bind at the former population (**62**; 5-HT_6 K_i = 98 n*M*), but not at 5-HT_3 (K_i > 10,000 n*M*) receptors, and possessed an affinity comparable to that of 5-HT (K_i = 75 n*M*). Hence, the standard series provided the first lead for a 5-HT_6-selective agent, and after testing one additional candidate, it seemed that a starting point for further study had been identified. Furthermore, not a single compound required synthesis in order to identify this lead structure.

Being a primary amine and prone to rapid metabolism in vivo and lacking significant lipophilicity, it was desired to examine amine-substituted analogs as well as other compounds that might be more lipophilic in order to more readily penetrate the blood–brain barrier. N,N-Dimethylation was tolerated, and the resultant compound, 5-methoxy-2-methyl-*N,N*-dimethyltryptamine (MMDT; K_i = 60 n*M*), retained the character of **62** *(86)*. Introduction of small N_1-alkyl substituents would also be expected to enhance lipophilicity, but these simply resulted in diminished 5-HT_6 receptor affinity *(86)*. It was found that the C_2-methyl group could be homologated to an ethyl group affording EMDT (**63**; *h*5-HT_6 K_i = 16 n*M*)—the first relatively selective 5-HT_6 agonist *(86)*. Further examination of C_2-substituents revealed that affinity decreased as the ethyl group was homologated to an *n*-propyl substituent (*see* structure **73**). Interestingly, and unexpectedly, a 2-phenyl substituent was tolerated; compound **64**, 2-phenyl-5-methoxy-*N,N*-dimethyltryptamine (PMDT; *h*5-HT_6 K_i = 20 n*M*) lacked agonist action, but behaved as a 5-HT_6 antagonist *(86)*.

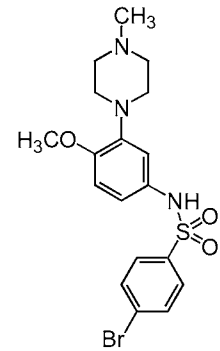
In the course of our investigation of 2-substituted derivatives of 5-methoxy-*N,N*-dimethyltryptamine, we prepared N_1-protected (i.e., 1-benzenesulfonyl) derivatives in order to functionalize the 2-position. Once the 2-position substituent was introduced, the N_1-protecting group would be removed *(86)*. One of the N_1-protected analogs, compound **65**, was submitted for binding and was



65



66a X = N
66b X = CH

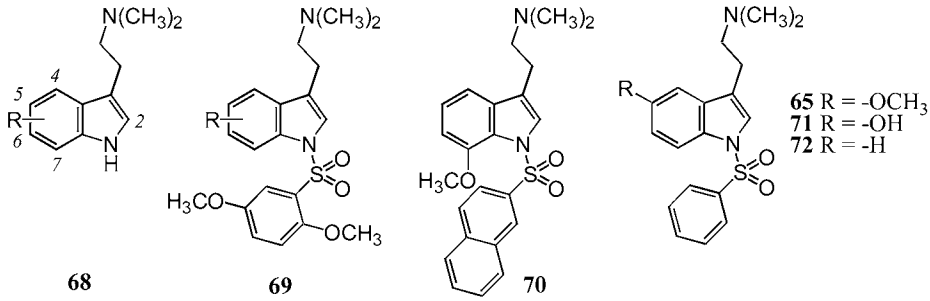


67

serendipitously found to bind with high affinity at 5-HT_6 receptors (**65**, K_i = 2.1 n*M*). Although certainly not a general principle, we have found over the years that when a compound binds with significantly higher affinity than the natural agonist ligand, more often than not it will be an antagonist. Compound **65**, MS-245, was subsequently found to behave as a 5-HT_6 antagonist *(87)*. Thus, within a very short period of time, EMDT **(63)** was identified as the first 5-HT_6 agonist, and PMDT **(64)** and MS-245 **(65)** were identified as 5-HT_6 antagonists. Before we could publish our findings on the latter two compounds, Hoffman-LaRoche and SmithKline/Beecham published their findings that **66** (**66a**: Ro 04-6790; **66b**: Ro 63-0563) and **67** represent 5-HT_6 antagonists *(88,89)*. These latter compounds, as did PMDT and MS-245, represented serendipitous discoveries originally identified from screening of available or library compounds. Subsequently, Merck-Sharp & Dohme independently published that **65** was a 5-HT_6 antagonist *(90,91)*.

Although each of the above compounds, or analogs thereof, is still under investigation (*see* ref. *6* for complete citations to the primary literature), it is rather curious that four of the first five 5-HT_6 antagonists possess an arylsulfonamide moiety. If nothing else, it raises the question of whether these agents bind in a similar manner.

One of the first tasks we undertook following the identification of MS-245 **(65)** was to determine if the benzenesulfonyltryptamines bind at 5-HT_6 receptors in a manner similar to that of the tryptamines lacking an N_1-substituent (i.e., simple tryptamines). Because a sulfonyl group was common to **65**–**67**, it was also of interest to determine if the sulfonyl moiety was a requirement for binding. Generally, if two series of compounds are binding in a similar manner, parallel substituent changes will often result in parallel shifts in affinity. Consequently, we examined a series of methoxytryptamine analogs **68** where the methoxy group was moved from the 5-position to the 4-, 6-, and

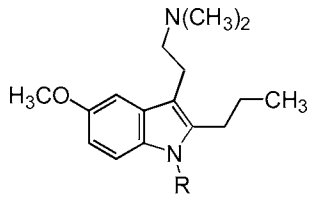


68 **69** **70**

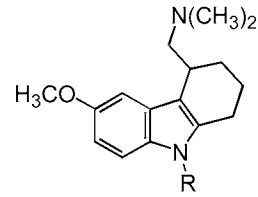
7-positions. The receptor affinities for these compounds were compared with those of their corresponding methoxy-substituted benzenesulfonyl analogs **(69)**. Moving the methoxy substituent around the ring of **68** from the 4- to the 7-position (K_i = 154 n*M*, 16 n*M*, 8000 n*M*, and 19600 n*M*, respectively) indicated that the 5-methoxy analog binds with the highest affinity and the 7-methoxy analog binds with substantially lower affinity. In the benzenesulfonyltryptamine series **(69)**, the corresponding methoxy analogs displayed higher affinity (K_i = 7.4 n*M*, 1.3 n*M*, 9.5 n*M*, and 183 n*M*, respectively); in addition, there seemed to be little difference in affinity regardless of whether the methoxy group was at the 4-, 5-, or 6-position. Whereas a 7-methoxy substituent resulted in low affinity for **68, 69** still displayed modest affinity. In fact, **70** (*h*5-HT_6 K_i = 5 n*M*) displayed an unexpectedly high affinity for a 7-methoxy-substituted tryptamine analog. This provided some of the first evidence that simple tryptamines and their N_1-arylsulfonyl counterparts might be binding differently at 5-HT_6 receptors *(87)*.

Because the location of the methoxy group had minimal influence on the affinity of the MS-245 analogs, it was assumed that it might not play a role in binding (or that independent of position, it might simply contribute to the overall enriched electronic character of the indole ring). The methoxy group of MS-245 (**65**; K_i = 2.3 n*M*) was demethylated to the hydroxy analog (**71**; K_i = 28 n*M*), and removed altogether (**72**; K_i = 4.1 n*M*). The hydroxy analog displayed 10-fold reduced affinity, whereas the affinity of the des-methoxy analog was essentially unaltered *(92)*. Russell et al. *(90)* published nearly identical K_i values for **65, 71**, and **72** (K_i = 2.3 n*M*, 19 n*M*, and 2.9 n*M*, respectively). Hence, the methoxy group is not a requirement for binding.

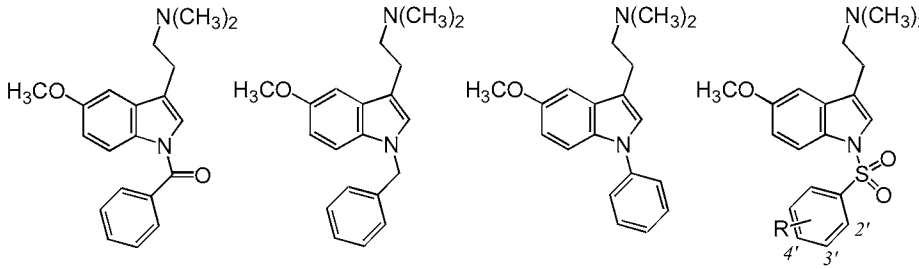
We had shown that homologation of the ethyl substituent of EMDT **(63)** to an *n*-propyl group (i.e., **73**; K_i = 185 n*M*) resulted in >10-fold decreased affinity. However, in the benzenesulfonyltryptamine series, affinity was not altered in the absence (i.e., MS-245; K_i = 2.3 n*M*) or presence (i.e., **74**; K_i = 2.5 n*M*) of a 2-*n*-propyl substituent *(93)*. This was another indication that simple tryptamines and benzenesulfonyltryptamines might be binding differently.



73 R = -H
74 R = -SO_2Ph



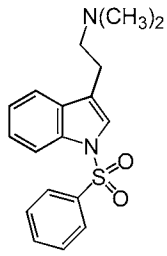
75 R = -H
76 R = -SO_2Ph



77 **78** **79** **80**

Compound **75** (K_i = 168) was examined as a conformationally constrained analog of **73**; like **73**, compound **75** displayed only modest affinity. However, incorporation of the benzenesulfonyl group enhanced the affinity of **75** (i.e., **76**; K_i = 1.5 n*M*) by about 100-fold *(93,94)*. Evidently, there was something about the presence of the arylsulfonyl moiety that was important for high-affinity binding. To determine whether the SO_2 moiety was specifically required or whether it was the presence of the oxygen functions or merely the distance between the N_1-position nitrogen atom and an aryl ring that was contributing to binding, we compared the affinity of MS-245 **(65)** with that of its benzoyl (i.e., **77**) and benzyl (i.e., **78**) counterparts. The SO_2 moiety was also removed altogether (i.e., **79**). Although the benzoyl (**77**; K_i = 18 n*M*) and phenyl (i.e., **79**; K_i = 33 n*M*) analogs retained affinity, they showed nearly 10- to 15-fold lower affinity than MS-245; in contrast, the affinity of benzyl derivative **78** (K_i = 6.5 n*M*) was only about 3-fold lower. So, although the presence of the sulfonyl moiety was optimal, it was not a requisite for 5-HT_6 binding *(93)*.

Having determined that the presence of an indolic methoxy group was tolerated but not necessary and that the sulfonyl moiety was optimal, we focused on the aryl portion of the benzenesulfonyl group. Substituents were introduced to the phenyl ring and, in general, had little effect on 5-HT_6 receptor affinity; that is, compounds **80** where R was an electron-withdrawing or electron-donating group (e.g., 4′-Cl, 4′-OCH_3, 2′,5′-di-OCH_3, and 4′-NH_2) failed to increase or



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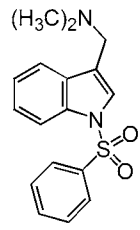

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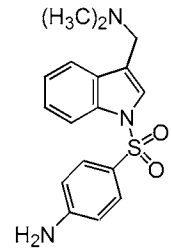

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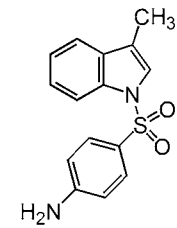


84

decrease affinity by more than 10-fold relative to MS-245 *(87)*. That the benzenesulfonyl group could be replaced by a 1- or 2-naphthylenesulfonyl group suggested that there might be at least some bulk tolerance in this region of the receptor *(87)*. The terminal amine was also examined. The terminal amine group of MS-245 could be replaced with a primary amine, *N*-monomethylamine, or *N*,*N*-diethylamine with <10-fold change in affinity *(92)*. A further increase in the size of the substituent (e.g., *N*-benzyl-*N*-methyl) decreased the affinity *(92)*; Russell et al. *(90)* also showed that replacement of the amine by large substituents (e.g., piperidinyl, morpholinyl) resulted in reduced affinity.

As mentioned earlier, we were struck by the structural similarity of **65–67**. The possibility exists that they might all share a common amine-binding site. Russell et al. *(90)* proposed that **65** and **67** might bind such that a piperidine amine atom of the latter mimics the tryptamine amine. This is more difficult to envision (comparing amine-to-sulfonamide distances) for **66** if the sulfonamido moieties are superimposed. A better superimposition of the structures might be achieved if the aminoalkyl chain of MS-245 was shortened by one methylene group. Hence, we prepared and evaluated compound **82**—a chain-shortened version of **81** (K_i = 4.1 n*M*). Interestingly, **82** (K_i = 3.1 n*M*) retained the affinity of **81** *(92)*. Compound **66** possesses four (or five) basic nitrogen atoms and it is not known with certainty which is the most important for binding *(95)* (presumably at the Asp-106 of transmembrane III [TM3] of the *h*5-HT_6 receptors). This problem does not exist with MS-245 and **82** because they possess only a single basic nitrogen atom.

We had shown (*vide supra*) that a 4′-amino group was tolerated by the benzenesulfonyltryptamines. It was also tolerated by gramine **82** (i.e., **83**; K_i = 6.9 n*M*). The basic amine of **82** could now be removed (note that at least one basic amine must be present in order to prepare a water-soluble salt). Skatole **84** (K_i = 12 n*M*), a 4′-monoaminergic analog of **83**, retained 5-HT_6 receptor affinity *(92)*. This was quite an interesting turn of events; the binding of skatole **84** indicated that the tryptamine "amine" is not required for binding! Does the

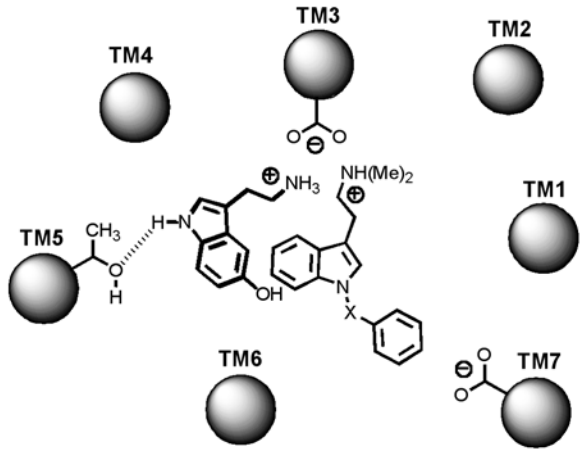
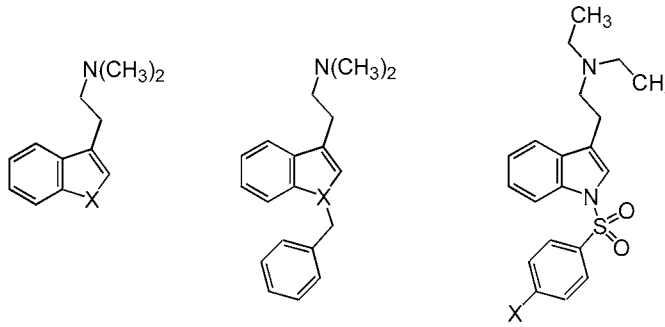


Fig. 7. Schematic representation of the interaction of simple tryptamines, such as serotonin, and N_1-substituted tryptamines, such as benzenesulfonyltryptamines where X = SO_2, with receptor features as identified using automated docking studies and a graphics model of the human 5-HT_6 receptors. Although both types of ligand might utilize the aspartate moiety of TM3, they seem to be oriented in different sterically accessible pockets. (Adapted from ref. *96*.)

aryl amine of skatole **84** now interact with Asp-106? Because both **82** and **84** bind at 5-HT_6 receptors, the presence of two basic amines in **83** is obviously unnecessary. This raised an additional question: Which amine function of **83** binds at Asp-106? These question remains to be answered.

We constructed a graphics model of the *h*5-HT_6 receptors to better understand how ligands might bind *(96)*. Using an automated docking method, two regions of steric accessibility were identified (*see* Fig. 7 for a schematic representation). Whereas simple tryptamines such as serotonin were found to dock in a manner that utilized the TM3 Asp-106 for an ionic interaction with the terminal amine, and a TM5 threonine residue (Thr-196) to form a hydrogen bond with the indolic N_1-H hydrogen atom (consistent with the results of mutagenesis studies), the arylsulfonyltryptamines were found to dock in an altogether different fashion. Although the latter interacted with Asp-106, they were situated more in the vicinity of TM6, TM7, and TM1. These results are consistent with the structure–affinity findings that simple tryptamines and aryl-X-tryptamines (where X = SO_2, CH_2, or CO) likely bind somewhat differently at 5-HT_6 receptors. A particularly satisfying test of this hypothesis derives from an investigation of some benzyl-substituted compounds. 5-Methoxy-N_1-benzyl-*N*, *N*-dimethyltryptamine (**78**; K_i = 6.5 n*M*) and N_1-benzyltryptamine (**87**; K_i = 6.0 n*M*) bind with high affinity; the latter has been shown to act as a 5-HT_6



85 X = NH
86 X = CH_2

87 X = N
88 X = CH

89 X = H
90 X = NH_2

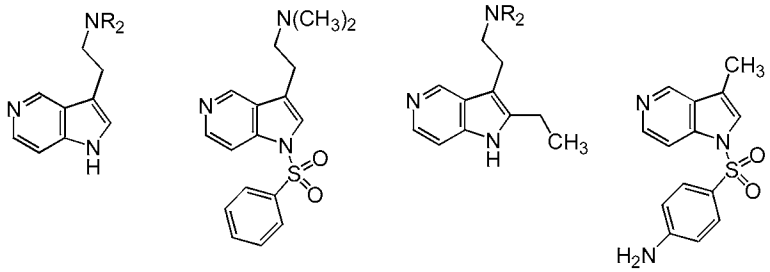
antagonist. The presence of a tryptamine N_1-H moiety seems important for the binding of simple tryptamines *(85,96)*. For example, replacement of the NH of tryptamine **85** (K_i = 30 n*M*) by CH_2 results in a dramatic decrease in affinity (i.e., **86**; K_i = 1900 n*M*). Presumably, **86** can no longer take advantage of the hydrogen-bond interaction at Thr-196. However, antagonists seem to bind in a fashion that does not require utilization of this threonine residue. Hence, it might be expected that benzyl indene **(88)** would bind with an affinity comparable to that of N_1-benzyltryptamine **(87)**. Indeed, benzyl indene **(88)** (K_i = 3 n*M*) retained high affinity for *h*5-HT_6 receptors *(96)* and binds with >600-fold higher affinity than indene **86**. Although these findings do not constitute "proof" of two different modes of binding, here is an instance where the results of graphics modeling studies and structure–affinity findings are consistent.

The graphics model identified another interesting receptor feature that had not been previously considered. In addition to Asp-106 in TM3, *h*5-HT_6 receptors also possess an aspartate moiety in TM7 (Asp-303) (Fig. 7) *(96)*. Upon docking, the benzenoid portion of the benzenesulfonyltryptamines is pointed toward this aspartate. It is conceivable, then, that an amino group at the 4′-position might interact with this aspartate to enhance affinity. Upon examination of several analogs, we found that the introduction of the 4′-amino group enhanced affinity from about 2-fold to as much as 20-fold relative to the parent compound. For example, **90** (K_i = 0.6 n*M*) binds with 10-fold higher affinity than its parent, **89** (K_i = 6.3 n*M*) *(92)*. On one hand, this suggests that a new interaction might be taking place between the 4′-amino group and Asp-303. On the other hand, formation of a new ionic bond might have been expected to result in greater affinity enhancement than was actually observed. Perhaps, the amine is not appropriately situated to take full advantage of this interaction. Current studies are examining this possibility further. Nonetheless, questions raised by this observation include the following: (1) For those ligands bearing

more than one amine, such as **66**, **83**, and **90**, which amine binds at which aspartate moiety? (2) For these same ligands, do both amine functions bind simultaneously at the two aspartate moieties? (3) For compounds such as **84**, does the amine bind at Asp-106 or Asp-303? Additional investigation is required to answer these questions.

It is not known how the arylsulfonyltryptamines bind relative to, for example, **66**; however, if they bind with their *N*-aryl benzenesulfonamide moieties strictly overlapped, incorporation of an aryl nitrogen atom might be tolerated by **65**. Consequently, we prepared several azaindole analogs to test this hypothesis *(97)*. Because **65** (K_i ~ 1–3 n*M*) already binds with higher affinity than **66** (K_i ~ 12–50 n*M*) and because it is not known if the aryl (i.e., pyridyl) nitrogen atom contributes to, or detracts from, the binding of **66b** at 5-HT_6 receptors, we were not necessarily anticipating aza-**65** to bind with higher affinity than **65**. On the other hand, if the aza analogs were completely lacking affinity (barring some unexpected effect of the "aza" nitrogen on the electronic character of the indole nucleus as a whole), it would suggest that the compounds are binding in a different manner. Azatryptamines **91** (K_i = 64 n*M*) and **92** (K_i = 100 n*M*) were found to bind with affinities not unlike that of serotonin. A first assumption is that they bind in a manner similar to 5-HT. However, whereas the affinity of certain tryptamines is enhanced by 50- to 100-fold upon the introduction of a benzenesulfonyl group, the same effect was not observed here; that is, N_1-substituted azatryptamine **93** (K_i = 84 n*M*) binds with an affinity similar to that of **92** and with about 20-fold lower than that of its corresponding N_1-substituted tryptamine **72** (K_i = 4.2 n*M*). Perhaps the azatryptamines already bind in a manner similar to that of the arylsulfonyltryptamines (or at least differently than that of the simple tryptamines) and the introduction of the N_1-substituent has no additional effect. Supporting evidence for this possibility is that 2-ethyl analogs of azatryptamine (i.e., **94** and **95**, K_i = 4900 n*M* and 3000 n*M*, respectively) rather than showing affinity similar to **91** or **92** (e.g., compare with **63**) bind with substantially reduced affinity *(97)*. Yet, azaskatole analog **96** (K_i = 41 n*M*) binds with less than fourfold lower affinity than skatole **84** (K_i = 12 n*M*) *(97)*. These differences in binding behavior argue that the N_1-substituted azatryptamines could be binding in a manner similar to the arylsulfonylindoles, but that the simple azatryptamines are not behaving like the simple tryptamines.

There is no guarantee that structurally similar agents will bind at 5-HT_6 receptors in a similar manner *(85)*. The tryptamines are a case in point. Upon casual inspection, because they share such gross structural commonalities as an indole nucleus separated from a basic terminal amine by a two-carbon-atom fragment, it might be expected that various tryptamines bind in a similar fashion at 5-HT_6 receptors. This turns out not to be the case. Sufficient examples



91 R = H
92 R = CH_3

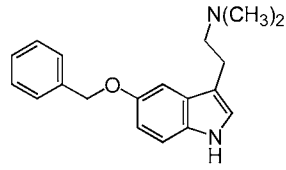
93

94 R = H
95 R = CH_3

96

were provided earlier to demonstrate this. Additional examples support this contention. Incorporation of an arylsulfonyl group generally enhances the 5-HT_6 receptor affinity of certain tryptamines by 50-fold to more than 100-fold. It might have been expected, then, that the introduction of such a substituent to tryptamines **97** (K_i = 18 n*M*) and **99** (K_i = 6.3 n*M*) would result in very high-affinity ligands. Actually, the benzenesulfonyl analogs of these two compounds (**98** and **100**, K_i = 14 n*M* and 17 n*M*, respectively) show no increase in affinity *(93)*. What can be surmised is that the introduction of the benzenesulfonyl group is insufficient to overcome the influence of certain 5-position substituents. It raises questions such as follows: (1) Do **97** and **99** already bind in a manner that mimics arylsulfonyltryptamines so that the introduction of the arylsulfonyl group has no additional effect? (2) Do **98** and **100** bind in a manner distinct from that of either the simple tryptamines or arylsulfonyltryptamines? In any event, it is apparent that parallel structural changes are not resulting in parallel shifts in affinity. The overall conclusion here is that caution must be used when attempting to extrapolate SAFIR or structure–activity relationship results from one series to another for purposes of drug design. With difficulties in understanding how variously substituted tryptamines bind, it is not surprising that it is even more difficult to understand how MS-245 **(65)** binds relative to **66** and **67**.

We have suggested that tryptamine derivatives lacking an N_1-substituent might orient differently at 5-HT_6 receptors than tryptamines bearing an N_1-benzyl or N_1-benzenesulfonyl substituent (*see* Fig. 7). *Parallel substituent changes commonly result in parallel shifts in receptor affinity if two series are binding in a similar manner.* Hence, we tested the above hypothesis by examining chiral tryptamine analogs either lacking or bearing an N_1-substituent under the premise that if they bind in a similar manner, parallel substituent changes should result in parallel affinity shifts. We have already demonstrated that the introduction of a methyl or ethyl group α to the terminal amine of the tryptamines results in somewhat decreased 5-HT_6 receptor affinity *(85)*. For example, whereas tryptamine



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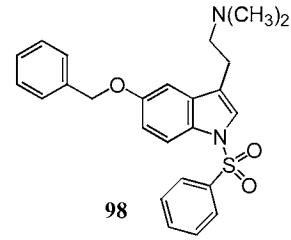

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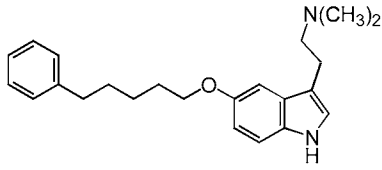

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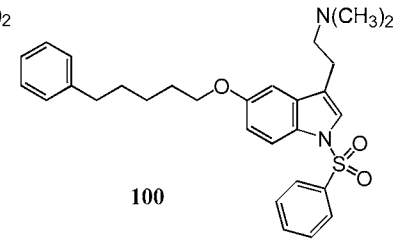
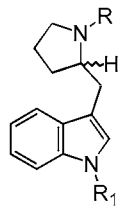


100

(K_i = 180 n*M*) binds with modest affinity *(85)*, (±)α-ethyltryptamine (K_i = 2000 n*M*) and its individual optical isomers (+)- and (−)-α-ethyltryptamine (K_i = 3100 and 1000 n*M*, respectively) bind with reduced affinity (Abate et al., unpublished data). However, it was felt that decreased affinity was an acceptable trade-off if such analogs would allow for enantiomeric potency comparisons. Rather than preparing simple α-methyl or α-ethyl analogs, however, we examined several pyrrolidinylmethylindoles because their absolute stereochemistry could be assigned. Specifically, we examined the isomers of **101** where R = H or Me and R_1 was either H or benzenesulfonyl. In the absence of an N_1-substituent, the *R*-isomer of **102** (R = H; K_i = 60 n*M*) displayed higher affinity than its *S*-enantiomer (K_i = 2400 n*M*). Similar results were seen with *N*′-methyl analogs (**103**, where R = CH_3; K_i = 9.1 n*M* and 640 n*M* for the R- and S-isomers, respectively) *(98)*. As seen with the structurally simpler *N*,*N*-dialkyltryptamines, introduction of an N_1-benzenesulfonyl moiety led to enhanced affinity as determined by examination of pyrrolidinylmethylindoles **(103)** (where R = H; K_i = 7.8 n*M* and 46 n*M* for the R- and S-isomers, respectively) and **103** (where R = CH_3; K_i = 0.3 n*M* and 1.7 n*M* for the R- and S-isomers, respectively). There is greater enantioselectivity in the absence of the benzenesulfonyl moiety (approx 50-fold) than in its presence (<6-fold). Furthermore, N′-methylation had dissimilar effects in the N_1-unsubstituted and N_1-substituted series; whereas N′-methylation enhanced the affinity of the N_1-unsubstituted compounds by about 5-fold, N′-methylation enhanced the affinity of the N_1-substituted series by about 25-fold *(98)*. Clearly, there are differences between the N_1-unsubstituted and N_1-substituted series. These results support the hypothesis that tryptamines bind differently depending on whether they bear an N_1-benzenesulfonyl group.



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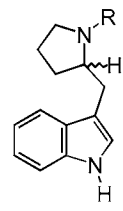

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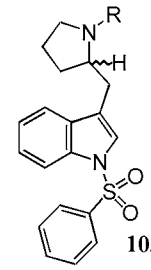

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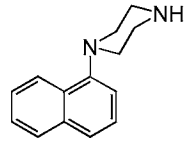

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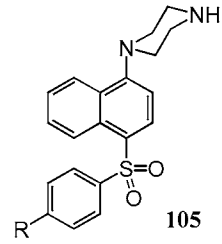
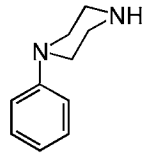


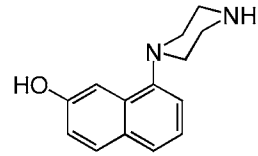
105

Years ago, we suggested that 1-(1-naphthyl)piperazine (1-NP; **104**) is a general tryptamine mimic that binds at multiple populations of 5-HT receptors in a relatively nondiscriminate manner. This raised the possibility that 1-NP **(104)** derivatives bearing the appropriate substituents should bind at 5-HT_6 receptors. To test this hypothesis, we prepared several substituted analogs of 1-NP bearing substituents known to enhance the 5-HT_6 receptor affinity of tryptamines. One initial concern was that because 1-NP lacked an indolic nitrogen atom, the derivatives might bind with low affinity; however, we had already demonstrated that the presence of this nitrogen atom is not a requirement for binding (e.g., *see* indene **88**). Hence, we prepared a series of such compounds. 1-NP (**104**; K_i = 120 n*M*) displayed an affinity comparable to that of 5-HT (**1**; K_i = 100 n*M*) itself. Compound **105** where R = H (K_i = 3.8 n*M*) displayed an affinity similar to that of MS-245 (**65**; K_i = 2.1 n*M*), and its 4′-amino analog (**105**; R = –NH_2; K_i = 0.9 n*M*) displayed an even higher affinity *(99)*. Evidently, 1-naphthylpiperazines represent a novel class of 5-HT_6 ligands that can be further exploited; in fact, for a small series of 1-NP analogs (n = 8), there was a significant correlation (r = 0.856) between their affinities and those of their corresponding tryptamine analogs *(99)*. Furthermore, because **105** is a sulfone, not a sulfonamide, these studies also identified 4-sulfonyl derivatives of arylpiperazines as a novel structure type with affinity for 5-HT_6 receptors.

When the 5-HT_6 project began, no ligands were available. We quickly identified EMDT using the standard series approach (although it might be noted that agonists with greater selectivity now have been reported by others; for



106



107

example, *see* ref. *100*) and, with the assistance of a little serendipity, PMDT and the arylsulfonyltryptamine MS-245. With these as novel lead structures, the deconstruction–reconstruction–elaboration approach coupled with classical medicinal chemistry techniques, receptor graphics molecular modeling, and QSAR studies (not described here), resulted in the identification of a host of ligands for further investigation, including carbazoles, indenes, gramines, skatoles, azaskatoles, and others. Hence, these approaches have been very useful for the identification and exploitation of lead structures.

2.7. Lack of Selective 5-HT$_{1E}$ and 5-HT$_{5A}$ Ligands

The standard series approach has proven to be effective in a number of instances. However, it is only fair to mention that there have been cases where the approach has not been successful. The series was examined for *h*5-HT$_{5A}$ serotonin receptor binding, and an extended series was examined at *h*5-HT$_{1E}$ receptors. With regard to the former, it was found that 5-HT (K_i = 170 n*M*) binds with modest affinity and that most structural modifications served only to reduce 5-HT$_{5A}$ affinity *(101)*. Where tryptamines retained reasonable affinity for the receptors, they were already known from previous studies with the standard series to bind at other populations of 5-HT receptors. Hence, the investigation provided no new clues to how selectivity might be achieved. In the course of these studies it was, however, shown that 1-phenylpiperazine (**106**, K_i > 10,000 n*M*) lacks affinity, that benz-fusion to 1-naphthylpiperazine (**104**, K_i = 40 n*M*) enhances affinity, and that incorporation of a 7-hydroxy group affords a high-affinity ligand (i.e., **107**, K_i = 3 n*M*) *(101)*. Because naphthylpiperazines are quite nonselective as serotonin ligands, **107** would not be expected to be a selective agent; however, the high affinity of **107** for *h*5-HT$_{5A}$ receptors provides a structural lead that now can be exploited.

Human 5-HT$_{1E}$ receptors remain an enigma; although 15 yr have passed since they were first identified *(102)*, no selective ligands are available. More than 40 tryptamine-related compounds were examined at *h*5-HT$_{1E}$ receptors and, remarkably, not one displayed substantially higher affinity than 5-HT itself (K_i = 10 n*M*) *(103)*. Even minor structural modifications of the 5-HT structure, such as O-methylation (5-methoxytryptamine; K_i = 945 n*M*) and

dehydroxylation (tryptamine; K_i = 500 n*M*) reduced affinity by at least 50-fold. Here too, where tryptamines retained reasonable affinity, they already had been demonstrated to bind at other 5-HT receptor populations. Few clues were obtained that might eventually result in tryptaminergic ligands with greater selectivity.

In summary, the standard series approach failed in these instances to offer indications as to how selectivity might be achieved. On the other hand, it was not necessary to synthesize any new compounds to obtain the required binding data. Furthermore, the investigations cannot be considered as failures; that is, the results (1) provided data for the formulation of structure–affinity relationships for each of the two 5-HT receptor subtypes, (2) provided information of the selectivity characteristics of a large number of known tryptamines (e.g., they showed that 5-HTQ lacks affinity for these receptors), and (3) afforded data that was used in QSAR studies to better understand what, and how, various structural features of the tryptamines contribute to their binding (or lack thereof) *(101,103)*.

3. Conclusion

Perhaps the best year for conducting research on selective serotonergic agents was 1980; at that time, one need only be concerned with selectivity for 5-HT_1 vs 5-HT_2 receptors. This situation has changed dramatically with the discovery of at least 14 different 5-HT receptor subpopulations. As entirely novel lead structures are developed, there is increasing possibility that an agent might bind at a nonserotonergic receptor.

For the most part, the vast majority of agents showing any degree of selectivity that are used in serotonin research were (or were derived from) chance discoveries. This is certainly true of the older agents such as DOM **(6)**, 8-OH DPAT **(9)**, ketanserin **(15)**, NAN-190 **(37)**, and many others. Synthesis of structurally related analogs of these serendipitously identified ligands to achieve enhanced selectivity became the norm. However, even here, little design rationale was actually employed (or at least the rationale was not always revealed in literature reports). Available agents were often used to identify pharmacological assays that then could be used, in turn, to screen compounds on a compound-by-compound basis and, more recently, via high-throughput screening methods. Nevertheless, although a number of *lead structures* or *structural templates* were identified over the years, additional efforts were frequently required to obtain ligands with the desired selectivity. Medicinal chemists working in the serotonin field have risen to the challenge and numerous selective agents are now available for many (although not all) 5-HT receptor types. In most instances, the key was identification of a "lead structure" using either a *structured research* approach or *serendipity*. Perhaps the first example of the purposeful

development of a selective serotonergic agent was that by Fozard and colleagues. Taking advantage of findings that cocaine and metaclopramide behaved as M-type serotonin receptor antagonists, they prepared hybrid structures that eventually represented the first 5-HT_3-selective antagonists (reviewed in ref. *18*). Two recent tales of ligand development are those leading to newer analogs of the 5-HT_6 antagonists **66** and **67**. Whereas **66** and **67** were originally identified via high-throughput screening, the pharmaceutical industry was able to take advantage of these new lead structures. Here, in addition to selectivity enhancement, Pharma needed to concurrently tackle optimization of bioavailability and other pharmacokinetic properties. These studies have been summarized and reviewed *(6,104)*.

This chapter describes some of the methods we have employed in our laboratory to develop selective serotonergic agents—in particular, the deconstruction–reconstruction–elaboration approach and the standard series approach—as pharmacological tools. The former is something that some medicinal chemists do (perhaps subconsciously) when, for example, formulating pharmacophore models (i.e., in determining the minimal base structure associated with a given activity); we have simply formalized the approach somewhat to systematically develop novel ligands with greater selectivity than the agents from which they were derived. The standard series approach has been especially useful in identifying new leads for subsequent development in the serotonergic area because there are so many 5-HT receptors, all of which utilize serotonin as the natural ligand. Lacking other viable starting points, the structure of serotonin is frequently the only structural template available to initiate such studies. In the course of our work, we have also applied graphics modeling, QSAR methods, and other techniques; as already mentioned, however, these typically require input that might not be available before a lead structure has been identified. Our investigations were also aided by a good dose of serendipity. In short, several different methods have been applied to the development of agents with selectivity for various serotonin receptors. Continued application of these methods should allow the further development of ligands with even greater selectivity and, possibly, the identification of agents selective for serotonin receptors for which selective agents do not currently exist.

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5

5-HT Receptor Signal Transduction Pathways

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Summary

The purpose of this chapter is to summarize the main features of various signal transduction pathways utilized by the G protein–coupled 5-hydroxytryptamine (5-HT) receptors. Herein, we discuss major and secondary signals emanating from the major subtypes of 5-HT receptors (5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7), as well as unique aspects of signaling for some of the subtypes. The 5-HT_3 receptors, which are 5-HT gated ion channels, will not be discussed in this chapter. This chapter highlights the complexity of signaling from the diverse G protein–coupled 5-HT receptors and underscores the fundamental importance of understanding the nuances of the determinants of signaling specificity for these receptors.

Key Words: Guanosine-5′-*O*-(3-thio)triphosphate; phosphorylation; serotonin (5-hydroxytryptamine); G protein; kinase; receptor; calmodulin; phospholipase.

1. Introduction

The receptors that are activated by serotonin (5-hydroxytryptamine, 5-HT) have been divided into seven families. Six of those families include at least 13 different genes that encode G protein–coupled 5-HT receptors. Those families have been characterized by pharmacological properties, amino acid sequences, gene organization, and second-messenger coupling pathways. Two key postgenomic modifications, alternative splicing of mRNA splicing and mRNA editing,

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create at least 20 more G protein–coupled 5-HT receptors. Thus, there are at least 30 distinct 5-HT receptors that signal through G proteins. The rich diversity of coupling of the 5-HT receptors to varied (and sometimes opposing) signaling pathways functions as a "fine-tuning" mechanism that allows the receptors to respond to the requirements of the specific cells and tissues in which they are expressed.

The basic structure of the G protein–coupled 5-HT receptors is similar to that proposed for nearly all of the G protein–coupled receptors (GPCRs). The 5-HT receptors are integral membrane proteins that possess seven hydrophobic transmembrane domains connected by three intracellular loops (termed i1–i3 loops) and three extracellular loops (termed e1–e3 loops). The amino terminus is oriented toward the extracellular space, whereas the carboxyl terminus is oriented toward the cytoplasm. In some cases, the carboxyl terminus is tethered to the plasma membrane through palmitoylation of cysteine residues. The 5-HT receptor proteins also possess common sites for posttranslational modifications. These include extracellular signal sequences, extracellular glycosylation domains, extracellular cysteine residues that might participate in structurally significant disulfide bonds, and intracellular domains for interacting with G proteins and other regulatory proteins, and sites for phosphorylation. There is a growing awareness that 5-HT receptors can interact with a large number of intracellular proteins through various motifs.

The purpose of this chapter is to review what is known about the second-messenger and effector linkages of 5-HT receptors in native tissues and cells and in heterologous expression systems (Table 1). We will review work that demonstrates an amazing diversity of signaling mechanisms for each 5-HT receptor subtype. The diverse array of signaling pathways for each of the receptors suggests that individual 5-HT receptor subtypes are capable of modulating many potential signals that could be affected by variables such as cell type, receptor number, protein–protein interaction, numbers and types of G proteins expressed in the target cells, and the specific agonist through which the receptor is activated.

2. The 5-Hydroxytryptamine$_1$ Receptors

The 5-HT$_1$ receptor family has five subfamilies, termed 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, and 5-HT$_{1F}$ receptors. The former 5-HT$_{1C}$ receptor has been reclassified as the 5-HT$_{2C}$ receptor based on similarities to other 5-HT$_2$ receptors in structure, gene organization, pharmacology, and second-messenger systems. The 5-HT$_1$ receptor genes are intronless, and the proteins couple primarily through $G_{i/o}$ proteins to the inhibition of adenylyl cyclase and to many other signaling pathways and effectors (Fig. 1). These will be reviewed in Sections 2.1 through 2.5.

2.1. The 5-Hydroxytryptamine$_{1A}$ Receptor

The 5-HT$_{1A}$ receptor is arguably the best characterized of the 5-HT receptors *(1,2)* because of the wide availability of specific ligands and because its cDNA and gene were cloned *(3,4)* nearly two decades ago. All 5-HT$_1$ receptors can be distinguished pharmacologically by their high affinities for 5-HT. The 5-HT$_{1A}$ receptor also has a high affinity for (±)-8-hydroxy-2-(di-*N*-propylamino)tetralin (8-OH-DPAT), a feature that was previously thought to be unique. However, it is now known that the 5-HT$_7$ receptor also has a relatively high affinity for 8-OH-DPAT. The 5-HT$_{1A}$ receptor has been implicated in diverse processes such as neuroendocrine regulation, vasoreactive headaches, thermoregulation, sexual behavior, food intake and appetite, memory, depression, aggression, anxiety, and immune function *(5–13)*. The 5-HT$_{1A}$ receptor was one of the first GPCRs to be cloned *(3,4,14,15)*. Like all 5-HT$_1$ receptors, the gene is intronless. Its mRNA and protein are expressed mainly in the brain, spleen, neonatal kidney, and gut *(3,4,16,17)*.

2.1.1. Major Signals of the 5-Hydroxytryptamine$_{1A}$ Receptor

The 5-HT$_{1A}$ receptor couples to the broadest panel of second messengers of any of the 5-HT receptors. The 5-HT$_{1A}$ receptor regulates diverse second messengers and target enzymes, channels, and kinases. Despite the diversity of signals emanating from the 5-HT$_{1A}$ receptor, all of the signals are almost completely sensitive to *pertussis* toxin, implicating $G_{i/o}$ proteins in the signals initiated by the 5-HT$_{1A}$ receptor. The primary coupling linkage of the 5-HT$_{1A}$ receptor (and of all 5-HT$_1$ receptors) is to the inhibition of adenylyl cyclase (AC)*(18,19)*. The 5-HT$_{1A}$ receptor inhibits AC in an extensive list of cells and tissues, including hippocampal and cortical neurons *(18,19)*, various cultured neuronal cells *(20–23)*, fibroblasts *(23–26)*, HEK293 cells *(14,27)*, HeLa cells *(28)*, LLC-PK$_1$ cells *(29)*, ventral prostate cells *(30)*, and Sf9 insect cells *(31)*.

Because the inhibition of AC by the 5-HT$_{1A}$ receptor is *pertussis* toxin sensitive, it is likely that the 5-HT$_{1A}$ receptor is coupled tightly to $G_{i/o}$ proteins. There is remarkable consistency across diverse expression systems in the ability of the 5-HT$_{1A}$ receptor to bind to most G proteins of the $G_{i/o}$ class. The receptor can bind to/activate $G_{i/o}$ proteins with a rank order of $G_{i\alpha3} > G_{i\alpha2} \geq G_{i\alpha1} \geq G_{o\alpha} > G_{z\alpha}$ *(29,31–40)*. Despite the apparently tight coupling to $G_{i/o}$, the 5-HT$_{1A}$ receptor does not universally inhibit AC in all cell types. For example, 5-HT$_{1A}$ receptors have not been shown to inhibit AC in the dorsal raphe despite being expressed in high density *(41)*, or in cultured astrocytes, despite the presence of mRNA for 5-HT$_{1A}$ receptor *(42)*.

Another major linkage of the 5-HT$_{1A}$ receptor is to activation of ERK (extracellular signal regulated protein kinase) in fibroblasts and neuronal cells *(43–49)*. ERK activation by the 5-HT$_{1A}$ receptor is initiated by βγ-subunits

Table 1
Signaling Linkages of 5-HT Receptors

Receptor	Major signaling linkages	Secondary signaling linkages	G Protein coupling
5-HT_{1A}	Inhibits adenylyl cyclase Stimulates ERK and related cascades Modulates K^+ channels	Inhibits Ca^{2+} conductances Activates PLC, PC-PLC, and PLA_2 Activates/inhibits NOS Activates NAD(P)H oxidase Activates NHE-1 Activates adenylyl cyclase (weak) Regulates apoptotic cascades Calmodulin	$G_{i\alpha3} > G_{i\alpha2} \geq G_{i\alpha1} \geq G_{o\alpha} > G_{z\alpha}$
5-HT_{1B}	Inhibits adenylyl cyclase Stimulates ERK and related cascades	Activates PLC and PLD Activates NOS Activates AC2 Activates K^+ Channels Regulates apoptotic cascades	$G_{i\alpha3} > G_{i\alpha1} \geq G_{i\alpha2} \geq G_{o\alpha}$
5-HT_{2B}	Activates PLC	Activates cell cycle Activates iNOS and cNOS Activates NAD(P)H oxidase Activates ERK and related cascades Activates NHE-1 Activates PLA_2 PDZ domain signals	$G_{q\alpha}$ and $G_{11\alpha}$ $G_{13\alpha}$
5-HT_{2C}	Activates PLC Activates PKC	Activates Na^+/Ca^{2+} exchanger PDZ domain signals Inhibits K^+ channels Inhibits GABA receptor PDZ domain signals	$G_{q\alpha}$ and $G_{11\alpha}$ $G_{o\alpha}$ and $G_{i\alpha1}$ $G_{13\alpha}$ Monomeric G proteins

5-HT_4	Activates adenylyl cyclase	Regulates various channels	$G_{s\alpha}$
	Activates PKA	Activates cAMP-GEF, Epac	$G_{13\alpha}$
		Inhibits NHE-2 and NHE-3	Rac, RhoA
		PDZ domain signals (5-HT_{4a} and 5-HT_{4e})	$G_{i/o\alpha}$ (5-HT_{4b})
5-HT_{5a}	Inhibits adenylyl cyclase	Inhibits ADP-ribosyl cyclase	$G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{0\alpha}$
		Activates PLC (transient)	
		Activates GIRK1 inwardly rectifying K^+ channel	
5-HT_{5B}	Unknown (likely inhibits adenylyl cyclase)	Unknown	Unidentified
5-HT_6	Activates adenylyl cyclase (AC5, but not AC1 or AC8)	Unknown	$G_{s\alpha}$
5-HT_7	Activates adenylyl cyclase) (AC5, AC1, and AC8)	Activates ERK	$G_{s\alpha}$
	Activates PKA	Activates T-type Ca^{2+} channels	
		Elevates intrcellular Ca^{2+}	
		Activates cAMP-GEF	
		PDZ domain signals (5-HT_{7b} and 5-HT_{7c})?	
5-HT_{1D}	Inhibits adenylyl cyclase	Inhibits Ca^{2+} conductances	$G_{i\alpha}$ and $G_{o\alpha}$
		Activates K^+ channels	
		Activates adenylyl cyclase (weak)	
5-HT_{1E}	Inhibits adenylyl cyclase	Activates adenylyl cyclase (weak)	$G_{i\alpha}$ and $G_{o\alpha}$
5-HT_{1F}	Inhibits adenylyl cyclase	Activates PLC	$G_{i\alpha}$ and $G_{o\alpha}$
5-HT_{2A}	Activates PLC	Activates NHE-1	$G_{q\alpha}$ and $G_{11\alpha} \geq G_{i\alpha}$
	Activates Ca^{2+} channels	Activates–inhibits adenylyl cyclase	$G_{12\alpha}$
	Activates PKC	Activates PLA_2	ARF-1, ARF-6, RhoA
		Activates Jak2/STAT3	
		Calmodulin	
		Stimulates ERK and related cascades	
		PDZ domain signals	

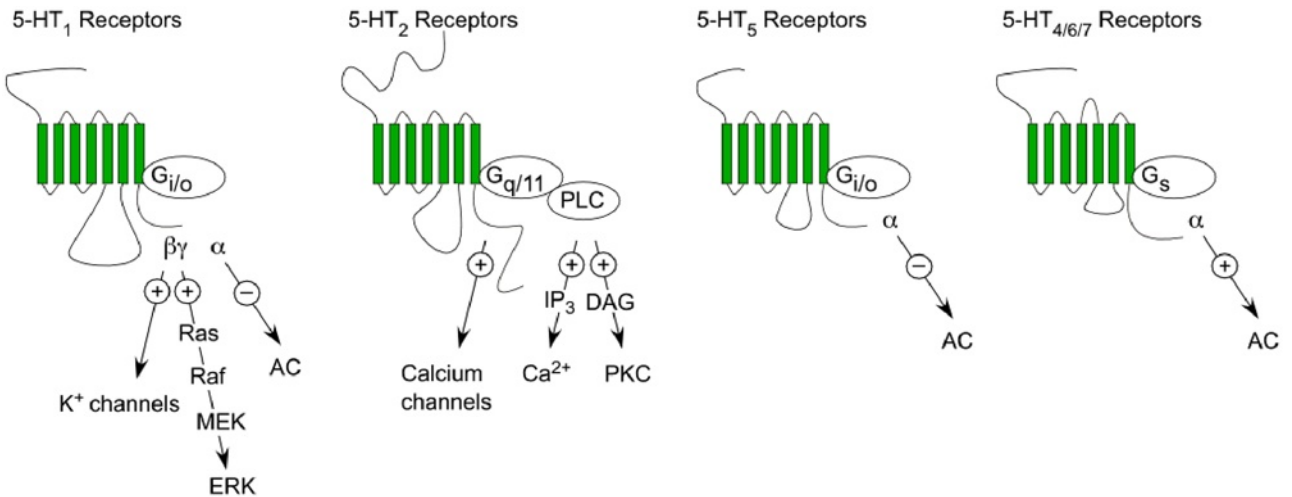

Fig. 1. Major signaling linkages of the subfamilies of G protein–coupled 5-HT receptors are shown. Stimulation is indicated by an arrow overlayed with a "+" sign. Inhibition is indicated by an arrow overlayed with a "–" sign. Abbreviations: AC, adenylyl cyclase; MEK, mitogen and extracellular signal regulated kinase; ERK, extracellular signal regulated kinase; PKC, protein kinase C; DAG, diacylglycerol; IP_3, inositol trisphosphate.

released from pertussis toxin-sensitive G proteins, starting a cascade that involves the formation of a signaling complex containing the nonreceptor tyrosine kinase (Src), tyrosine phosphorylated Shc (a docking platform), phosphatidylinositol-3′ kinase (PI3′-K) Grb2 (an adapter protein), and an activator of Ras (Sos). Activation of Ras results in sequential activation of Raf, MEK (mitogen and extracellular signal-regulated kinase), and ERK *(44–46,48)*. A number of other elements might be involved in the activation ERK by the 5-HT_{1A} receptor, including phosphatidylcholine-specific phospholipase C (PC-PLC) *(44)*, Ca^{2+}/calmodulin (CaM)-dependent endocytosis *(47)*, protein kinase C-α (PKC-α) *(43)*, and the production of reactive oxygen species *(49)*.

The 5-HT_{1A} receptor can regulate a number of ERK-related effectors including activation of PI3′-K *(43–46)*, stimulation of NF-KB (nuclear factor-KB) *(50)*, and inhibition of caspase 3 *(43,51)*. The inhibition of caspase 3 is crucial for 5-HT_{1A}-mediated attenuation of anoxia-induced apoptosis *(51)*, suggesting that the 5-HT_{1A} receptor could play a potential role in neuronal survival.

The importance of ERK activation by the 5-HT_{1A} receptor is highlighted by the observation that the receptor can stimulate proliferation of T-lymphocytes *(13)*, pancreatic carcinoid tumor cells *(52)*, human small cell lung carcinoma cells *(53)*, and transfected cell lines *(25,54)*. In contrast, endogenous lymphocyte 5-HT_{1A} receptors have been implicated in the inhibition of mitogenesis *(55)*.

The modulation of K^+ channels is a major signal of the 5-HT_{1A} receptor. It has been known for nearly 20 yr that the 5-HT_{1A} receptor stimulates neuronal G protein–gated inwardly rectified (GIRK) K^+ channels *(56–60)*. Those channels typically mediate hyperpolarizing postsynaptic potentials in the heart and nervous system during activation of $G_{i/o\alpha}$ coupled receptors. The regulation of GIRK channels by receptors requires interaction of G protein βγ-subunits with the channels *(60,61)*. When the 5-HT_{1A} receptor is expressed in primary cultures of rat atrial myocytes, it stimulates an endogenous atrial inward rectifier K^+ current *(62)*. When rat atrial RNA, GIRK1 RNA or rat brain GIRK3 RNA are expressed with 5-HT_{1A} receptor RNA in *Xenopus* oocytes, the 5-HT_{1A} receptor stimulates GIRK activity *(60,63,64)*. The efficiency of this coupling can be regulated by various regulators of G protein signaling (RGS proteins; RGS1, RGS3, and RGS4, but not RGS2) *(60)*. In some cell types, the 5-HT_{1A} receptor does not stimulate inward rectified K^+ (IRK) channels; when coexpressed by transfection into COS-7 cells, the 5-HT_{1A} receptor inhibits the cloned rat IRK-type inwardly rectifying K^+ channel IRK1 (Kir 2.1). The inhibition appears to involve protein kinase A (PKA)-mediated phosphorylation of the channel, suggesting that the 5-HT_{1A} receptor might stimulate cAMP production and/or PKA in COS-7 cells *(65–67)*.

2.1.2. Secondary Signals of the 5-Hydroxytryptamine$_{1A}$ Receptor

The 5-HT$_{1A}$ receptors couple to multiple secondary signaling pathways, which can vary depending on the cell type and other factors. For purpose of this chapter, secondary signals are signals that have not been documented to occur in most host cells or tissue types for the particular receptor. One of the most interesting and elusive secondary signaling pathways is to the activation of AC. The 5-HT$_{1A}$ receptor has been reported to stimulate AC in the hippocampus and ventral prostate *(30,65,66,68,69)*. Some of these responses might be the result of the 5-H$_7$ receptor subtype, which can be stimulated by the 5-HT$_{1A}$ receptor-selective ligand, 8-OH-DPAT *(65)*. Nevertheless, the possibility that the 5-HT$_{1A}$ receptor can stimulate cAMP accumulation is interesting in that the wild-type receptor has not been shown to couple to cAMP accumulation or to G_s in transfected cells *(35,70)*. Malmberg and Strange demonstrated that the carboxyl-terminal region of the i3 loop of the 5-HT$_{1A}$ receptor could be mutated to induce a very weak coupling to G_s *(70)*. Therefore, if the 5-HT$_{1A}$ receptor couples positively to AC, it might require a highly specific cellular milieu, perhaps necessitating the expression of particular subtypes of AC. In that regard, three different studies have suggested that adenylyl cyclase 2 (AC2) is critical for the positive coupling of the 5-HT$_{1A}$ receptor to cAMP accumulation. In *Xenopus* oocytes, β_2-adrenergic receptor-mediated increases in cAMP could be enhanced by activation of coexpressed 5HT$_{1A}$ receptors. The additional activation by the 5HT$_{1A}$ receptor was enhanced by coexpression of AC2, but not AC3 *(71)*. Interestingly, depletion of $G_{i\alpha1}$ from GH4 pituitary cells reversed the effect of agonist stimulation of the 5-HT$_{1A}$ receptor on cAMP accumulation from inhibitory to stimulatory *(72)*. The same group showed evidence supporting the contribution of the 5-HT$_{1A}$ receptor to agonist-independent increases in basal cAMP accumulation *(27)*. In both cases, the effect seemed to be mediated by $G_{i/o}$ proteins, suggesting that release of $\beta\gamma$-subunits from pertussis toxin-sensitive G proteins might be critical for the effects. The 5-HT$_{1A}$ receptor also might regulate other cyclic nucleotides in that it inhibits cGMP production in rat cerebellum *(73)*.

The 5-HT$_{1A}$ receptors can activate phosphatidylinositol specific phospholipase C (PI-PLC) in some host cells, including HeLa and Ltk$^-$ fibroblasts. Fargin and colleagues first demonstrated that human 5-HT$_{1A}$ receptors could activate PLC in HeLa cells *(28)*. The stimulation was similar in magnitude to that induced by endogenous histamine H_1-like receptors, but was not as efficient as 5-HT$_{1A}$ receptor-mediated inhibition of AC *(74–76)*. Liu and Albert definitively demonstrated the cell-specific nature of PLC activation by the 5-HT$_{1A}$ receptor, showing that the rat 5-HT$_{1A}$ receptor stimulated phosphatidylinositol hydrolysis and release of Ca^{2+} from intracellular stores in Ltk$^-$ fibroblasts, but not in BALB/c-3T3 cells or in GH_4C_1 pituitary cells *(23,54)*. The human 5-HT$_{1A}$

receptor stimulates PLC when expressed in *Xenopus* oocytes *(77)*. The endogenous 5-HT_{1A} receptor in a human Jurkat T-cell-like line stimulates PLC *(78)*, whereas the 5-HT_{1A} receptor does not stimulate PLC when transfected into CHO, COS-7, or NIH-3T3 cells *(25,28,50,79)*. Surprisingly, the 5-HT_{1A} receptor also has been shown to inhibit PLC activation in rat hippocampus *(80)*, although this effect was not sensitive to pertussis toxin. Thus, regulation of PLC by the 5-HT_{1A} receptor seems to depend on cell-specific factors. In cells where the 5-HT_{1A} receptor stimulates PLC, this activation is associated with stimulation of protein kinase C (PKC) *(23,75)*. Stimulation of PKC by the 5-HT_{1A} receptor almost certainly will depend on the expression of βγ-regulated PLC in the target cell or tissue *(74)*. The activation of PLC and PKC is relevant in that the 5-HT_{1A} receptor acutely stimulates Na^+/K^+-ATPase (important in cell volume defense) through a Ca^{2+}-mediated pathway *(75)*, and Na^+-dependent phosphate uptake (important for cellular energy metabolism) through a PKC-mediated pathway *(74)* in HeLa cells.

There are several reports that the 5-HT_{1A} receptor can regulate phospholipases other than PLC, including stimulation of PC-PLC in CHO cells *(50)*, and of phospholipase A_2 (PLA_2) in hippocampus and in transfected HeLa and CHO cells *(76,80,81)*. In contrast, 5-HT_{1A} receptors appear to inhibit *N*-methyl-D-aspartic acid (NMDA) receptor-induced arachidonic acid release in adult rat hippocampus, suggesting that 5-HT_{1A} receptor can inhibit PLA_2 in some settings *(82)*.

Multiple methods have been used to demonstrate that the 5-HT_{1A} receptor can stimulate production of at least two reactive oxygen species, H_2O_2 and superoxide *(49)*. The production of these reactive oxygen species requires an NAD(P)H oxidase-like enzyme and occurs at a point upstream of Src. Moreover, production of reactive oxygen species is essential in the activation of ERK by the 5-HT_{1A} receptor *(49)*. The 5-HT_{1A} receptor also regulates the production of nitric oxide (NO), although there is no consensus on whether the major effect is to stimulate or inhibit its production. The 5-HT_{1A} receptor increases NO production in several circumstances. The 5-HT_{1A} receptor can activate NO synthase in rat ventral prostate cells *(30)*. Physiological effects of the 5-HT_{1A} receptor such as renal microvascular dilation and 8-OH-induced hyperphagia can be blocked by NO inhibitors *(83–87)*. Putative endogenous 5-HT_{1A} receptors in rat ventral prostate cells stimulate NO synthase activity *(30)*. In contrast, 5-HT_{1A} receptors inhibit NMDA-induced NO production in the adult rat hippocampus and in human neocortical slices *(82,88)*, suggesting that the regulation of NO synthesis by the 5-HT_{1A} receptor is complex and cell-specific.

The 5-HT_{1A} receptor inhibits Ca^{2+} currents in neuronal cell lines *(21)* and in neurons *(89)*, probably by inhibiting both N- and P/Q-type Ca^{2+} channels *(90–92)*. When transfected into GH_4C_1 cells, rat 5-HT_{1A} receptors inhibit Bay K8644-mediated Ca^{2+} influx through a pathway that requires $G_{o\alpha}$ *(33)*. The

5-HT_{1A} receptor in *Xenopus laevis* spinal neurons inhibits Ω-agatoxin-IVA-sensitive (P/Q-type) Ca^{2+} channels in a voltage-independent manner *(92)*. 5-HT_{1A} receptor-mediated inhibition of Ca^{2+} currents in dorsal raphe neurons can be inhibited by a peptide inhibitor of G protein βγ subunits *(93)*. In aggregate, these results support a role for the 5-HT_{1A} receptor in the inhibition of distinct Ca^{2+} channels through the intermediary actions of $G_{o\alpha}$ and G protein βγ subunits.

The 5-HT_{1A} receptor has also been shown to regulate several other channels in transfected cells, including activation of the endogenous high-conductance anion channel in CHO cells through either $G_{i\alpha2}$ or $G_{i\alpha3}$, stimulation of an oscillatory Ca^{2+}-activated Cl^- current, and cystic fibrosis transmembrane regulator (CFTR) Cl^- channels in *Xenopus* oocytes *(71,77)*. The effect on CFTR likely involves G protein βγ subunits and was enhanced by coexpression of AC2 and $G_{s\alpha}$ *(71)*. The 5-HT_{1A} receptor expressed by transfection in CHO cells inhibits an endogenous high-conductance anion channel through either $G_{i\alpha2}$ or $G_{i\alpha3}$ *(94)*.

The 5-HT_{1A} receptor regulates a number of important transport processes in transfected cells and native tissues. In addition to the previously mentioned activations of Na^+/K^+-ATPase and Na^+-dependent phosphate uptake, the 5-HT_{1A} receptor stimulates a Na^+/H^+ exchanger through a signaling pathway that requires $G_{i\alpha2}$ and/or $G_{i\alpha3}$, PI3′-K and Src *(40)* and accelerates mediator-facilitated electron fluxes across the plasma membrane *(49)*. The 5-HT_{1A} receptor regulates neuronal 5-HT, norepinephrine, and acetylcholine release. The release of 5-HT involves presynaptic receptors *(95,96)*, whereas the release of acetylcholine and norepinephrine involves postsynaptic receptors *(97–99)*.

2.1.3. Novel Aspects of 5-Hydroxytryptamine$_{1A}$ Receptor Signaling

The multiplicity of signaling pathways modulated by the 5-HT_{1A} receptor, like other GPCRs, is likely to be mediated by cell-specific factors that affect coupling of receptors to G proteins, alter the cellular trafficking or metabolism of the receptors, or induce G protein-independent receptor regulation. In that regard, there has been considerable recent interest in identifying nonclassical 5-HT receptor-interacting proteins (Fig. 2; Table 2). Thus far, one nonclassical 5-HT_{1A} receptor-interacting protein has been identified. Turner and colleagues demonstrated that CaM interacts with the third intracellular loop of the 5-HT_{1A} receptor at two distinct sites. Those are located in the juxtamembrane amino- and carboxyl-terminal regions of the i3 loop of the receptor *(100)*. They showed that the receptor coimmunoprecipitates with CaM in transfected CHO cells. They also used bioluminescence resonance energy transfer to show that interactions between the receptor and CaM can occur in living cells. Both sites possess putative α-helical structures with positively charged amino acids located on the face of the helix opposite the hydrophobic residues. The affinities of CaM for those synthetic peptides derived from those regions of the receptor are appox 100 n*M*

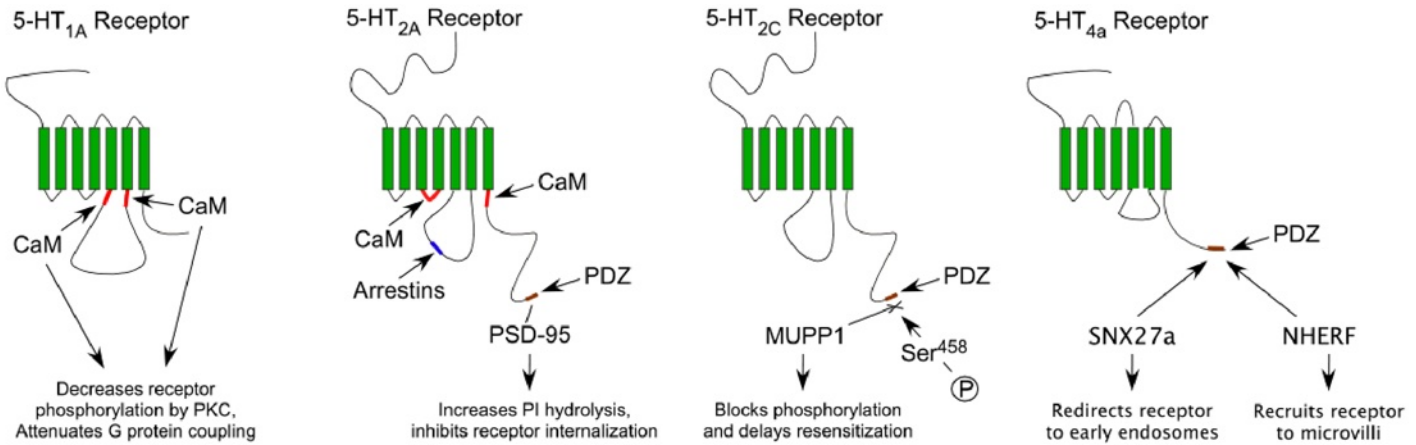

Fig. 2. Novel receptor-interacting partners of selected 5-HT receptors, their interaction domains on the receptors, and consequences of the interactions. Abbreviations: CaM, Ca^{2+}/calmodulin; PDZ, a protein interaction domain termed postsynaptic density 95/discs large/ZO-1; PSD-95, a key protein component of the excitatory postsynaptic density; MUPP1, multi-PDZ-domain protein; SNX27a, a member of the new sorting nexin family of proteins; NHERF, NHE regulatory factor. MUPP1 prevents phosphorylation of serine-458 of the 5-HT_{2C} receptor.

Table 2
5-HT Receptor-Interacting Proteins

Receptor	Interacting protein	Function(s)	Interacting region(s) of the receptor	Ref.
5-HT_{1A}	CaM	Blunts G protein coupling, blunts phosphorylation	i3 loop	*100*
5-HT_{2A}	MUPP1	?	Carboxyl terminus PDZ domain	*238*
	RhoA	PLD activation	N^{376}PLVY	*214*
	ARF-1, ARF-6	PLD activation	N^{376}PLVY, carboxyl terminus, i3 loop	*214, 215*
	CaM	Blunts G protein coupling, blunts phosphorylation	i2 loop, carboxyl terminus	*199*
	Caveolin-1	Increases coupling to $G_{q/11a}$		*235*
	Arrestins	Desensitization	i3 loop	*234*
	PSD-95	Increase coupling to $G_{q/11a}$, blunts internalization	Carboxyl terminus PDZ domain	*236, 237, 239*
	ARIP-1 (MAGI-2); SAP97; MPP3 (Digh3), CIPP	?	Carboxyl terminus PDZ domain	*239*
	AOP-2	?	Carboxyl terminus	*239*
5-HT_{2B}	MUPP1	?	Carboxyl terminus PDZ domain	*238*
5-HT_{2C}	MUPP1	Facilitates receptor phosphorylation and resensitization	Carboxyl terminus PDZ domain	*238, 240, 241*
	Veli-3-CASK-MINT1 ternary complex	?	Carboxyl terminus PDZ domain	*238, 239, 416*

		PSD-95	?	Carboxyl terminus PDZ domain	*416*
		MPP3 (Digh3)	?	Carboxyl terminus PDZ domain	*239, 416*
		CaM; CAPZ-α2 and CAPZ-β; PKCθ-interacting protein (PICOT); β-actin; 2810409H07Rik protein; dynamin 1; α-fodrin	?	Carboxyl terminus	*416*
		ARIP-1, SAP97, SAP102	?	Carboxyl terminus	*239*
	5-HT_{4a}	New sorting nexin (SNX27)	Trafficking and targeting of the receptor	Carboxyl terminus PDZ domain	*340*
		NHERF (EBP50)	Trafficking and targeting of the receptor	Carboxyl terminus PDZ domain	*340*
		ARIP-1 (MAGI-2); MPP3 (Digh3); guanine aminase; Veli-1–3; peroxiredoxin 5	?	Carboxyl terminus PDZ domain	*340*
		Ulip2	?	Carboxyl terminus	*340*
	5-HT_{4e}	nNOS, CIPP	?	Carboxyl terminus PDZ domain	*340*
		SEC23	?	Carboxyl terminus	*340*

and approx 1.7 μ*M*, respectively, suggesting that the interaction between CaM and the 5-HT_{1A} receptor is relevant. CaM binding to the receptor both inhibits G protein coupling and decreases phosphorylation of 5-HT_{1A} receptor i3 loop peptides. Those findings suggest that CaM might dampen both signal initiation (G protein coupling) and desensitization (phosphorylation). Obviously, CaM is potentially a critical mediator of 5-HT_{1A} receptor function.

2.2. *The 5-Hydroxytryptamine$_{1B}$ Receptor*

The existence of the 5-HT_{1B} receptor was suspected on the basis of a low-affinity ^{3}H-spiperone binding site *(1,101)*, distinguishing it from the high-affinity 5-HT_{1A} receptor. 5-HT_{1B} receptors are widely expressed in brain tissue, probably in both presynaptic and postsynaptic locations *(102,103)*. The highest levels of 5-HT_{1B} receptor mRNA are in the striatum, nucleus accumbens, olfactory tubercle, hypothalamus, cortex, hippocampus, thalamus, amygdala, dorsal raphe, and cerebellum *(104)*. 5-HT_{1B} receptors are expressed peripherally as well, particularly in diverse vascular beds *(105–109)* and in both endothelial *(110)* and vascular smooth muscle cells *(111,112)*.

Because there is some confusion regarding the identities of the 5-HT_{1B} and 5-HT_{1D} receptors, we will briefly review historical aspects of the two closely related receptor subtypes. The two receptors have very similar (but not identical) pharmacological profiles. The most significant difference is that the 5-HT_{1B} receptor binds to antagonists of the β-adrenergic receptor with high affinity, whereas the 5-HT_{1D} receptor does not. It was originally thought that the 5-HT_{1B} receptor was expressed primarily or exclusively in rodents (hamsters, mice, and rats), whereas the 5-HT_{1D} receptor was expressed in nonrodent species (humans, cows, dogs, and guinea pigs). Additionally, significant overlap in the tissue expression patterns of the receptors supported the idea that the two were species homologs *(113)*.

The identification of two intronless human receptor genes encoding 5-HT receptors with pharmacological characteristics similar to the 5-HT_{1D} receptor resulted in their temporary designation as 5-HT_{1D} receptors (5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$) *(114–116)*. The subsequent cloning and identification of the rodent 5-HT_{1B} receptor cDNA and gene revealed that the human 5-$HT_{1D\beta}$ receptor has strikingly higher homology with the rodent 5-HT_{1B} receptor than with the human 5-$HT_{1D\alpha}$ receptor *(117–120)*. The cloning of a rat gene that encodes a 5-HT receptor with pharmacological characteristics more closely resembling the 5-HT_{1D} than the 5-HT_{1B} receptor further supported the emerging awareness that the 5-HT_{1B} and 5-HT_{1D} receptors represent separate gene products *(121)*. In that regard, a single amino acid difference between the rodent 5-HT_{1B} receptors and the human 5-$HT_{1D\beta}$ receptor was demonstrated to account for the unique ability of the rodent 5-HT_{1B} receptors to bind β-adrenergic receptor antagonists with

high affinity *(122–124)*, lending further credence to the idea that the two are species homologs. The cloning of rabbit homologs of the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors further supported the distinctions between those receptor subtypes *(125)*. The weight of evidence was sufficiently compelling that the nomenclature of these receptors was changed such that the human 5-$HT_{1D\beta}$ and all 5-HT_{1B} receptors were reclassified as 5-HT_{1B} receptors, whereas the human 5-$HT_{1D\alpha}$ and rodent 5-HT_{1D} receptors were reclassified as 5-HT_{1D} receptors *(126)*.

2.2.1. Major Signals of the 5-Hydroxytryptamine$_{1B}$ Receptor

All of the 5-HT_1 receptor subtypes share many of the signaling linkages with the 5-HT_{1A} receptor in that their signals are conveyed predominantly through pertussis toxin-sensitive G proteins *(127)*, although there might be subtle differences in coupling efficiencies to G proteins between 5-HT_{1A} and 5-HT_{1B} receptors *(39,48)*. Mammalian 5-HT_{1B} receptors expressed exogenously in insect Sf9 cells couple primarily to pertussis toxin-sensitive G proteins with a rank order of $G_{i\alpha3} > G_{i\alpha1} \geq G_{i\alpha2} \geq G_{o\alpha}$, as measured by high-affinity agonist binding *(39)*. Not surprisingly, the best characterized signaling linkage of the 5-HT_{1B} receptor is to the inhibition of AC in diverse tissues *(128)*, native cells *(110,112, 129–131)*, and transfected cell models *(27,116,118,132–134)*.

The 5-HT_{1B} receptor has also been closely linked to proliferative cascades. Endogenous 5-HT_{1B} receptors in fibroblasts were reported well over a decade ago to stimulate DNA synthesis through pertussis toxin-sensitive G proteins *(135)*. Similar effects have been shown in transfected C6 glioma cells *(136)*. Not surprisingly, the 5-HT_{1B} receptor can activate ERK in multiple native and transfected cell types *(48,137–139)*. In CHO fibroblast cells, ERK activation involves PI3′-K and MEK *(48,139)*, resulting in activation of p70 S6 kinase *(137)*.

2.2.2. Secondary Signals of the 5-Hydroxytryptamine$_{1B}$ Receptor

Much like the 5-HT_{1A} receptor, the 5-HT_{1B} receptor couples to multiple signaling pathways, which can vary depending on the cellular context. The 5-HT_{1B} receptor also has been documented to increase cAMP in HEK293 cells through a process that requires the coincidental involvement of both AC2 and G_i proteins *(27)*. The 5-HT_{1B} receptor activates PLC in native and transfected cells, resulting in pertussis toxin-sensitive increases in intracellular Ca^{2+} and hydrolysis of phosphoinositides *(22,112,130,131,140)*. 5-HT_{1B} receptors in rabbit mesenteric artery stimulate phospholipase D (PLD) via a pathway that requires activation of PKC and influx of extracellular Ca^{2+}, but which is independent of PLC activation *(111)*. 5-HT_{1B} receptors increase the production of NO by activating NO synthase (NOS) in cultured human coronary artery rings *(141)* and in cultured bovine aortic endothelial cells *(137)*. The pathway through which 5-HT_{1B} receptors stimulate NO production is pertussis toxin sensitive and relies on increasing intracellular levels of Ca^{2+} *(142)*.

Presynaptic 5-HT_{1B} receptors inhibit neuronal 5-HT release *(95,143–146)*. Human 5-HT_{1B} receptors transfected into C6 glioma cells activate Ca^{2+}-dependent K^+ channels *(147)*. 5-HT_{1B} receptors in BE_2-C neuroblastoma cells also activate Akt kinase, suggesting a possible role in apoptosis *(138)*. 5-HT_{1B} receptors can form homo-oligomers and can form hetero-oligomers when coexpressed with 5-HT_{1D} receptors *(148)*; however, the functional significance of oligomerization on 5-HT receptor signaling is currently not known.

2.3. The 5-Hydroxytryptamine$_{1D}$ Receptor

The 5-HT_{1D} receptor gene was discovered by homology screening using the canine RDC4 gene. That screen revealed two similar genes that encode pharmacologically similar 5-HT receptors. Those receptors were originally called 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors but were later reclassified respectively as the 5-HT_{1D} and 5-HT_{1B} receptors, as described in Section 2.2.1. The 5-HT_{1D} receptor gene is intronless, encoding a protein of 377 amino acids, and it shares 43% and 59% sequence identity with the 5-HT_{1A} and 5-HT_{1B} receptors, respectively *(114,115,121)*. Binding sites for the 5-HT_{1D} receptor have been localized in the substantia nigra, globus pallidus, and caudate, with lower levels in the cortex and hippocampus putamen *(149–151)*. However, the same group was unable to detect 5-HT_{1D} receptor mRNA in the globus pallidus or substantia nigra *(149)*. These results could be rectified by the idea that 5-HT_{1D} receptors might be transported along axon terminals after their synthesis. Another group demonstrated 5-HT_{1D} receptor mRNA in the olfactory tubercle, dorsal raphe, entorhinal cortex, cerebellum, mesencephalic trigeminal nucleus, and the trigeminal ganglion *(104)*. 5HT_{1D} receptor immunoreactivity has been detected on trigeminal sensory neurons with peripheral and central projections to dural blood vessels and to the discrete areas of the medulla associated with the trigeminal sensory system *(109)*. 5-HT_{1D} receptors are also expressed in the peripheral vascular beds *(106)* and in the circular muscle of the human small intestine *(152)*.

2.3.1. Major Signals of the 5-Hydroxytryptamine$_{1D}$ Receptor

Like the other 5-HT_1 receptors, the major signaling pathway of the 5-HT_{1D} receptor is to the inhibition of AC through pertussis toxin-sensitive G proteins in the substantia nigra *(153)*, in MDCK cells *(154)*, and in transfected C6 glioma and NIH-3T3 fibroblast cells *(116,134,155,156)*.

2.3.2. Secondary Signals of the 5-Hydroxytryptamine$_{1D}$ Receptor

The 5-HT_{1D} receptor regulates K^+ and Ca^{2+} channels. The 5-HT_{1D} receptor inhibits Ω-conotoxin-GVIA-sensitive (N-type) Ca^{2+} channels in a voltage-independent fashion in *Xenopus* spinal neurons *(92)* and stimulates Ca^{2+}-dependent K^+ channel C6 glioma cells *(147)*. When the 5-HT_{1D} receptor is expressed at high levels in CHO or Y1 adrenal cells, the receptor can induce a

weak increase in cAMP accumulation *(157–159)*. Not surprisingly, the 5-HT_{1D} receptor can stimulate DNA synthesis/cell growth in native cells (three small cell lung carcinoma cell lines: SCLC, GLC8, and NCI-N-592), and when transfected into C6 glioma cells *(53,160,161)*. The 5-HT_{1D} receptor inhibits neuronal 5-HT release *(96,146)* and inhibits glutamate release from neocortical glutamatergic nerve terminals in the human brain cortex *(162)* and rat cerebellum *(73)*. Presynaptic 5-HT_{1D} receptors inhibit norepinephrine release in the human atrium *(163)*.

2.4. The 5-Hydroxytryptamine$_{1E}$ Receptor

The 5-HT_{1E} receptor was originally characterized as the portion of ^{3}H-5-HT binding to human brain cortex homogenates that was not competed by a cocktail of antagonists of 5-HT_{1A}, 5-$HT_{1B/D}$, and 5-HT_2 receptors *(164)*. The 5-HT_{1E} receptor gene, which is intronless, was subsequently cloned from the human, rat, and guinea pig *(165–168)*, with the human gene encoding a protein of 365 amino acids. Comparatively little is known about the signaling linkages of the 5-HT_{1E} receptor. Like all 5-HT_1 receptors, it appears to couple primarily through pertussis toxin–sensitive G proteins. Indeed, low concentrations of agonist inhibit AC in several cell types transfected with the 5-HT_{1E} receptor *(167,169)*. The 5-HT_{1E} receptor was also shown to stimulate AC in BS-C-1 cells, but this effect required high concentrations to promote increases in cAMP *(169)*. It is likely that this response is conditional, in much the same manner as 5-HT_{1B} receptor coupling to AC2 *(27)*. Regarding other signaling linkages, the 5-HT_{1E} receptor has not yet been demonstrated to regulate phosphoinositide hydrolysis, intracellular Ca^{2+} levels, or arachidonic acid mobilization in BS-C-1 cells *(169)*.

2.5. The 5-Hydroxytryptamine$_{1F}$ Receptor

When it was originally cloned from the mouse, the 5-HT_{1F} receptor was referred to as the 5-$HT_{1E\beta}$ receptor because of the pharmacological similarities with the 5-HT_{1E} receptor *(170)*. As is the case for all 5-HT_1 receptors, the coding region of the 5-HT_{1F} receptor gene is intronless *(171)*. However, the 5-HT_{1F} receptor is encoded by at least three transcripts in the mouse brain, and the presence of an intron splice junction in the 5′-untranslated region of the rat and mouse gene suggests that the transcripts could be differentially regulated *(172)*. The 5-HT_{1F} receptor gene encodes a predicted protein of 366 amino acids with ≥60% homology with the 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1E} receptors. 5-HT_{1F} receptor mRNA has been detected in the human brain, uterus, and mesentery, but not in the liver, spleen, kidney, pancreas, heart, or testes *(173)*. When stably expressed in NIH-3T3 cells, the 5-HT_{1F} receptor negatively couples to AC *(170,173)*.

The 5-HT_{1F} receptor can also stimulate PLC, but this linkage is cell-specific. 5-HT induces rapid increases in intracellular Ca^{2+} and hydrolysis of phosphoinositides when the 5-HT_{1F} receptor is transfected in $LMTK^{-}$ cells, but not in

NIH-3T3 cells. Those effects were blocked by pertussis toxin, supporting a role for $G_{i/o}$ proteins *(174)*. The relevance of the coupling to PLC is unclear because the transfected cells used in this study expressed very high levels of receptors (4.4 pmol/mg of protein).

3. 5-Hydroxytryptamine$_2$ Receptors

There are three members of the 5-HT_{2A} receptor family, termed 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors *(175)*. The 5-HT_{2A} receptor is probably the 5-HT M receptor described by Gaddum and Picarelli *(176)*. The 5-HT_{2B} receptor was formerly referred to as the 5-HT_{2F} receptor (or SRL [serotonin receptor-like]) *(177)*, and the 5-HT_{2C} receptor was formerly referred to as the 5-HT_{1C} receptor. The 5-HT_2 receptors couple primarily to the PLC second-messenger pathway *(178)* in native tissues *(179)* and heterologous cells *(180)*. Like the 5-HT_1 receptors, the 5-HT_2 receptors can couple to other second-messenger pathways in a cell-specific manner. Unlike the 5-HT_1 receptors, the 5-HT_2 receptor genes have introns. Although the 5-HT_2 receptors are similar in structure, pharmacology, and signaling pathways, there are a few differences in their signaling properties *(181,182)*. These differences will be discussed in Sections 3.1–3.3.

3.1. The 5-Hydroxytryptamine$_{2A}$ Receptor

The 5-HT_{2A} receptor was originally identified as a low-affinity 5-HT binding site that could be detected with ^{3}H-spiperone *(183,184)*. The subsequent cloning of the rat and human 5-HT_{2A} receptor genes unequivocally established the binding site as a bona fide receptor *(180,185)*. The 5-HT_{2A} receptor shares considerable homology with the 5-HT_{2B} and the 5-HT_{2C} receptors. The 5-HT_{2A} receptor is widely distributed in the brain (cortex, caudate nucleus, hippocampus, nucleus accumbens, and olfactory tubercle) *(186)*, kidney *(187,188)*, skeletal muscle *(189–191)*, smooth muscle *(192,193)*, and platelets *(194)*.

3.1.1. Major Signals of the 5-Hydroxytryptamine$_{2A}$ Receptor

The primary signaling linkage of the 5-HT_{2A} receptor is to the activation of PLC-β in nearly all tissues and cells in which it is expressed. Activation of PLC results in hydrolysis of phosphoinositides and elevations of intracellular Ca^{2+} *(181,182,195,196)*. These effects can result in activation of CaM, Ca^{2+}-activated signaling enzymes, and L-type Ca^{2+} channels *(197)* and stimulation of PKC *(198)*.

CaM is a major or primary signaling target of Ca^{2+} mobilization in most cells types. There are four lines of indirect evidence that the 5-HT_{2A} receptor signals through CaM. First, the 5-HT_{2A} receptor has two CaM-binding sites in the second intracellular loop and carboxyl terminus, and these can regulate coupling of the receptor to G proteins *(199)*. Second, agonist-induced upregulation of the 5-HT_{2A} receptor depends on CaM and Ca^{2+} CaM-dependent kinase 2 *(200)*.

Third, Berg et al. showed that the 5-HT_{2A} receptor induces CaM-dependent increases in cAMP formation in A1A1 cells *(201)*. Fourth, a CaM-dependent pathway is involved in 5-HT_{2A} receptor-induced cyclo-oxygenase 2 mRNA expression in renal mesangial cells *(202,203)*.

Hydrolysis of phosphoinositides usually leads to increases in intracellular Ca^{2+}. The 5-HT_{2A} receptor increases intracellular Ca^{2+} levels through several mechanisms, including the liberation of intracellular Ca^{2+} stores and/or by activating Ca^{2+} channels. The 5-HT_{2A} receptor has been closely linked to the activation of L-type Ca^{2+} channels *(197,204–207)*, although this link should be carefully established in each cell type in that there is considerable overlap in the pharmacology of L-type Ca^{2+} channels and the 5-HT_{2A} receptor *(208)*. The 5-HT_{2A} receptor couples to both voltage-dependent and voltage-independent Ca^{2+} channels *(204,206,209,210)*. 5-HT_{2A} receptor-mediated increases in intracellular Ca^{2+} have been linked to stimulation of Ca^{2+}-activated potassium channels in C6 glial cells and rat cortical astrocytes *(205)* and to Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes.

3.1.2. Secondary Signals of the 5-Hydroxytryptamine$_{2A}$ Receptor

The 5-HT_{2A} receptor can modulate many secondary signals. For example, the 5-HT_{2A} receptor activates phospholipases such as PLD and PLA_2. The 5-HT_{2A} receptor has been shown to couple to PLD in cultured rat renal mesangial cells *(212)*, but this effect is not universal in that the receptor does not couple to PLD in guinea pig trachea *(213)*. A sequence (NPLVY) at the junction of the seventh transmembrane region and carboxyl terminus of the 5-HT_{2A} receptor has been shown to be responsible for binding of small G proteins ADP-ribosylating factor-1 (ARF-1) and RhoA to the receptor and for activation of PLD *(214)*. ARF-1 binds to the carboxyl-terminal tail of the 5-HT_{2A} receptor and to a lesser extent to the i3 loop. ARF-6 also binds to those regions, albeit less avidly *(215)*. The 5-HT_{2A} receptor is coupled to the mobilization of polyunsaturated fatty acids and arachidonic acid in C6 glial cells *(216)*. In CHO and 1C11 cells, 5-HT_{2A} receptors activate PLA_2 and stimulate arachidonic acid release *(181,217)*. The potential physiological relevance of PLA_2 activation is illustrated by the observations that 5-HT_{2A} receptor-induced prostaglandin release plays a major role in accelerating gastrointestinal transit *(218)* and that the 5-HT_{2A} receptor stimulates shedding of the amyloid precursor protein ectodomain through a PLA_2-dependent pathway *(219)*.

The 5-HT_{2A} receptor can regulate cAMP formation in certain cells, although this effect is cell-specific *(220)*. In renal mesangial cells, the 5-HT_{2A} receptor inhibits forskolin-stimulated cAMP formation through a pertussis toxin-sensitive mechanism *(187)*. The inhibition of cAMP apparently occurs through direct interaction with $G_{i/o}$ in that it was present in washed membranes and did not

involve PLC, Ca^{2+}, or PKC. The 5-HT_{2A} receptor can stimulate cAMP formation in FRTL-5 thyroid cells through a pertussis toxin-sensitive mechanism *(195)* and in A1A1 cells through an indirect pathway that involves PKC-α and/or PKC-δ and CaM *(201)*.

The 5-HT_{2A} receptor can also activate mitogenic cascades. The 5-HT_{2A} receptor activates ERK mitogen-activated protein kinases (MAPKs) in vascular smooth muscle cells *(197,207,221,222)* and in renal mesangial cells *(188,223)*. The signaling pathways linking the 5-HT_{2A} receptor to ERK are complex. In renal mesangial cells, the pathway requires both stimulation of PKC and production of reactive oxygen species production via an NAD(P)H oxidase-like enzyme *(188,223)*. In vascular smooth muscle cells, the activation of ERK involves PLC, L-type Ca^{2+} channels, transactivation of the epidermal growth factor (EGF) receptor, NHE-1, and MEK1 *(197,207,222,224)*. The activation of the ERK pathway by the 5-HT_{2A} receptor is relevant in that it is involved in the contraction of vascular smooth muscle cells as well as in phosphorylation of the retinoblastoma gene product *(221)*, whereas the activation in mesangial cells results in the induction of early-response genes such as cyclo-oxygenase-2 and Egr-1 *(203)*, cellular proliferation *(198)*, and upregulation of the pro-fibrotic cytokine, transforming growth factor-β (TGF-β) *(188)*.

The 5-HT_{2A} receptor in rat skeletal muscle myoblasts stimulates myogenic differentiation and rapidly induces tyrosine phosphorylation of Jak2 (Janus 2) kinase and STAT3 (signal transducers and activators of transcription), resulting in translocation of STAT3 into the nucleus. Because the 5-HT_{2A} receptor coprecipitates with Jak2 and STAT3, there is evidence that they are physically associated within a signaling complex *(189)*. The 5-HT_{2A} receptor in mesangial cells also activates the monomeric G protein, Rho, through which it regulates the dynamics of the actin cytoskeleton *(225)*.

The 5HT_{2A} receptor regulates the production of reactive oxygen and nitrogen species. In renal mesangial cells, the receptor stimulates the production of H_2O_2 and superoxide through the action of an NAD(P)H oxidase-like enzyme *(188)*, which is downstream of PKC. The production of H_2O_2 and superoxide serves an important role in the activation of ERK by the 5-HT_{2A} receptor in those cells *(223)*. The 5-HT_{2A} receptor can either stimulate or inhibit NO synthesis. 5-HT_{2A} receptors inhibit cytokine-stimulated inducible nitric oxide synthase in C6 glioma cells *(226)*. In contrast, 5-HT_{2A} receptor-induced release of NO regulates gastrointestinal transit *(218)*.

Like the 5-HT_{1A} receptor (*see* Section 2.1), the 5-HT_{2A} receptor can regulate several transport processes. The 5-HT_{2A} receptor activates the type 1 sodium-proton exchanger (NHE-1) in renal mesangial cells *(187,227)* and vascular smooth muscle cells *(222)*, the Na^+K^+-ATPase (sodium pump) in airway smooth muscle cells *(228)*, and the $Na^+/K^+/2Cl^-$ cotransporter when 5-HT_{2A} receptor transfected

into NIH/3T3 fibroblasts *(229)*. In rat renal mesangial cells, the 5-HT_{2A} receptor accelerates electron fluxes across the plasma membrane *(188)*. The 5-HT_{2A} receptor stimulates the release of dopamine and norepinephrine (but not 5-HT) in the frontal cortex of rats *(230)*. Both 5-HT_{2A} and 5-HT_{2C} receptors stimulate amyloid precursor protein ectodomain secretion through a pathway that involves PLA_2 *(219)*. The 5-HT_{2A} receptor also increases glucose uptake in skeletal muscle myotubes through a process that requires increases in the plasma membrane content of glucose transporters GLUT1, GLUT3, and GLUT4 *(191)*.

3.1.3. Unique Aspects of 5-Hydroxytryptamine$_{2A}$ Receptor Signaling

The 5-HT_{2A} receptor has an interesting feature in that it is internalized in response to both agonists and antagonists *(231)*. This feature of the 5-HT_{2A} receptor serves as an important illustration that ligand binding to GPCRs might induce subtle conformational changes that could make an antagonist for one signal (e.g., Ca^{2+} mobilization) function as an agonist in another (internalization). The ability of the 5-HT_{2A} receptor to internalize in response to both antagonists and agonists might play important roles in its signaling and in the actions of antipsychotic medications. Bhatnagar et al. *(231)* showed that both agonist- and antagonist-induced internalization of the 5-HT_{2A} receptor were dynamin dependent, but insensitive to dominant-negative arrestins. In contrast, binding of agonists (but not antagonists) to the 5-HT_{2A} receptor induced more robust translocation of arrestin-3 than arrestin-2 to the plasma membrane and resulted in differential sorting of arrestins and 5-HT_{2A} receptors into distinct plasma membrane domains and intracellular compartments *(231)*. These differences in the intracellular trafficking of the various signaling components might be important in determining which signals are generated by the 5-HT_{2A} receptor. This possibility is nicely demonstrated by the observation that 5-HT binding to the 5-HT_{2A} receptor induces translocation of PKC and increases intracellular Ca^{2+}, whereas lysergic acid diethylamide (LSD) induces PKC translocation without stimulating Ca^{2+} mobilization *(232)*.

The importance of the aforementioned observations was brought into sharp focus by the stunning observation by Elphick and colleagues that the human polyomavirus, JCV, infects cells by using the 5-HT_{2A} receptor *(233)*. Infection of glial cells could be blocked by 5-HT_{2A} receptor antagonists and neutralizing antibodies. HeLa cells, which are naturally deficient in 5-HT_{2A} receptors, could not be infected by JCV. Transfection of HeLa cells with 5-HT_{2A} receptors rendered the cells susceptible to JCV infection, after which JCV was colocalized with 5-HT_{2A} receptors in early endosomes. Thus, 5-HT_{2A} receptors can facilitate cellular entry of JCV and might therefore play a role in the development of progressive multifocal leukoencephalopathy. The signaling effects of JCV binding to the 5-HT_{2A} receptor have not been elucidated yet.

The 5-HT_{2A} receptors have recently been demonstrated to bind to a number of proteins that can alter their function (Table 2). For example, arrestins are colocalized with 5-HT_{2A} receptors in vivo and have been shown to bind to the middle portion of the i3 loop of the 5-HT_{2A} receptor, a region that is composed of α-helical and β-loop, β-turn, and β-sheet regions. Binding of arrestins to this portion of the receptor is likely to be mediate desensitization of the receptor and possibly to participate in the propagation of some signals *(234)*. Surprisingly, Bhatnagar and colleagues recently demonstrated that caveolin-1 complexes with 5-HT_{2A} receptors in C6 glioma cells, rat synaptic membranes, and transfected HEK-293 cells *(235)*. This interaction was significant in that caveolin-1 appeared to facilitate the interaction of 5-HT_{2A} receptors with $G_{q\alpha}$, as measured by increases in intracellular Ca^{2+}. This interaction was specific in that caveolin-2 did not increase 5-HT_{2A} receptor signaling. Moreover, caveolin-1 knockdown greatly diminished both 5-HT_{2A} and P_{2Y} purinergic receptor signaling without altering PAR-1 thrombin receptor signaling. The mechanism through which caveolin-1 enhances the 5-HT_{2A} receptor coupling to $G_{q\alpha}$ has not been fully elucidated. However, this interaction highlights the potential roles of nonclassical receptor-interacting proteins in 5-HT receptor signaling.

Turner and Raymond reported that the 5-HT_{2A} receptor possesses two CaM-binding domains in the i2 loop and proximal juxtamembrane region of the carboxyl terminus *(199)*. Both sites possess putative α-helical structures with positively charged amino acids located on the face of the helix opposite from hydrophobic residues, consistent with CaM-binding motifs. The affinities of CaM for those synthetic peptides derived from those regions of the receptor are approx 65 n*M* and approx 170 n*M*, respectively, suggesting that the interaction between CaM and the 5-HT_{2A} receptor is relevant. CaM coimmunoprecipitates with the receptor and binds avidly to fusion proteins containing each of the putative CaM-binding regions of the 5-HT_{2A} receptor. CaM inhibits G protein coupling by the 5-HT_{2A} receptor and decreases phosphorylation of the 5-HT_{2A} receptor carboxyl-terminal peptide. Those findings suggest that CaM might dampen G protein coupling primarily through binding to the i2 loop and attenuate phosphorylation through binding to the carboxyl terminus. Coupled with the findings for the CaM binding to the 5-HT_{1A} receptor, CaM appears to be a critical modulator of 5-HT receptor function. Interestingly, there are putative CaM-binding domains in the 5-HT_{1B}, 5-HT_{1F}, and 5-HT_{6} receptors (proximal and distal i3 loop), 5-HT_{1D} receptor (i2 loop and proximal i3 loop), 5-HT_{1E} receptor (i2 loop and distal i3 loop), 5-HT_{2C} receptor (i2 loop and proximal carboxyl terminus), 5-HT_{4} receptor (proximal carboxyl terminus), and 5-HT_{7} receptor (distal i3 loop and proximal carboxyl terminus) (Turner and Raymond, unpublished). Thus, CaM binding to nearly all of the 5-HT receptors could play critical regulatory roles, although further study will be required.

Xia et al. reported that a canonical type I PDZ-binding; postsynaptic density 95/Discs large/ZO-1) domain composed of the last four amino acids (VSCV) on the carboxyl terminus of the 5-HT_{2A} receptor is important for the preferential targeting of the receptor to the dendritic compartment of cultured pyramidal cells. Disruption of this domain by deletion or masking with green fluorescent protein (GFP) greatly attenuated the targeting of 5-HT_{2A} receptors to dendrites without affecting the sorting to axons *(236)*. Further, they showed that the 5-HT_{2A} receptor PDZ domain associates with a PDZ domain-containing protein, PSD-95, which is a key component of the excitatory postsynaptic density. Moreover, PSD-95 induces complex changes in various 5-HT_{2A} receptor functions. PSD-95 increases the efficiency of 5-HT_{2A} receptor-mediated activation of hydrolysis of inositol phosphates, inhibits receptor internalization, and induces clustering of the receptors at the plasma membrane. In contrast, PSD-95 has no effect on 5-HT_{2A} receptor desensitization *(237)*.

One group has used proteomic methods to demonstrate that the 5-HT_{2A} receptor (or fusion protein derived from it) can bind to multiple cellular proteins (Table 2) *(238,239)*. Many of those proteins are important for subcellular targeting or trafficking. Putative 5-HT_{2A} receptor-interacting proteins include multi-PDZ-domain protein (MUPP1), activin receptor-interacting protein 1, SAP97, PSD-95, membrane protein palmitoylated 3 (MPP-3), and channel-interacting PDZ protein (CIPP), all of which interact through the PDZ domain on the carboxyl terminus of the receptor, and antioxidant protein 2, which interacts with the carboxyl terminus of the 5-HT_{2A} receptor through a PDZ-independent mechanism *(238,239)*. The functional significance of these interactions remains to be defined, although it is known that MUPP1 binding to the 5-HT_{2C} receptor regulates receptor phosphorylation and resensitization *(240,241)*.

3.2. The 5-Hydroxytryptamine$_{2B}$ Receptor

The 5-HT_{2B} receptor was first described as the receptor that mediates contraction of the gastric fundus *(242)* and was subsequently linked to contraction of the longitudinal muscle layers of the human small intestine *(152)*. It was formerly referred to as either the SRL *(243)* or the 5-HT_{2F} receptor. The receptor was cloned from the mouse and rat in 1992 *(177,243)* and from humans in 1994 *(244)*. The human receptor gene has two introns similar to the 5-HT_{2A} and 5-HT_{2C} receptors. The predicted 5-HT_{2B} receptor protein is 481 amino acids in length *(244)*. The human receptor has 45% homology with the 5-HT_{2A} receptor and 42% homology with the 5-HT_{2C} receptor *(245)*.

Although originally discovered as a gut receptor, mRNA encoding the 5-HT_{2B} receptor is expressed with greatest abundance in the human liver and kidney. Lower levels of expression have been detected in the pancreas and

spleen *(246)*. The expression of 5-HT_{2B} receptor mRNA in brain appears to be relatively limited *(244,247)*. 5-HT_{2B} receptor immunoreactivity has been reported in neurons in the cerebellum, dorsal hypothalamus, lateral septum, and medial amygdala *(248)*.

3.2.1. Major Signals of the 5-Hydroxytryptamine$_{2B}$ Receptor

The primary signaling linkage of the 5-HT_{2B} receptor is to the activation of PLC-β in nearly all tissues and cells in which it is expressed, either endogenously or heterologously *(232,244,247,249,250)*. There is scant evidence that the 5-HT_{2B} receptor can couple to other phospholipases. The 5-HT_{2B} receptor has been shown in one report to activate PLA_2 in IC11 cells *(217)*. However, the 5-HT_{2B} receptor does not couple to PLD in rat stomach fundus *(251)*. Because the 5-HT_{2B} receptor activates PLC, it would be expected that the receptor would signal through Ca^{2+}, CaM, and PKC, although only a few studies have provided experimental evidence to support those linkages. The 5-HT_{2B} receptor activates Ca^{2+} oscillations in *Xenopus* oocytes through inositol trisphosphate-dependent release of internal Ca^{2+} stores and Ca^{2+} influx, resulting in the activation of a Ca^{2+}-dependent Cl^- channel *(243,252)*. In contrast, the 5-HT_{2B} receptor induces release of Ca^{2+} in human pulmonary artery endothelial cells from ryanodine-sensitive intracellular stores through a pathway that is independent of hydrolysis of inositol phosphates *(253)*.

3.2.2. Secondary Signals of the 5-Hydroxytryptamine$_{2B}$ Receptor

The 5-HT_{2B} receptor can modulate a number of secondary signals, the most prominent of which are linked to mitogenic and morphogenic cascades. 5-HT_{2B} receptors stably expressed in mouse fibroblast $LMTK^-$ cells rapidly activate Ras and ERK1/ERK2 MAPKs through $G_{\alpha q}$ and $G_{\beta\gamma}$. Activation of this pathway also stimulates 5-HT-dependent proliferation and foci formation. When injected into nude mice, the foci cause tumors *(254)* through the Ras–ERK pathway.

The 5-HT_{2B} receptor stimulates cell cycle progression through an ERK-dependent pathway that results in hyperphosphorylation of Rb through upregulation and activation of cyclin E/cdk2 and cyclin D1/cdk4 kinases. The 5-HT_{2B} receptor also rapidly transactivates the platelet-derived growth factor (PDGF) receptor kinase. Cellular proliferation, activation of the ERK, activation of the PDGF receptor, and upregulation and activation of both cyclin D1 and cyclin E depend on the nonreceptor tyrosine kinase, Src *(255)*.

The 5-HT_{2B} receptors regulate embryonic morphogenesis, probably by modulating the differentiation of myocardial precursor cells and cranial neural crest cells. 5-HT_{2B} receptor antagonists interfere with cranial neural crest cell migration and induce their apoptosis, and they cause abnormal sarcomeric organization in the subepicardium and induce the specific absence of the trabecular cell

layer in the ventricular myocardium *(256)*. The specific signaling pathways involved in the regulation of cellular migration, apoptosis, and morphogenesis by the 5-HT_{2B} receptor have not been completely defined, but a study by Nebigil et al. suggested that ErbB-2 might be involved *(257)*. 5-HT_{2B} receptor knockout mice suffer from embryonic and neonatal death caused by specific heart defects *(257)*. Knockout embryos lack cardiac trabeculae, resulting in mid-gestation lethality. Surviving newborn mice display severe ventricular hypoplasia associated with impaired cardiomyocyte proliferative capacity. Surviving adult knockout mice show severe cardiac histopathological changes, including ventricular dilation and myocyte disarray. They also found that there was a specific reduction in the expression levels of the receptor tyrosine kinase, ErbB-2. Their data suggest that the 5-HT_{2B} receptor probably requires ErbB-2 in the signaling pathway for cardiac differentiation *(257)*. Thus, tyrosine kinases such as Src, ErbB-2, and the PDGF receptor might be important signaling partners of the 5-HT_{2B} receptor.

The 5-HT_{2B} receptor increases production of NO in human coronary artery endothelial cells *(142)* and in porcine cerebral artery, where this results in vasorelaxation *(207)*. 5-HT_{2B} receptors endogenously expressed in a variety of cell types (IC11 cells, *Mastomys natalensis* carcinoid cells, or $LMTK^-$ fibroblasts) increase intracellular cGMP levels through dual activation of constitutive nitric oxide synthase (cNOS) and inducible NOS (iNOS). Increases in cGMP levels require the presence of a PDZ motif in the carboxyl-terminal tail of the 5-HT_{2B} receptor *(258)*. Furthermore, activation of iNOS, but not cNOS, appears to be mediated through $G_{13\alpha}$. Thus, the 5-HT_{2B} receptor, through a carboxyl-terminal PDZ domain, can activate NO production through two overlapping pathways: 5-HT_{2B} receptor → PDZ domain → cNOS → NO/cGMP and 5-HT_{2B} receptor → PDZ domain + $G_{13\alpha}$ → iNOS → NO/cGMP *(258)*.

The 5-HT_{2B} receptor stimulates bicarbonate secretion in cultured rat epididymal epithelium through a pathway that is dependent on prostaglandin synthesis *(259)*. Because the 5-HT_{2B} receptor has been reported to stimulate phosphorylation of the serotonin transporter, SERT, and of the Na^+/K^+-ATPase *(260)*, the receptor might also regulate those transporters. The 5-HT_{2B} receptor does not typically regulate cAMP production, but when heterologously expressed in AV12 cells, it induces a modest increase in cAMP levels *(220)*.

Like the 5-HT_{2A} and 5-HT_{2C} receptors, the 5-HT_{2B} receptor can interact with MUPP1, although functional significance of this interaction is unclear *(238)*.

3.3. The 5-Hydroxytryptamine$_{2C}$ Receptor

The 5-HT_{2C} receptor was originally termed the 5-HT_{1C} receptor and was identified as a high-affinity ^{3}H-5-HT binding site in the choroid plexus *(186)*. The cloning of the receptor from several species *(185,261–263)* led to the

realization that it is much more closely related to the 5-HT_2 receptor family than to the 5-HT_1 receptor family. The 5-HT_{2C} receptor is expressed nearly exclusively in the brain, with high levels in the choroid plexus, nucleus accumbens, cortex, amygdala, hippocampus, caudate nucleus and substantia nigra *(264–266)*. The 5-HT_{2C} receptor gene has three introns *(148,262,267)*. Despite the presence of three introns, only a single splice variant of the 5-HT_{2C} receptor has been reported, and this is a truncated nonfunctional variant *(148,268)*. The 5-HT_{2C} receptor generates multiple functional receptor variants through a process called mRNA editing *(269)*. This unique feature will be discussed in Section 3.3.3.

3.3.1. Major Signals of the 5-Hydroxytryptamine$_{2C}$ Receptor

The major linkage of the 5-HT_{2C} receptor is to the activation of PLC in the brain *(270,271)* and transfected cells *(181,182,196,272)*. The activation of PLC is probably mediated through $G_{q/11\alpha}$ proteins in mammalian cells, although Chen et al. presented evidence that the 5-HT_{2C} receptor could couple to $G_{o\alpha}$ and $G_{i\alpha1}$ in *Xenopus* oocytes *(273)*. Tohda et al. also suggested that low-molecular-weight monomeric G proteins are important in the inositol phosphate hydrolysis induced by the 5-HT_{2C} receptor in COS-7 cells *(274)*.

The 5-HT_{2C} receptor regulates a number of effectors through PLC-, Ca^{2+}-, or PKC-dependent mechanisms. The majority of the work in this area has used the *Xenopus* oocyte system. 5-HT_{2C} receptors expressed in *Xenopus* oocytes activates Ca^{2+}-gated Cl^- channels through stimulation of Ca^{2+} release from inositol trisphosphate-sensitive intracellular stores *(275,276)*. When coexpressed in *Xenopus* oocytes, the 5-HT_{2C} receptor inhibits Kv1.3, a type n K^+ channel, through elevation of intracellular Ca^{2+} but not through PKC, CaM, or phosphatases *(277)*, and inhibits the activity of the voltage-activated K^+ channel, Kv1.5, through a PLC-dependent pathway *(278)*. 5-HT_{2C} receptors also inhibit GABA-A receptor channels by a Ca^{2+}-dependent, phosphorylation-independent mechanism in *Xenopus* oocytes *(279)*. This effect occurs through endogenous G_o proteins in the *Xenopus* oocytes and can be reproduced by coexpression of mammalian G protein α-subunits ($G_{o\alpha}$, $G_{o\beta}$, $G_{q\alpha}$, $G_{11\alpha}$) along with the 5-HT_{2C} receptor. The effect was mediated by a PLC-β endogenous to the *Xenopus* oocytes. The 5-HT_{2C} receptor, when coexpressed in *Xenopus* oocytes with rat brain mRNA, also inhibits an inwardly rectified, Ba^{2+}-sensitive K^+ conductance through a PKC-dependent pathway *(276)*.

3.3.2. Secondary Signals of the 5-Hydroxytryptamine$_{2C}$ Receptor

The 5-HT_{2C} receptor can modulate a variety of secondary signals. The 5-HT_{2C} receptor inhibits K^+ channels in the choroid plexus *(280)* and when expressed in cell lines *(281,282)*. The 5-HT_{2C} receptor stimulates an apical Cl^- conductance in mouse choroid plexus *(280)*. It can modulate the production of NO, although the

effects are variable. The 5-HT_{2C} receptor attenuates both NMDA-stimulated cGMP production and increases in NO in rat cerebellum *(283)* and in human neocortical slices *(88)*. In contrast, the 5-HT_{2C} receptor stimulates cGMP and NO production in the choroid plexus through a pathway that requires Ca^{2+}, PLA_2, and lipoxygenase activity *(284)*.

The 5-HT_{2C} receptor activate PLA_2-mediated arachidonic acid release *(181)*. It can also regulate cAMP production under some circumstances. When the receptor is expressed at very high density (>10 pmol/mg membrane protein) in stably transformed AV12 cells, it has been shown to inhibit forskolin-stimulated cAMP production through $G_{i/o}$ proteins. Pretreatment with pertussis toxin also unmasked a small stimulatory effect of the 5-HT_{2C} receptor on cAMP accumulation. When expressed at low density, the 5-HT_{2C} receptor was shown to potentiate forskolin-stimulated cAMP production by about twofold, probably through the release of G protein βγ-subunits *(220)*.

Like the 5-HT_{2A} and 5-HT_{2B} receptors, the 5-HT_{2C} receptor participates in mitogenic signaling. When expressed in NIH-3T3 cells, the recombinant $5HT_{2C}$ receptor stimulates the formation of transformed foci, and injection of cells derived from the foci into nude mice results in the generation of tumors *(285)*. However, when expressed in CCL36 fibroblasts, the 5-HT_{2C} receptor mediates only a very weak transforming activity *(286)*.

The 5-HT_{2C} receptor can modulate a number of distinct transport processes. Recombinant 5-HT_{2C} receptors expressed in 3T3 cells can stimulate amyloid precursor protein processing through a pathway that involves PKC and PLA_2 *(219)*. The 5-HT_{2C} receptor activates an electrogenic Na^+/Ca^{2+} exchanger in histaminergic neurons in the tuberomammillary nucleus or the hippocampus, resulting in increased firing rates of the neurons and depolarization *(287)*. The 5-HT_{2C} receptor has been implicated in the secretion of prolactin *(288)*, corticosterone, and ACTH *(289)*. The 5-HT_{2C} receptor inhibits the neuronal release of norepinephrine and dopamine, but not 5-HT *(290)*.

3.3.3. Unique Aspects of 5-Hydroxytryptamine$_{2C}$ Receptor Signaling

There are two unique aspects of 5-HT_{2C} receptor signaling. The receptor has been shown in a yeast two-hybrid screen to interact with a novel multivalent PDZ protein called MUPP1. The C-terminus of the 5-HT_{2C} receptor selectively interacts with the 10th PDZ domain of MUPP1, which might serve as a signaling scaffold *(238)*. The authors used a number of techniques to validate the interaction between 5-HT_{2C} receptors and MUPP1 in the rat choroid plexus and in transfected cells, including coimmunoprecipitation, immunohistochemistry, and confocal microscopy. The functional significance of the interaction between MUPP1 and the 5-HT_{2C} receptor is supported by studies showing that the deletion of the 5-HT_{2C} receptor, PDZ recognition motif prevents phosphorylation

of the receptor and delays its resensitization *(240)*. Indeed, agonist-induced phosphorylation of Ser^{458} decreases the association of the receptor with MUPP1 *(241)*. Thus, protein interactions through the 5-HT_{2C} receptor PDZ domain could modify receptor signaling.

Becamel et al. *(239)* used a proteomic approach to identify a number of other putative 5-HT_{2C} receptor-interacting proteins. These include proteins that interact with the carboxyl-terminal PDZ domain such as activin receptor-interacting protein-1 (ARIP-1), membrane associated guanylate kinase (MAGUK) with inverted domain structure, SAP97, SAP102, PSD-95, MPP-3, P55 MAGUK subfamily member, and the Veli-3–CASK–MINT1 ternary complex. Other proteins interacted with the carboxyl terminus independent of the PDZ domain. These included CaM, CAPZ-α2, CAPZ-β, PICOT, β-actin, 2810409H07Rik protein, dynamin 1, and α-fodrin (*see* Table 2) *(239)*. The functions of these proteins to modulate 5-HT_{2C} receptor signaling remain to be defined.

The 5-HT_{2C} receptor exhibits a novel mechanism for generating multiple functional receptor variants through a process called mRNA editing *(269)*. Burns et al. *(269)* observed that five adenosines (referred to as sites A–E) within the coding sequence of the putative i2 loop of the 5-HT_{2C} receptor were converted to guanosines at the RNA level. The mRNA editing probably occurs by deamidation of the adenosines to inosines, which are then read at the RNA level as guanosines *(291)*. This phenomenon appears to be unique for the 5-HT_{2C} receptor among GPCRs. Indeed, neither the 5-HT_{2A} or 5-HT_{2B} receptors or other GPCRs has been shown to undergo mRNA editing *(292)*. In the human 5-HT_{2C} receptor, combinations of A → G conversions can result in at least 21 discrete mRNA species encoding 14 different editing variants of the 5-HT_{2C} receptor. The genomic (unedited) receptor expresses amino acids INI (in the sequence IRNPI in the i2 loop), whereas conversion of all three amino acids results in VNV, VSV, VGV, or VDV *(232,292,293)*. These are referred to as 5-$HT_{2C\text{-}INI}$, 5-$HT_{2C\text{-}VNV}$, 5-$HT_{2C\text{-}VSV}$, 5-$HT_{2C\text{-}VGV}$, and 5-$HT_{2C\text{-}VDV}$ receptors. Other human receptor variants are referred to as 5-$HT_{2C\text{-}VNI}$, 5-$HT_{2C\text{-}VSI}$, 5-$HT_{2C\text{-}ISI}$, 5-$HT_{2C\text{-}INV}$, 5-$HT_{2C\text{-}ISV}$, 5-$HT_{2C\text{-}IGI}$, 5-$HT_{2C\text{-}VGI}$, 5-$HT_{2C\text{-}IDI}$, and 5-$HT_{2C\text{-}IDV}$ receptors. In rats, only four A → G conversion sites (termed sites A–D) have been identified, resulting in 11 discrete mRNAs and 7 different receptor protein isoforms *(293,294)*.

The functional significance of the various edited isoforms of the 5-HT_{2C} receptor has been underscored by their differential abundances of expression in total brain and hypothalamic mRNA populations *(293)* and distinct functional properties. Moreover, the variant edited receptors exhibit differential abilities to bind various ligands, to mobilize intracellular Ca^{2+}, and to stimulate hydrolysis of inositol phosphates *(232,292,293)*. Burns and colleagues showed that mRNA editing of the 5-HT_{2C} receptor leads to a 10- to 15-fold reduction in the efficacy of the interaction between receptors and their

G proteins *(269)*. Those observations led the authors to speculate that mRNA editing played important roles in determining the specificity of signaling by the 5-HT_{2C} receptor. Indeed, the authors subsequently demonstrated that the nonedited 5-$HT_{2C\text{-}INI}$ receptor variant functionally couples to $G_{q\alpha}$ and $G_{13\alpha}$, resulting in an actin cytoskeleton rearrangement, whereas the extensively edited 5-$HT_{2C\text{-}VSV}$ and 5-$HT_{2C\text{-}VGV}$ receptor variants did not couple to $G_{13\alpha}$ and did not induce cytoskeletal rearrangements. Thus, RNA editing of the 5-HT_{2C} receptor selectively receptor regulates coupling to G proteins and downstream signals *(295)*.

4. 5-Hydroxytryptamine$_4$ Receptors

Three subtypes of 5-HT receptors couple primarily to the activation of AC through G_s, including the 5-HT_4, 5-HT_6, and 5-HT_7 receptors *(296)*. Unlike the 5-HT_6 and 5-HT_7 receptors, which were cloned prior to being characterized, the 5-HT_4 receptor was well characterized pharmacologically and functionally *(297,298)* prior to its cloning *(299–304)*. The primary functions of the 5-HT_4 receptor are prokinetic gastrointestinal actions and positive isotropy, chronograph, and lusitropy in the cardiac atria *(305)*.

The human 5-HT_4 gene is highly complex, containing multiple introns *(303,304)* and thus could have splice variants. Indeed, Gerald et al. reported the isolation of two rat brain 5-HT_4 receptors, which were originally termed 5-HT_{4S} and 5-HT_{4L} to indicate differences in their predicted lengths (387 vs 406 amino acids) *(302)*. The putative protein products share sequence identity from amino acids 1–358 and diverge in amino acid sequence after L^{358}. After the cloning of murine cDNA homologs of these receptors *(301)*, the subtypes were redesignated as the 5-HT_{4a} and 5-HT_{4b} receptors to be more in keeping with the recommendations of the Nomenclature Subcommittee of the International Union of Pharmacology (NC-IUPHAR) *(175)*. Two subsequent reports suggested that the correct length of the longer 5-HT_{4b} receptor is actually 388 amino acids *(304,306)*. There are currently 10 known splice variants of the 5-HT_4 receptors. These include 5-HT_{4a} to 5-HT_{4i} receptors, which differ only in the length of their intracellular carboxyl-terminal tails *(300,307–309)*, and the 5-HT_{4n} receptor, which extends only one amino acid beyond the splice site *(310)*. All of the 5-HT_4 receptors activate AC in an agonist-dependent fashion and many of the splice variants exhibit a high degree of constitutive activity.

The 5-HT_4 receptors are expressed in the brain and periphery. There might be subtle differences in the distribution of 5-HT_4 receptor splice variants depending on species *(301,307,308,311–314)*. 5-HT_{4a}, 5-HT_{4b}, 5-HT_{4c}, and 5-HT_{4d} receptors are expressed variably in the brain, atrium, and gut *(308,311, 312,315)*. In contrast, mouse and rat 5-HT_{4d} receptors as well as mouse 5-HT_{4e} and 5-HT_{4f} receptors appear to be brain-specific *(307)*.

4.1. Major Signals of the 5-Hydroxytryptamine$_4$ Receptor

The primary signaling pathway of 5-HT$_4$ receptors is the stimulation of AC in tissues *(297,298,315–318)* or when heterologously expressed in cells *(299–302,307,308,311,312,319)*, and all of the splice variants appear to efficiently stimulate AC.

Not surprisingly, many of the effects of 5-HT$_4$ receptors are linked to the activation of PKA, which is activated by increases in cAMP. PKA-mediated effects of the 5-HT$_4$ receptor include inhibition of K^+ channels *(320)*, relaxation of the colonic circular smooth muscle *(321)*, positive lusitropy, chronotropy, and inotropy in the cardiac atria *(322,323)*, facilitation of dopamine release in the striatum *(324)*, regulation of a hippocampal Ca^{2+}-activated potassium current *(325)*, and activation of atrial L-type Ca^{2+} channels *(326)*. In addition, exogenously expressed 5-HT$_{4b}$ receptors have been shown to stimulate ERK in HEK293 cells through a pathway that is PKA, Raf, and Ras dependent, but which is independent of Rap1 *(327)*. However, not all effects of the 5-HT$_4$ receptors are mediated by PKA. For example, the human 5-HT$_{4g}$ receptor, when transfected into CHO cells, regulates the metabolism of the amyloid precursor protein APP695 through a cAMP-dependent and PKA-independent signaling pathway *(328)*. In that regard, the 5-HT$_{4e}$ receptor induces ectodomain shedding of a nonamyloidogenic form of amyloid precursor protein, sAPPα, through the cAMP-regulated guanine nucleotide exchange factor, epac, and the small GTPase Rac *(329)*.

4.2. Secondary Signals of the 5-Hydroxytryptamine$_4$ Receptor

The 5-HT$_4$ receptors modulate the activities of channels and transporters by increasing cAMP levels. These include activation of L-type Ca^{2+} channels *(326)*, chloride currents in human jejunal mucosa and rat distal colon *(330,331)*, and the I_f pacemaker current in atrial myocytes *(332)* and stimulation of aldosterone release from the adrenal glands *(333,334)*, striatal dopamine release *(324)*, hippocampal and frontal cortex acetylcholine release *(335,336)*, and hippocampal 5-HT release *(337)*. 5-HT$_4$ receptors also inhibit various channels, including a KV3.2-like delayed rectifier K^+ channel *(303)*, a voltage-activated K^+ channel in colliculi neurons *(320,338)*, a Ca^{2+}-activated, afterhyperpolarizing, and K^+ current in hippocampus *(325)*.

Not all 5-HT$_4$ receptor effects are mediated by cAMP. 5-HT$_4$ receptors inhibit the sodium-proton exchanger isoforms NHE-2 and NHE-3 in human intestinal epithelial cells and T-84 cells through Src-dependent phosphorylation of PLC-γ, elevation of intracellular Ca^{2+} levels, and subsequent activation of PKC-α *(339)*. 5-HT$_{4e}$ receptors increase phosphoinositide hydrolysis in CHO cells *(329)*. 5-HT$_{4a}$ receptors in neuroblastoma × glioma NIE-115 cells cause $G_{13\alpha}$ and RhoA-dependent neurite retraction and cell rounding.

4.3. Unique Aspects of 5-Hydroxytryptamine$_4$ Receptor Signaling

Although 5-HT$_4$ receptor splice variants uniformly are able to stimulate AC activity in the presence of 5-HT *(308)*, some interesting differences in the behavior of the splice variants have been observed *(307)*. The 5-HT$_{4e}$ and 5-HT$_{4f}$ receptors induce significantly more agonist-independent increases in AC activity than do some other 5-HT$_4$ receptor splice variants. A model has been presented in which a serine- and threonine-rich portion of the carboxyl terminus (residues 346–359), which is shared by all of the splice variants, is believed to hold the receptor in an inactive conformation. In this model, longer splice variants such as 5-HT$_{4a}$ and 5-HT$_{4b}$ receptors augment the inhibitory function of residues 346–359, whereas shorter splice variants like the 5-HT$_{4e}$ and 5-HT$_{4f}$ do not, resulting in increased basal cAMP levels for the shorter variants *(307)*. This fascinating story is likely to become more interesting, as the 5-HT$_4$ receptor splice variants differ in their expression of distinct PDZ domains *(296,307)*, which might alter their signaling properties or other functions.

In that regard, proteomic methods have been used to identify repertoires of proteins that selectively associate with the carboxyl termini of 5-HT$_4$ receptor splice variants *(340)*. Ten proteins were identified as potential binding partners of the 5-HT$_{4a}$ receptor, including seven with at least one PDZ domain (Table 2). These include ARIP-1 (which is also called membrane-associated guanylate-kinase inverted-2 [MAGI-2]), MPP-3 (a member of the P55 membrane-associated guanylate-kinase [MAGUK]) family, new sorting nexin SNX27a (also called Mrt1a), ezrin/radixin/moesin-binding phosphoprotein 50 (EBP50 or NHERF), Veli1-Veli3, guanine aminase, and peroxiredoxin 5. Ulip2 also bound to the 5-HT$_{4a}$ receptor carboxyl terminus, although its sequence lacks an obvious PDZ domain and/or PDZ ligand *(340)*. It is noteworthy that many of these proteins are known to be involved in membrane targeting or protein trafficking. In that regard, the authors demonstrated by coimmunoprecipitation and confocal microscopy that the 5-HT$_{4a}$ receptor (but not the 5-HT$_{4b}$ or 5-HT$_{4e}$ receptors) interacts with both SNX27 and NHERF. Moreover, SNX27 appeared to be responsible for targeting the 5-HT$_{4a}$ receptor, whereas the 5-HT$_{4a}$ receptor was colocalized to microvilli with NHERF and ezrin *(340)*.

In contrast, only three proteins were associated with the 5-HT$_{4e}$ receptor carboxyl terminus (Table 2). These included two PDZ domain-containing proteins, the neuronal isoform of nitric oxide synthase (nNOS), and CIPP. The 5-HT$_{4e}$ receptor carboxyl terminus interacted with Sec23 in a non-PDZ-associated fashion. Sec23 is involved in the endoplasmic reticulum (ER) vesicular budding and ER-to-Golgi transport of proteins. Interestingly the 5-HT$_{4b}$ receptor carboxyl terminus (which lacks a PDZ domain), did not interact with any of the 13 proteins that interacted selectively with the 5-HT$_{4a}$ or 5-HT$_{4e}$ receptors. It seems likely

that 5-HT_4 receptor-interacting proteins can selectively participate in membrane localization, trafficking, and possibly signal transduction of the various isoforms.

Receptor splice variants could differ in desensitization properties, as Mialet and colleagues detected subtle differences in the rates of desensitization between the 5-HT_{4d} and 5-HT_{4e} receptors *(341)*. Other subtle differences between the isoforms might exist. For example, two highly potent putative 5-HT_4 receptor antagonists, SB204070 and RS39604, possess partial agonist properties at the human 5-HT_{4d} receptor *(312)*. Pindon and colleagues detected interesting differences between 5-HT_{4a} and 5-HT_{4b} receptors transfected in HEK293 cells *(342)*. They used [^{35}S]GTP-γ-S binding and cAMP assays to establish that the 5-HT_{4b} receptor couples to $G_{i/o\alpha}$ proteins, in addition to $G_{s\alpha}$, whereas the 5-HT_{4a} receptor couples only to $G_{s\alpha}$. Surprisingly, the 5-HT_{4a} receptor was able to trigger an increase in the intracellular Ca^{2+} concentration, independent of inositol phosphate formation, cAMP, or PKA *(342)*. Thus, it seems likely that the differences in the carboxyl termini of the 5-HT_4 receptor isoforms might influence their functional properties in other ways that have not yet been elucidated.

5. 5-Hydroxytryptamine$_5$ Receptors

The 5-HT_5 receptor family has two known members, designated as 5-HT_{5A} and 5-HT_{5B} receptors *(343)*. The 5-HT_{5A} and 5-HT_{5B} receptors were first cloned from the mouse *(344,345)* and then from the rat *(346)*. The human 5-HT_{5A} receptor homolog was subsequently cloned *(347)*, but the 5-HT_{5B} receptor does not seem to be functionally expressed in humans *(347)*. The 5-HT_5 receptors are among the least studied of the 5-HT receptors, especially regarding pharmacology and signaling. Most of the work on the 5-HT_5 receptors has focused on the 5-HT_{5A} receptor because the 5-HT_{5B} receptor is not expressed in the human *(343)*.

The mouse, rat, and human 5-HT_5 receptors have been expressed in various cell lines. Initial reports did not divulge the signal transduction systems of the 5-HT_5 receptors *(345,346)*, although agonist binding to the recombinant receptor was found to be guanine nucleotide sensitive, suggesting that the 5-HT_5 receptors couple to G proteins *(344)*. Inhibition of forskolin-stimulated AC activity was first reported for the rat 5-HT_{5A} receptor expressed in C6 glioma cells *(348)*. Agonist-induced inhibition of AC activity has also been demonstrated for the human 5-HT_{5A} receptor expressed in HEK293 cells *(349,350)*. The coupling of the 5-HT_5 receptors to the inhibition of AC suggests that the receptors couple through G_i, and/or G_o, protein α-subunits. Indeed, agonist-induced stimulation of ^{35}S-GTPS binding by the human 5-HT_{5A} receptors expressed in HEK293 cells was shown to be pertussis toxin sensitive *(349)*. Francken et al. used the baculovirus system to coexpress the human 5-HT_{5A} receptor in sf9 insect cells with

various G protein α-subunits and showed that the receptor could couple to $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha}$, but not to $G_{z\alpha}$, $G_{s\alpha}$, $G_{12\alpha}$, or $G_{13\alpha}$ *(351)*. Coexpression of receptors and $G_{i\alpha}$ subunits in the absence of exogenous G protein βγ subunits produced increases in both agonist affinity and maximum G protein activation that were smaller than those in the presence of G exogenous G protein βγ subunits, suggesting that coexpression of each three subunits of the G protein heterotrimers improves 5-HT_{5A} receptor–G protein coupling.

Although it has now been clearly established that the primary coupling mechanism of the 5-HT_5 receptors is to inhibit AC, the receptors also participate in other signaling pathways. Noda et al. expressed 5-HT_{5A} receptors in undifferentiated C6 glioma cells, documenting inhibition of AC *(352)*. They also documented that 5-HT_{5A} receptors inhibited ADP-ribosyl cyclase and increased outward currents by whole-cell patch clamp and that those effects were sensitive to pertussis toxin. They further demonstrated that 5-HT_{5A} receptors induced a transient increase in phosphoinositide hydrolysis and that the increase was significant in that current activation could be attenuated by blocking the inositol triphosphate (IP_3) receptor *(352)*. Based on those effects, the authors concluded that 5-HT_{5A} receptor mobilizes Ca^{2+}; however, they did not measure Ca^{2+} levels. The 5-HT_{5A} receptor also has been demonstrated to couple to inwardly rectified potassium (GIRK) channels in *Xenopus* oocytes *(353)*.

6. 5-Hydroxytryptamine$_6$ Receptors

The rat 5-HT_6 receptor DNA was cloned by two groups in 1993 *(354,355)*. The human 5-HT_6 receptor was cloned in 1996 *(356)* and the murine receptor was cloned in 2001 *(357)*. The 5-HT_6 receptor gene contains two introns *(354,355)*, and the human gene encodes a 440-amino-acid protein *(356)*. The 5-HT_6 receptor has high affinities for typical and atypical antipsychotic, antidepressant, and psychotropic drugs. It is expressed primarily in the brain, with lower levels in the stomach and adrenal gland *(354,355)*. Antibodies, antisense oligonucleotides, and radioligand-binding studies suggest that the 5-HT_6 receptor is abundant in several brain regions, including cortical and limbic brain regions, the olfactory tubercle, caudate nucleus, hippocampus, nucleus accumbens, and striatum *(358–362)*. Interestingly, the 5-HT_6 receptor regulates cholinergic and glutamatergic neuronal activity (rather than dopaminergic) and has been proposed as a regulator of feeding, cognition, emotion, learning, and memory *(363–368)*.

The primary signaling linkage of the 5-HT_6 receptors is to the stimulation of AC *(355,356,360,369)*. Native 5-HT_6 receptors stimulate AC activities in pig caudate membranes and in striatal neurons *(370)*. 5-HT_6 receptors also increase cellular levels of cAMP when transfected into JEG-3, COS-7, and HEK293 cells *(357,371)*. In HEK293 cells, the increases in cAMP levels are

mediated primarily through stimulation of AC Type 5 (AC5, a G_s-sensitive isoform), but not through CaM-regulated AC isoforms, AC1 and AC8 *(369)*. In aggregate, the aforementioned signaling studies strongly support elevation of cAMP through G_s as the primary signaling linkage of the 5-HT_6 receptor. Mutagenesis studies have provided further evidence of strong coupling between the 5-HT_6 receptor and G_s, documenting that the carboxyl-terminal portion of the third intracellular loop of the 5-HT_6 receptor is critical in coupling to G_s, in a manner similar to the coupling of 5-HT_2 receptors to G_q *(372,373)*. In the mouse receptor, coupling to G_s occurs through a critical region spanning amino acids 264–268 at the carboxyl-terminal end of the third intracellular loop of the receptor.

Several possible splice variants of the 5-HT_6 receptor have been described, but the functional significance of those variants of 5-HT_6 receptors has not been established *(354,374)*. Messenger RNA for at least one of the splice variant is expressed in the human caudate, although transfection studies into COS-7 cells demonstrated membrane expression of the truncated protein, without apparent function *(374)*.

7. 5-Hydroxytryptamine$_7$ Receptors

The 5-HT_7 receptors have high affinities for atypical antipsychotic agents and might mediate some of their effects *(375)*. 5-HT_7 receptors have been cloned from a number of mammalian species *(375–382)*. Likely homologs have been cloned from *Xenopus laevis (383)*, *Caenorhabditis elegans* (ser-7B) *(384)*, *Aedes aegypti (385)*, and *Drosophila melanogaster* (5-HT_{dro1}) *(386)*. The human 5-HT_7 receptor gene contains two introns *(379,387,388)* and was initially reported to encode a protein of 445 amino acids *(376)*. The presence of the introns suggested that splice variants could exist. Indeed, four splice variants (5-HT_{7a}, 5-HT_{7b}, 5-HT_{7c}, and 5-HT_{7d}) have been described.

The recent development of selective 5-HT_7 receptor antagonists such as SB269970-A and SB656104-A has facilitated the linkage of 5-HT_7 receptors to specific effects in various brain regions *(389,390)*. The 5-HT_7 receptors are highly expressed in the brain, particularly in the neocortex, hippocampus, and hypothalamus, as well as in the suprachiasmatic nucleus, where they have been proposed to participate in the regulation of circadian rhythm *(377,391)*. There is increasing evidence that 5-HT_7 receptors play a role in the control of circadian rhythms and sleep, anxiety, cognitive disturbances, and possibly vasoactive headaches *(390)*. 5-HT_7 receptors have been demonstrated to inhibit serotonin release in slices of rat raphe nuclei *(392)*. 5-HT_7 receptors are expressed endogenously in astrocytes and three splice variants (5-$HT_{7/a/b/d}$) are expressed in various human glioblastoma cell lines *(393,394)*, where they mediate increases in cAMP levels. 5-HT_7 receptors also increase progesterone production by human

granulosa-lutein cells *(395)*. In addition to being expressed in neurons, the 5-HT_7 receptor is expressed in glial cells *(394,396)*. Autoradiographic studies demonstrate that 5-HT_7 receptors are expressed most abundantly in the anterior thalamus and dentate gyrus. There are intermediate levels of receptor expression in the hypothalamus, anterior cingulated gyrus, hippocampus, amygdala, and various brainstem nuclei. This pattern of distribution suggests involvement in affective behavior and cognition *(389)*.

The 5-HT_7 receptors also are expressed in the periphery, where their best characterized function is to mediate relaxation of vascular smooth muscle *(397)*. In particular, 5-HT_7 receptors are expressed throughout the gut *(398)* and stomach *(399)*, as well as in vasculature *(400,401)*. 5-HT_7 receptors are also expressed in adrenal glomerulosa cells *(402,403)* and corneal epithelial cells *(404)*. In the adrenal gland, 5-HT_7 receptors stimulate the release of aldosterone from adrenal glomerulosa cells *(402,403)* by increasing cAMP levels.

7.1. Major Signals of the 5-Hydroxytryptamine$_7$ Receptor

Like the 5-HT_4 and 5-HT_6 receptors, 5-HT_7 receptors *(405)* primarily couple to the stimulation of AC through G_s *(375–379,395)*. Transfection of the 5-HT_7 receptor into insect *(397,406)* and various mammalian cells *(375–380,384,388, 393,400,407,408)* clearly demonstrates that the major coupling mechanism of the 5-HT_7 receptors is to stimulate AC. The 5-HT_7 receptors almost certainly stimulate AC primarily through activation of G_s, a hypothesis that is supported by the demonstration of strong precoupling of 5-HT_{7a} receptors (but not of 5-HT_{4b} receptors) to G_s *(409)*. In particular, several charged residues in the carboxyl portion of the third intracellular loop of the 5-HT_{7a} receptor are critical for coupling to G_s *(406)*.

7.2. Secondary Signals of the 5-Hydroxytryptamine$_7$ Receptor

There is growing awareness that 5-HT_7 receptors can signal through additional pathways. It is noteworthy that the 5-HT_{7a} receptor is capable of stimulating several distinct forms of AC, including G_s-sensitive AC5 and CaM-sensitive AC1 and AC8, when expressed in HEK293 cells *(369)*. The observation that the 5-HT_{7a} receptor (but not the 5-HT_6 receptor) can stimulate CaM-sensitive AC isoforms suggests that 5-HT_7 receptors could theoretically couple to G proteins other than G_s. Consistent with that idea, 5-HT_{7a} receptors can increase intracellular Ca^{2+} *(369)*, but the effect appears to be independent of phosphoinositide metabolism, G_i proteins, and PKC. 5-HT_7 receptors have been shown to increase intracellular Ca^{2+} levels in adrenal glomerulosa cells, and this occurs through PKA-dependent activation of T-type Ca^{2+} channels *(403)*. Those results are consistent with observations that 5-HT can activate Ca^{2+}-stimulated AC in the rat hippocampus and cerebral cortex *(410)*.

The 5-HT_7 receptors can activate mitogenic kinases. 5-HT_7 receptors activate p38 MAPK and protein PKC-ε in human U373 MG astrocytoma cells *(411)*. Although it is clear that 5-HT_7 receptors can activate ERK in several cell types, there is not a consensus on the pathway(s) through which this occurs *(327,412, 413)*. Lin and colleagues showed that in cultured hippocampal neurons and transfected PC12 cells, 5-HT_7 receptors increase cAMP through AC, activate PKA, and stimulate ERK *(413)*. Although activation of ERK by neuronal G_s-coupled receptors typically is mediated through a PKA-dependent pathway, stimulation of ERK was insensitive to PKA inhibition in the study by Lin and colleagues *(413)*. In addition, the authors presented evidence that ERK activation involves AC, elevations of cAMP, and cAMP-guanine nucleotide exchange factor(s) (cAMP-GEF), possibly Epac1 or Epac2 *(413)*. In contrast, exogenously expressed 5-HT_{7a} receptors stimulate ERK in non-neuronal HEK293 cells through a pathway that requires PKA, Raf, and Ras, but which is independent of Rap1 *(327)*. The differences might be the result of distinct signaling milieus present in neuronal and epithelial cell types.

Interestingly, all four splice variants stem from intron 2, which corresponds to the carboxyl terminus of the 5-HT_7 receptor. No splice variants have been reported to arise from the first intron, which is in the putative i2 loop of the receptor. The first clue that functional splice variants existed came from the observation that the sequence identified by Lovenberg et al. was truncated by 13 amino acids when compared with other cloned sequences and that the truncation occurred at the second intron *(375–377)*. Subsequently, the four splice variants were identified by several groups *(388,400,407,408)*.

The 5-HT_{7a} and 5-HT_{7b} receptors are expressed in rats and humans, whereas the 5-HT_{7c} receptor is expressed in rats and the 5-HT_{7d} receptor is expressed in humans *(400)*. The three human splice variants encode proteins of 448 *(5-HT_{7a})*, 435 *(5-HT_{7b})*, and 479 *(5-HT_{7d})* amino acids. All of the individual splice variants can stimulate AC *(400,407,408)*. To date, no clear functional differences between the splice variants have been reported in that coupling to AC, pharmacology, constitutive activity, and inverse agonist effects have been reported to be virtually indistinguishable *(414,415)*. However, it is possible that the splice variants could differ with respect to phosphorylation, desensitization, or trafficking. One noteworthy difference in the splice variants is the presence or absence of PDZ motifs in the distal carboxyl terminus. 5-HT_{7a} and 5-HT_{7d} receptor do not have a PDZ motif, whereas the 5-HT_{7b} and 5-HT_{7c} have group II and group I PDZ motifs, respectively *(296)*.

8. Conclusions

The variety of signaling mechanisms of the 5-HT receptors has become broadly accepted as a mechanism of “fine-tuning” receptor response to the

requirements of the specific cells and tissues in which they are expressed. The diversity of signaling is mediated by tissue- and cell-specific expression of a large number of 5-HT receptors and their postgenomic variants, by regulatory covalent modifications such as glycosylation, palmitoylation, and phosphorylation, by protein–protein interactions, by differential subcellular localization and trafficking, and by cell-specific and ligand-specific variables. This chapter has highlighted some insights into 5-HT receptor signaling diversity and has hinted at the promise of new development in the area of receptor-specific protein–protein interactions. Undoubtedly, the next few years hold promise that significant new discoveries will further add to the complex nature of 5-HT receptor signaling diversity.

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6

Agonist-Directed Trafficking of 5-HT Receptor-Mediated Signal Transduction

Kelly A. Berg and William P. Clarke

Summary

For decades, pharmacologists have maintained that drugs have two properties: affinity, which describes a drug's ability to bind to a receptor, and intrinsic efficacy, which describes the ability of a drug to change the behavior of a receptor toward cellular signaling machinery. Both affinity and intrinsic efficacy were defined to be ligand constants independent of response mechanisms and, thus, were of great predictive value for drug discovery efforts. Recently, clear evidence from many seven-transmembrane-spanning receptor systems has accumulated to indicate that ligands might not obey the tenets of classical receptor theory. In fact, intrinsic efficacy appears to be variable (changes with cell phenotype and physiological state) and dependent on cellular signaling machinery (response dependent), and ligands appear to have multiple intrinsic efficacies. Drugs can no longer be labeled as agonists (partial or full), inverse agonists (partial or full), or antagonists without reference to the receptor system (receptor and signaling pathway/response) and experimental conditions used. This newly discovered ligand behavior has been given many names by different groups: "agonist-directed trafficking of receptor stimulus," "functional selectivity," "stimulus trafficking," and "biased agonism," to name a few. Although the pharmacological actions of drugs have become considerably more complex, our pharmacological treasure chest might be much richer than previously thought, possibly heralding new and improved pharmacotherapy. In this chapter, we discuss the evidence for agonist-directed trafficking of serotonin receptor stimulus.

Key Words: Intrinsic efficacy; signal transduction; receptor theory; serotonin receptors.

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1. Introduction

1.1. Traditional Receptor Theory

The pioneering work of Clark *(1)*, Ariëns *(2)*, Stephenson *(3)*, and Fuchgott *(4)* provided a foundation for modern drug discovery based on the concepts embodied in classic occupation theory as presented in Equation 1:

$$E = f(S) = f\left(\frac{\varepsilon_A R_T}{1 + K_A/[A]}\right) \quad (1)$$

where the magnitude of an effect (E) produced when an agonist, at a specified concentration ([A]), interacts with a receptor population is a function (f) of the stimulus (S) produced by the agonist. The magnitude of the receptor stimulus, in turn, depends on four factors. Two of these are chemical properties of the ligand itself: the affinity-related parameter, K_A, and intrinsic efficacy, ε. The remaining two parameters are cell- or tissue-dependent properties: receptor density, R_T, and a monotonic and continuous function, f [frequently considered to be hyperbolic $\{S/(S+a)\}$], that describes the efficiency of signal transduction.

The drug-dependent properties of affinity and intrinsic efficacy are the most important to drug discovery. Affinity is the property of a drug that describes its capacity to bind to a receptor. In physicochemical terms, affinity can be described as the reciprocal of the equilibrium dissociation constant (K_A) of the drug for the receptor, which is defined as the rate constant for offset (k_{off}) divided by the rate constant for onset (k_{on}) of binding. Differential affinity for different receptors is a basis for drug selectivity, a valuable property of a drug because therapeutic specificity has been attributed to selective activation of receptor subtypes. Conversely, adverse drug effects have been attributed to poor selectivity of drugs and to the activation of receptors other than the one believed to be responsible for therapeutic effect. Consequently, a prime goal in drug discovery has been to identify or develop drugs with high affinity and high selectivity for individual receptor subtypes (however, *see* ref. *5* for an opposing viewpoint).

Intrinsic efficacy is defined as the capacity of a drug to elicit a stimulus from the activation of a single receptor. The concept of receptor stimulus was introduced by Stephenson to reflect the signal generated by a receptor population, in response to an agonist, which is converted into a response by the signal transduction machinery of a cell. The nonlinear nature of the stimulus–response function provided an explanation for why submaximal occupancy of a receptor population by an agonist could generate a maximal response, a phenomenon often called “receptor reserve” or “spare receptors.” In Furchgott’s model, intrinsic efficacy, like affinity, was a unique property of a drug–receptor pair that is

independent of the cellular signal transduction machinery and, thus, response independent. Intrinsic efficacy described the ability of a drug to activate a receptor and, because it was response independent, could theoretically be used to predict the efficacy of a drug in any system. In molecular terms, intrinsic efficacy can be thought of as the capacity of a drug to promote a change in receptor conformation that leads to increased affinity/efficacy of the activated receptor for the next molecule in the signal transduction cascade (e.g., G protein).

Although Furchgott's intrinsic efficacy cannot be measured directly, it can be assessed with relative measures that nullify tissue- or system-dependent factors (R_T and f) involved with the production of a measured response. For partial agonists, relative efficacy can be measured as the ratio of the maximal response of the agonist to the maximal response of a reference agonist, which is equal to the ratio of the intrinsic efficacy (ε) of the agonist to that of the reference agonist ($\varepsilon_A/\varepsilon_{Aref}$). For full agonists, the measurement of relative efficacy requires measurement of the occupancy–response curves (comparison of K_A/EC_{50} ratios) because of complications of receptor reserve. Because ε, as originally defined by Furchgott, is a unique drug property (with reference to a particular receptor) that is independent of the nature of the signaling components coupled to the receptor, $\varepsilon_A/\varepsilon_{Aref}$ and, therefore, agonist relative efficacy must also be independent of the signaling components of the cell and, therefore, be response independent. Thus, two agonists with relative efficacies of 0.3 and 0.6 as measured for stimulation of cAMP accumulation in one tissue should have the same values for measurement of hormone secretion in another tissue. Consequently, the ligand property of intrinsic efficacy was of considerable value for drug discovery, because it permitted the prediction of relative efficacy in any tissue from measurement in a test system.

1.2. Agonist-Directed Trafficking of Receptor Stimulus

In 1995, Kenakin presented an hypothesis for a new concept of agonist action that challenged the classical theory that intrinsic efficacy was response independent and hinted that drugs might have a far richer pharmacology than previously imagined. He termed this new concept "agonist-directed trafficking of receptor stimulus" *(6–8)*, but it has been also called "ligand-specific signaling," "functional selectivity," "biased agonism," or "differential engagement" by others (*see* refs. *9* and *10*). Kenakin's hypothesis stated that agonist interaction with a receptor promotes the formation/stabilization of *agonist-specific receptor conformational states*. For a receptor that couples to multiple signal transduction pathways within a cell, one consequence of agonist-specific receptor states could be differential activation of effectors. The phrase "agonist-directed trafficking of receptor stimulus" stems from the concept, developed by Stephenson (*see* above), of a receptor stimulus that is produced by the interaction of an agonist

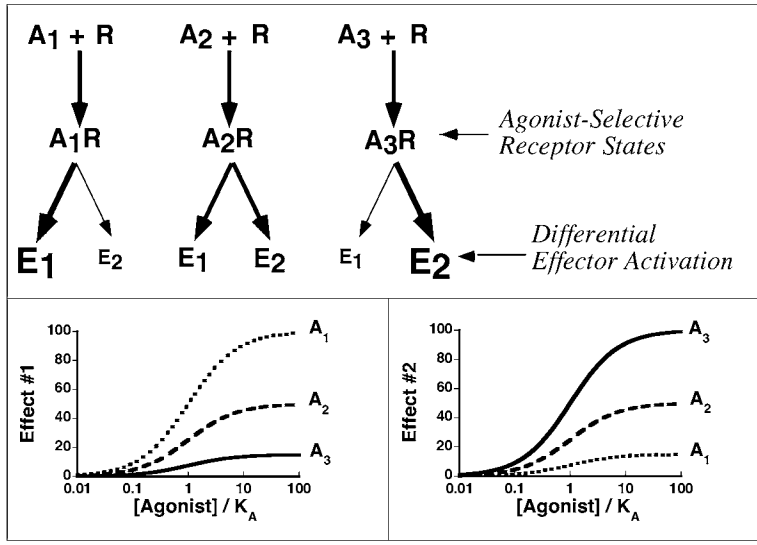

Fig. 1. Model of agonist-directed trafficking of receptor stimulus. **Top:** The interaction of agonist #1 (A_1) with a receptor (R) promotes the formation of an agonist-specific receptor conformation/state that more effectively activates effector pathway #1 (E_1) than E_2. When agonist #2 (A_2) interacts with the *same receptor on the same cell*, an agonist-specific receptor state is formed in which both effector pathways are activated equally. When A_3 interacts with R, E_2 is favored over that of E_1. **Bottom:** Concentration–response curves derived from a computer simulation with three agonists measuring two effector processes coupled to the same receptor. For effect #1, agonist #1 has greater efficacy than agonist #3, whereas for effect #2, A_3 has greater efficacy than A_1. For effect #1, rank order of agonist efficacy is $A_1 > A_2 > A_3$, whereas for effect #2, the rank is $A_3 > A_2 > A_1$. Because potency of full agonists is dependent on efficacy as well as affinity, stimulus trafficking can result in potency differences of an agonist measured for different responses.

with a receptor. An agonist is able to "traffic" the receptor stimulus differentially to various cellular effector pathways in an agonist-dependent manner. Figure 1 illustrates the stimulus trafficking concept and shows results predicted with a computer simulation. Because the pattern of biochemical responses within the cell elicited by agonist #1 ($E_1 \gg E_2$) differs markedly from that elicited by agonist #3 ($E_2 \gg E_1$), the ultimate net effect on cell function produced by agonist #1 would be different from that produced by agonist #3, even though both agonists activate the same receptor.

Initial support for Kenakin's hypothesis stemmed from reports of agonist effects that could not be explained with traditional receptor theory. Spengler et al. *(11)* found that $PACAP_{1–27}$ had a slightly greater potency than $PACAP_{1–38}$

for cAMP accumulation, whereas $PACAP_{1–38}$ was considerably more potent for phosphatidylinositol hydrolysis than $PACAP_{1–27}$ acting at the pituitary adenylyl cyclase–activating polypeptide (PACAP) receptor expressed in LLC PK1 cells. Robb et al. *(12)* reported that tyramine was almost two orders of magnitude more potent than octopamine in inhibiting cAMP accumulation from activation of *Drosophila* octopamine–tyramine receptor expressed in Chinese hamster ovary (CHO) cells, whereas octopamine was more potent than tyramine when the kinetics of changes in $[Ca^{2+}]_i$ were measured. Meller et al. *(13)* found reversal of the relative efficacies of dopamine D_2 receptor agonists (quinpirole and 3-PPP) in the striatum and anterior pituitary. Reversal of relative efficacy and reversal of potency (which is due to reversal of relative efficacy) is not consistent with traditional receptor theory.

Although there are many examples of differential agonist effects on different responses, before agonist-directed trafficking is invoked as an explanation it is important that differential effects be shown to result from the activation of a single receptor. In this regard, heterologous receptor systems are especially useful because they permit verification of the lack of agonist effect in the absence of the target receptor (by testing for effects of agonists in the parent cell line in the absence of the receptor under study). Also important is to rule out that apparent differential agonist effects on multiple responses are the result of "strength of stimulus" differences. For example, given two responses coupled to a receptor where one response is a consequence of the other [e.g., increases in intracellular calcium stemming from phospholipase C (PLC) activation], the second response is often amplified with respect to the first. In such a situation, an agonist with high efficacy would activate both responses, whereas an agonist with low efficacy might not produce a measurable response from the first, but because of amplification, it could at the second response. It would thus appear, falsely, that the weaker agonist is response-selective.

The most sound evidence for agonist-directed trafficking of receptor stimulus is to demonstrate response-dependent differences in agonist relative efficacy using maximal response ratios of partial agonists (where receptor reserve is not an issue). In as much as potency of agonists depends on efficacy as well as affinity *(3)*, response-dependent differences in agonist potency (especially reversal of agonist potency order) might also be indicative of stimulus trafficking.

Receptors, like all proteins, are known to spontaneously adopt a variety of conformations, and current concepts of receptor function (e.g., the extended and cubic ternary complex models) are based on the allosteric transitions between multiple conformational states. Some of these conformations are called "active" because they are able to interact with cellular signal transduction mechanisms (and are the basis of "constitutive receptor activity"). If one assumes that different receptor conformations might present different intracellular portions of a

receptor to transducer molecules (e.g., G proteins), then it is possible that different receptor conformations might differentially activate different signaling cascades by virtue of differential coupling/activation affinity for transduction molecules. In other words, constitutive receptor activity might be response dependent. The addition of a ligand can bias the spectrum of receptor conformations through selective affinity. Thus, a ligand can stabilize a conformation, or group of conformations, enriching that population while depleting others. Kenakin has coined the phrase "conformational cafeteria" to describe the process whereby ligands enter receptor space and selectively stabilize certain conformations for which they have the highest affinity *(14,15)*. As a consequence, ligand-selective receptor conformations might mediate ligand-selective signaling via a single receptor subtype.

There are several important implications for agonist-directed trafficking of receptor stimulus. From a receptor theory point of view, the notion that intrinsic efficacy is a drug property that is independent of the response measured is incorrect. In fact, ligands might have multiple intrinsic efficacies that differ with different signaling systems. Thus, rather than a single stimulus that differs only in strength (as proposed by Stephenson and Furchgott), ligands might deliver multiple stimuli of different intensities. Models of receptor function must be extended to include multiple active states (R*, R**, R***, etc.) with differential effector activity.

Furthermore, ligand effects might be cell phenotype dependent. It is possible that the capacity of a receptor protein to adopt different conformations could be restricted when the receptor is in different microenvironments associated with different cells. Also, given different stoichiometries and quality of signaling molecules expressed in different cells, the same receptor conformation in different cells might signal differently. This makes it especially important that the system chosen for drug discovery be therapeutically relevant.

Perhaps the most important implication of stimulus trafficking is that our pharmacological treasure trove might be much richer than previously thought. A central theme of drug discovery is to increase therapeutic selectivity (and minimize adverse effects) by developing drugs with high affinity for a single receptor subtype. Thus, ß-adrenoceptor agonists have a wide range of physiological activities, but agonists that are selective for the $ß_2$ subtype are better suited for treatment of asthma, as they are devoid of cardiostimulator properties. Within this framework, agonist activation of receptor subtypes differ only in strength of stimulus. Agonist-directed trafficking of receptor stimulus, however, suggests that ligands have more selectivity than that afforded by differential affinity for different receptor subtypes. By developing drugs that are not only receptor subtype-selective but also signal pathway-selective, it might be possible to increase therapeutic selectivity even further.

2. Agonist-Directed Trafficking of Receptor Stimulus and Serotonin Receptors

2.1. 5-HT$_{2C}$ Receptors

The 5-hydroxytryptamine$_{2C}$ (5-HT$_{2C}$) receptor subtype is a member of the seven-transmembrane-spanning (7-TMS) receptor superfamily, more commonly known as G protein–coupled receptors and has been shown to couple to a variety of cellular signal transduction systems. The 5-HT$_{2C}$ receptor, like all members of the 5-HT$_2$ receptor family, is best known for its capacity to activate second-messenger signal transduction cascades via pertussis toxin-insensitive G proteins of the $G_{q/11}$ and $G_{12/13}$ families *(16–18)*. Activation of the 5-HT$_{2C}$ receptor leads to both phospholipase A$_2$ (PLA$_2$)-mediated arachidonic acid (AA) release and PLC-phosphatidylinositol (PI) hydrolysis in the brain *(19,20)* and in heterologous expression systems *(21,22)*. Although it has been established that coupling to PLC is via $G_{\alpha q/11}$ *(16)*, the signal transduction molecule(s) mediating PLA$_2$-AA signaling is unknown. In addition, phospholipase D (PLD) is activated via G_{13} by the 5-HT$_{2C}$ receptor expressed naturally in choroid plexus epithelial cells and when expressed heterologously in NIH-3T3 cells *(23)*. This receptor can also couple to $G_{\alpha i}$ *(24,25)* and mitogen-activated protein kinase (MAPK) signaling *(26)* and can activate a variety of desensitization mechanisms *(27–29)*.

2.1.1. Agonist-Directed Trafficking of 5-HT$_{2C}$ Receptor Stimulus

Our studies with the 5-HT$_{2C}$ receptor provided the first solid evidence to support the agonist-directed trafficking of receptor stimulus hypothesis *(22,30)*. Using an unambiguous measure of agonist efficacy (maximal response to partial agonists), reversal of efficacy order for 5-HT$_{2C}$ agonists occurred for stimulation of PLC-mediated hydrolysis of inositol phosphates (PLC-PI) vs PLA$_2$-mediated AA release (PLA$_2$-AA). In CHO cells, stably expressing low levels of the human 5-HT$_{2C}$ receptor, the relative efficacies of a series of 5-HT$_{2C}$ agonists was measured by conducting full concentration–response curves to test agonists and to 5-HT as a reference agonist. Lack of receptor reserve for 5-HT in the cells was established using irreversible receptor alkylation with phenoxybenzamine. Importantly, both the PLC-PI and PLA$_2$-AA responses were independent of each other under the conditions of the experiment and were measured simultaneously from the same cells, thereby obviating possible differences in experimental conditions in interpretation of the results. Figure 2 shows concentration–response curves of 5-HT$_{2C}$ agonists expressed as the percentage of the maximal response of 5-HT (curves for 5-HT were run in each experiment). When expressed in this way, the plateaus of the curves represent the relative efficacy of the test agonist. Traditional receptor theory predicts that

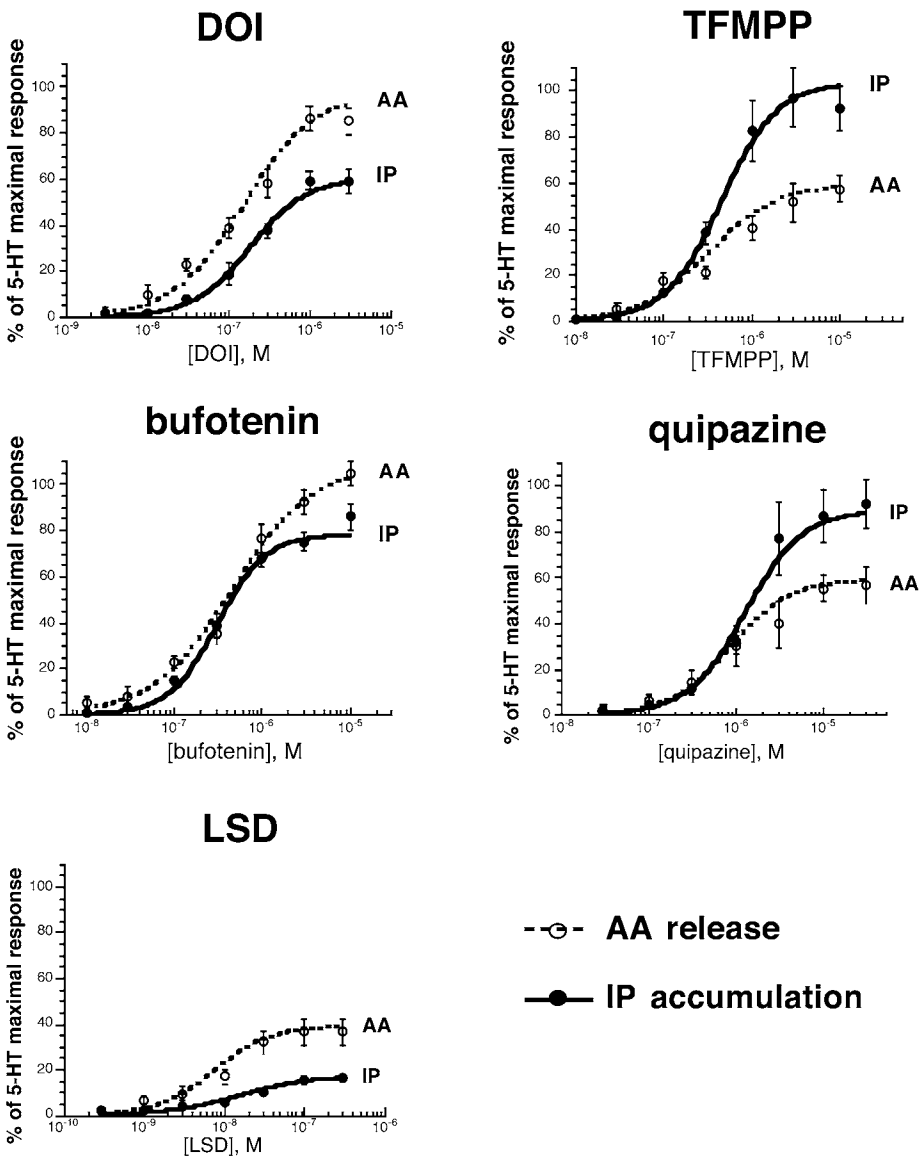

Fig. 2. Agonist-directed trafficking of 5-HT_{2C} receptor stimulus. Concentration–response curves for 5-HT2C agonists measuring AA release (PLA_2-AA) and IP accumulation (PLC-PI) in CHO-1C19 cells expressing the human 5-HT_{2C} receptor (approx 200 fmol/mg protein). Cells, in serum-free medium, were labeled with 1 μCi/mL [^{3}H]-*myo*-inositol (10–25 Ci/mmol) for 24 h and with 0.1 μCi/mL [^{14}C]-arachidonic acid (57 mCi/mmol) for 4 h at 37°C. Measurements of PLC-mediated IP accumulation and PLA2-AA release were made from the same multiwell, simultaneously, after 10 min of

the curves for both responses for each agonist should be superimposable—no difference in relative efficacy between responses. It is clear, however, that agonist relative efficacy differed depending on whether the PLC-PI or the PLA_2-AA response was measured, thus supporting stimulus trafficking for these ligands. More striking, however, is that there was a reversal of efficacy order. Some agonists (DOI, lysergic acid diethylamide [LSD], bufotenin) preferentially activated the PLA_2-AA response, whereas others (TFMPP and quipazine) favored the PLC-IP response. The rank order of agonist relative efficacy was dependent on the response measured. For the PLC-PI response, the rank order of relative efficacy was TFMPP = quipazine > bufotenin = DOI > LSD, whereas when the measure was AA release, the rank order of relative efficacy was bufotenin = DOI > quipazine = TFMPP > LSD. As expected for responses elicited from the activation of a single receptor in the absence of receptor reserve, the potency of the agonists did not differ between responses. Data such as these indicate that agonists are capable of providing multiple stimuli, of different intensity, upon receptor activation.

There have been other reports that also suggest that ligands might differentially signal via the 5-HT_{2C} receptor. Werry et al. *(31)* found that the relative efficacy of DOI and quipazine was reversed when measured for the PLC-PI and calcium responses vs phosphorylation of extracellular signal-related kinases 1 and 2 (ERK1/2) via the 5-HT_{2C} receptor expressed in CHO cells. Interestingly, it appeared that ERK1/2 phosphorylation was dependent on the PLC-PI signaling pathway. Because stimulus trafficking cannot occur between two responses that are sequentially connected (*see* Section 2.2 and ref. *22*), this result is somewhat surprising. It is likely, therefore, that DOI and quipazine recruit additional or alternative pathways to activate ERK, as the authors suggest. LSD also might elicit unique signaling via the 5-HT_{2C} receptor *(32)*. In NIH-3T3 cells expressing the 5-HT_{2C} receptor, it was reported that although LSD was equally efficacious as 5-HT in stimulating PLC-PI, it does not elicit increases in intracellular calcium or cause phosphorylation of the 5-HT_{2C} receptor. Although LSD is known to have a weaker efficacy than 5-HT, it does not appear that this differential signaling could be the result of

Fig. 2. *(continued)* agonist exposure. Data are expressed as the percentage of the maximal response to 5-HT, concentration–response curves for which were run in each experiment. Data were fit to a three-parameter logistic equation using nonlinear regression analysis to obtain estimates of E_{max}, EC_{50}, and slope parameters. Because of the absence of receptor reserve, the maximal agonist response represents agonist relative efficacy for each effector. Note that the drugs do not differ in potency between responses (AA vs IP), as expected for drugs acting at a single receptor in the absence of receptor reserve. Reproduced with permission from ref. *22*.

the "strength of stimulus" issue (*see* above) as the relative efficacies of other weak agonists (DOI, DOB, and m-CPP) were similar to that of 5-HT. However, strength of stimulus does appear to underlie ligand-specific coupling of the 5-HT_{2C} receptor (5-$HT_{2C\text{-}VSV}$ isoform, *see* Section 2.1.2) to $G_{\alpha q/11}$ vs $G_{\alpha i}$ *(33)*.

2.1.2. mRNA-Edited 5-HT_{2C} Receptor Isoforms and Stimulus Trafficking

The 5-HT_{2C} receptor is unique among 7-TMS receptors in that mRNA transcripts of the rat and human 5-HT_{2C} receptor have been found to undergo adenosine-to-inosine editing events at five sites that encompass amino acids 156–160 within the putative second intracellular loop of the encoded human receptor, resulting in the production of 14 receptor isoforms *(34,35)*. In the human brain, the nonedited receptor contains the amino acids isoleucine, asparagine, and isoleucine (i.e., INI) at positions 156, 158, and 160, respectively, and two of the principal edited isoforms expressed have valine, serine, and valine (i.e., VSV) or valine, glycine, and valine (i.e., VGV) corresponding to these amino acid positions (156, 158, and 160, respectively). Interestingly, RNA editing of the 5-HT_{2C} receptor is reduced in the brains of schizophrenic patients *(36)*, enhanced in the brains of suicides *(37,38)*, and regulated by various drug treatments *(37,39,40)*.

There are considerable differences in the signaling properties of edited 5-HT_{2C} isoforms. Although there are differences among the individual edited isoforms, editing can lower the affinity and potency of 5-HT (but not all ligands) and appears to reduce constitutive receptor activity toward PLC-PI *(34,41)*. We have found that the capacity of RNA-edited 5-HT_{2C} isoforms to subserve stimulus trafficking is also altered. Agonist relative efficacy was not different between PLC-PI and PLA_2-AA responses for the fully edited 5-$HT_{2C\text{-}VSV}$ and 5-$HT_{2C\text{-}VGV}$ isoforms compared with the nonedited 5-$HT_{2C\text{-}INI}$ receptor *(42)*. It is important to note that 5-HT was able to stimulate both PLC-PI and PLA_2-AA pathways, and although potency was reduced for both responses, as expected on the basis of reduced affinity of 5-HT *(34,41,43)*, the potency did not differ between the two responses. The lack of effector pathway dependence of agonist relative efficacy for the 5-$HT_{2C\text{-}VSV}$ and 5-$HT_{2C\text{-}VGV}$ isoforms indicates that the potential for agonists to traffic receptor stimuli differently to effector mechanisms is missing from these edited 5-HT_{2C} receptor isoforms. Because the 5-$HT_{2C\text{-}VSV}$ and 5-$HT_{2C\text{-}VGV}$ receptor isoforms differ from the nonedited isoform, 5-$HT_{2C\text{-}INI}$, in three amino acids located in the second intracellular loop, the lack of agonist-directed signaling by these edited isoforms suggests that the second intracellular loop plays an important role in transmitting agonist-specific information to the cellular signaling systems. The loss of stimulus trafficking suggests that either (1) these fully edited receptor

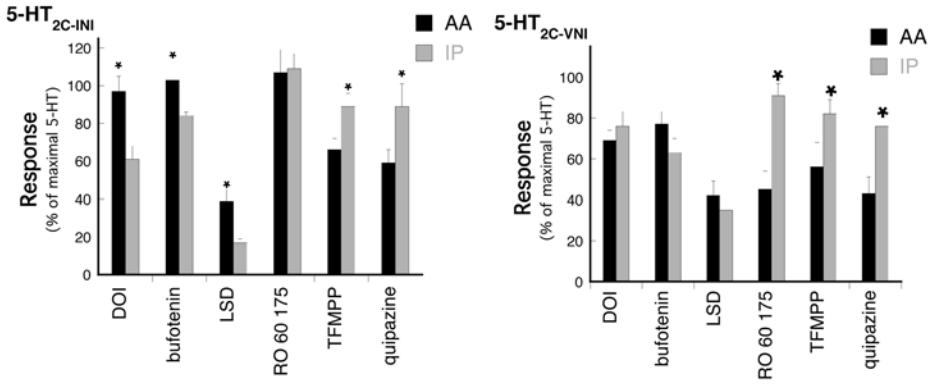

Fig. 3. Preferential activation of the PLA_2-AA response (AA) is lost at the 5-$HT_{2C\text{-}VNI}$ receptor isoform. Cells were incubated with maximal concentrations of the indicated agonists for 10 min and PLA_2-AA and PLC-PI responses were measured from the same cells, simultaneously. Data were normalized to the maximal 5-HT-mediated response determined in each individual experiment. Some data from the 5-HT_{2C}-INI cell line (shown on left) are taken from ref. 22. $*p < 0.05$; comparison of relative efficacy between AA and IP.

isoforms are not capable of adopting ligand-specific conformations or (2) the capacity of ligand-specific conformations to differentially interact with signaling molecules is impaired.

Recent data from our lab suggest that even a single, conservative amino acid change in the second intracellular loop that occurs as a result of mRNA editing (valine for isoleucine at position 156 of the human 5-HT_{2C} receptor; I156V; 5-$HT_{2C\text{-}VNI}$), alters, but does not abolish, stimulus trafficking. As shown in Fig. 3, agonists with greater relative efficacy for PLA_2-AA vs PLC-PI at the nonedited receptor (e.g., DOI and bufotenin) lost preferential efficacy toward PLA_2 in cells expressing the 5-$HT_{2C\text{-}VNI}$ receptor such that agonist relative efficacy for PLC-PI and PLA_2-AA was the same. However, agonists with preferential activity toward PLC-PI (e.g., TFMPP and quipazine) still retained their PLC-PI signaling preference. The reduction in agonist relative efficacy toward PLA_2-AA as a result of the I156V substitution was not due simply to the reduced general capacity of the edited isoforms to signal to PLA_2-AA, as the capacity for serotonin to stimulate PLC-PI and PLA_2-AA was not different for the 5-$HT_{2C\text{-}VNI}$ vs the 5-$HT_{2C\text{-}INI}$ isoforms. These data suggest that the I156V substitution, which occurs naturally as a result of mRNA editing, can dramatically alter the signaling profile of the 5-HT_{2C} receptor and underscore the need to study the other signaling pathways as well as the signaling capacity of each of the 5-HT_{2C} receptor isoforms.

2.1.3. Desensitization of 5-HT_{2C} Receptor System and Stimulus Trafficking

It is well known that prolonged exposure to some ligands can alter the responsiveness of a receptor system. The best known example of this is desensitization (reduced responsiveness) that occurs in response to treatment with agonists. Ligands can activate multiple mechanisms that result in uncoupling of receptors from effectors, alter responsiveness of signaling molecules and effectors and/or downregulate receptors and/or signaling molecules *(44,45)*. In the sense that these mechanisms are activated in response to receptor activation, they can be considered "effectors" in the same way that PLC or adenylyl cyclase are considered effectors. There are many examples where the relative efficacy of a ligand to elicit a classical response (e.g., stimulation of adenylyl cyclase or PLC) differs from that to elicit desensitization. For example, although methadone and 1-α-acetyl-methadone are weaker at inhibiting adenylyl cyclase than morphine, Yu et al. *(46)* found that methadone and l-α-acetyl-methadone were more efficacious at causing phosphorylation and producing desensitization of μ-opioid receptors than morphine. Also, Lewis et al. *(47)* reported that D_1-dopamine agonist efficacy to stimulate adenylyl cyclase activity was not a good predictor of the capacity of D_1 dopamine agonists to desensitize that system. For serotonin receptors, it has been recognized for many years that prolonged treatment with some nonagonist ligands (antagonists or inverse agonists, as measured with the PLC-PI response) can downregulate 5-HT_{2A} and 5-HT_{2C} receptors, a process that has been referred to as "anomalous downregulation" (for a review, *see* ref. *48*). Actions such as these could be the result of ligand-directed trafficking of the receptor stimulus differentially to desensitization/downregulation mechanisms, whereby a ligand could act as an antagonist for activation of PLC, but as an agonist for activation of downregulation mechanisms. For example, morphine promotes μ-opioid receptor conformations that are capable of activating $G_{\alpha i}$ for inhibition of adenylyl cyclase but that are not targets for desensitization mechanisms *(49)*. Agonist-specific receptor conformations of the μ-opioid receptor have been suggested on the basis of the differential capacity of protein kinase A to phosphorylate morphine-occupied vs DAMGO-occupied MOR in vitro *(50)*.

Based on the differential efficacies for morphine vs DAMGO and methadone to activate the μ-opioid receptor (as assessed by measurement of K^+ channel [GIRK] activation) and elicit internationalization of the μ-opioid receptor (which correlates negatively with efficacy to promote physiological tolerance), Whistler et al. *(51)* put forth the term RAVE (relative activity versus endocytosis) as a measure that might provide insight into an opiate drug's ability to produce tolerance and dependence. Thus, morphine has a high RAVE value (it is more efficacious at activating than internalizing the μ-opioid receptor) whereas methadone

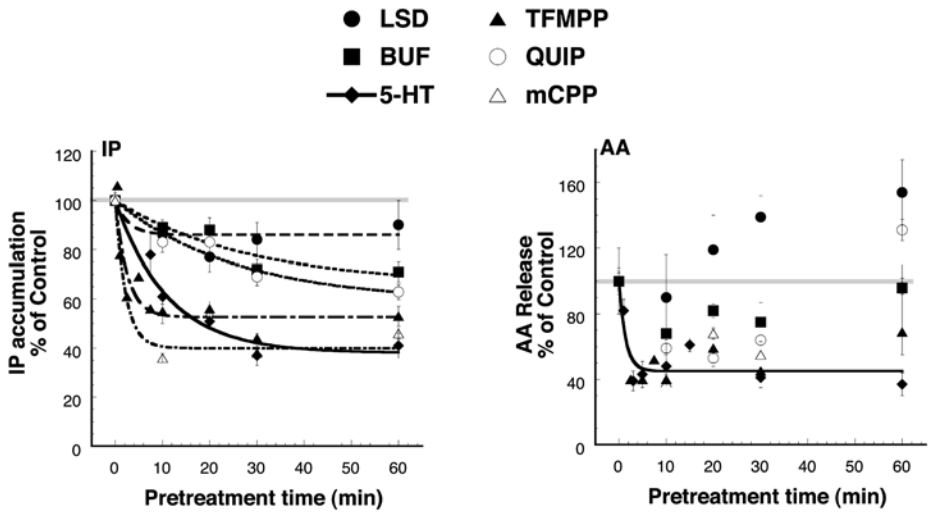

Fig. 4. Agonist dependence of desensitization of 5-HT_{2C} receptor-mediated IP accumulation and AA release. Cells were pretreated for 0–60 min with maximal concentrations (at least 20X K_A) of the agonists 5-HT (10 μ*M*), quipazine (30 μ*M*), LSD (100 n*M*), TFMPP (10 μ*M*), m-CPP (20 μ*M*) or bufotenin (10 μ*M*), washed, and AA release and IP accumulation measured in response to 10 μ*M* 5-HT for 10 min. AA release and IP accumulation were measured simultaneously, from the same cells. Data are expressed as a percentage of control 5-HT stimulation and are reproduced from ref. *85* with permission.

and DAMGO have low RAVE values. As pointed out by Whistler et al. *(51)*, RAVE values alone are unlikely to completely underlie mechanisms of physiological tolerance and dependence. However, development of this term highlights the significance of specifically studying differential drug efficacy at responses from activation of receptors vs desensitization/downregulatory mechanisms and the general importance of understanding effector pathway-dependent intrinsic efficacy.

We have also found that 5-HT_{2C} ligands differentially traffic the receptor stimulus to mechanisms associated with rapid desensitization vs activation of PLC-PI and PLA_2-AA. Figure 4 shows the capacity of a series of ligands to elicit desensitization of 5-HT_{2C} receptor-mediated PLC-PI and PLA_2-AA responses, measured simultaneously from the same cells. 5-HT produces a rapid and similar reduction in responsiveness of both systems. However, desensitization elicited by pretreatment with other ligands was quite varied and did not seem to be related to their relative efficacy to activate PLC-PI or PLA_2-AA (Fig. 5). For example, the magnitude of desensitization of the AA and IP

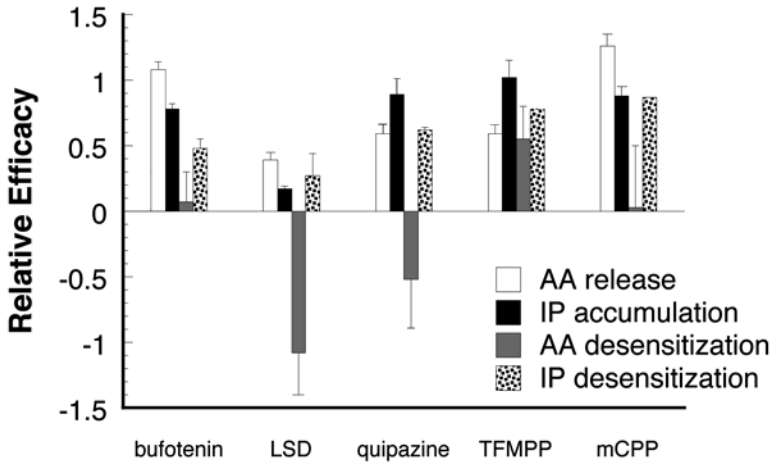

Fig. 5. Relative efficacies of agonists for stimulation and desensitization of AA release and IP accumulation. Relative efficacy values are with reference to the effect produced by maximal concentration of 5-HT. For stimulation of AA release and IP accumulation, values are from ref. *22*, with the exception of m-CPP. Values for desensitization are derived from the 60-min time-point (Fig. 4). Negative values for desensitization indicate that pretreatment with the ligand resulted in enhancement of 5-HT-stimulated AA release (LSD and quipazine). Reproduced with permission from ref. *85*.

responses elicited by TFMPP, an agonist with relative efficacy values of 1 and 0.6 for PLC and PLA_2, respectively, was similar to that produced by 5-HT. However, desensitization elicited by quipazine, a drug with relative efficacy for PLC and PLA_2 similar to that of TFMPP (0.9 and 0.6, respectively), was markedly different from that produced by TFMPP. Pretreatment with quipazine produced less desensitization of the PLC response and enhanced the responsiveness of the PLA_2 pathway. Bufotenin, an agonist with efficacies similar to that of 5-HT to activate PLC and PLA_2 (0.8 and 1, respectively), produced much less desensitization of both responses than did 5-HT. Interestingly, pretreatment with LSD produced a small reduction in responsiveness of the PLC-PI pathway, but increased responsiveness of the PLA_2-AA cascade.

Ligand-dependent differences in relative efficacy to promote desensitization of different responses coupled to the same receptor could be the result of differences in the ability to activate desensitization mechanisms for each response. Alternatively, ligand-specific receptor conformations could serve differentially as targets for desensitization mechanisms that target the receptor. It is known that some desensitization mechanisms, such as G protein–coupled receptor kinase (GRK) and arrestin, appear to be sensitive to receptor conformation

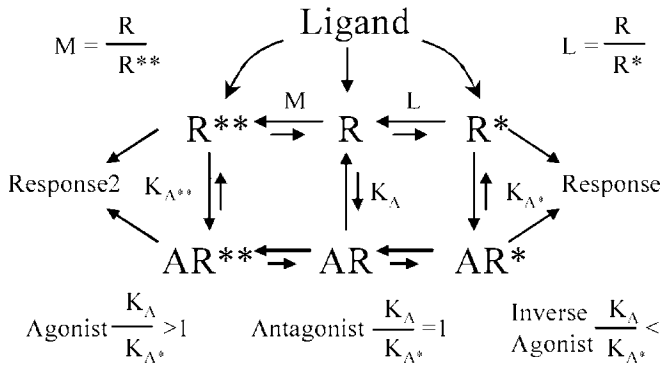

Fig. 6. A multistate model of receptor function with three states. The receptor population consists of an inactive receptor conformation (R) in equilibrium with two (or more) active receptor conformations (R* and R**). Each active conformation can differentially activate effector mechanisms, leading to response1 or response2 in the absence of an agonist. Two isomerization constants (L and M) define the propensity of the receptor to adopt an active conformation in the absence of a ligand. Agonists can differentially stabilize R* vs R** depending on the value of the equilibrium dissociation constants KA* and KA** relative to KA. Inverse agonists can also have differential effects on response1 vs. response2 depending upon the relative values of L and M and of the affinity constants. Additional active states with additional isomerization and affinity constants can be added. Adapted from Leff et al. (*86*) and Berg et al. (*22*).

because they preferentially interact with agonist-activated receptors *(52)*. When occupied by morphine, but not DAMGO, the μ-opioid receptor is not a target for arrestin-mediated uncoupling from G protein *(49)*. Moreover, such effects could be cell typed dependent. Gray et al. *(53)* found different mechanisms for 5-HT_{2A} receptor desensitization and resensitization in HEK 293 vs C6 glioma cells. Thus, ligands might produce a receptor conformation(s) with differential capacity to activate, vs serve as a targets for, desensitization mechanisms in a cell-dependent manner.

2.1.4. Constitutive 5-HT_{2C} Receptor Activity and Stimulus Trafficking

As mentioned earlier, current models of receptor function provide for receptor conformations that interconvert between inactive and active states. The agonist-directed trafficking of receptor stimulus hypothesis suggests that there are multiple active conformations of a receptor that differ in their capacity to couple/activate effector pathways. Such multistate models predict that, like agonist-stimulated responses, constitutive receptor activity and the relative efficacy of inverse agonists should also be response dependent (Fig. 6).

We measured the relative efficacy of inverse agonists for four nonedited 5-$HT_{2C\text{-}INI}$ responses: PLC-PI, PLA_2-AA, $G_{\alpha i}$ activation (as measured with GTP[γ^{35}S] binding) and sensitization of the PLC-PI response. Sensitization of the PLC-PI response is the result of an action of inverse agonists to reduce constitutive 5-HT_{2C} receptor activity toward desensitization mechanisms (*see* ref. *54*). Like agonist-stimulated activity, constitutive receptor activity can also continuously activate desensitization mechanisms such that a receptor system exists in a partially desensitized state. Reducing constitutive receptor activity with prolonged treatment with an inverse agonists (>4 h) turns off activation of desensitization mechanisms and allows the receptor system to resensitize, which is visualized by enhanced constitutive receptor activity and enhanced responsiveness to agonist stimulation *(54)*. For the 5-HT_{2C} receptor expressed in CHO cells, this effect is very sensitive to constitutive receptor activity (i.e., it occurs in cells expressing low levels of the 5-HT_{2C} receptor, for which inverse agonists behave as competitive antagonists for PLC-PI) and is response dependent (i.e., it occurs for the PLC-PI but not the PLA_2-AA response).

Figure 7 shows that, indeed, the relative efficacy of inverse agonists differs markedly depending on the response measured. Note especially the markedly different relative efficacies of SB 242084 and SB 243213. SB 242084 displays a relatively strong inverse agonism toward PLA_2, $G_{\alpha i}$ activation, and the PLC sensitization response; however, for the basal PLC response, SB 242084 is an agonist (*also see* Fig. 8). SB 243213 also is a relatively strong inverse agonist for all responses except the basal PLC response, where it is an antagonist. These data support the contention that constitutive activity and inverse agonist relative efficacy are response dependent and that the receptor stimulus from constitutively active receptors is also different for different responses.

2.1.5. Protean 5-HT_{2C} Ligands

Protean ligands are ligands that can behave as agonists or inverse agonists at the same receptor, depending on the response measured *(7,55)*. The word "protean" stems from the mythological Greek god Proteus, who was said to be able to change form at will. Protean ligand behavior can result from actions of ligands in different systems with different levels of constitutive receptor activity or within the same system after a change in receptor system function [e.g., desensitization *(7,55)*]. Protean ligand behavior can also occur as a result of agonist-directed trafficking of receptor stimulus *(56)*. A number of examples of protean behavior by ligands have been reported *(57–62)*. Figure 8 shows the effect of SB 242084 on 5-HT_{2C}-mediated responses in CHO cells *(63)*. SB 242084 displays agonist behavior toward 5-HT_{2C}-PLC-PI, but it is an inverse

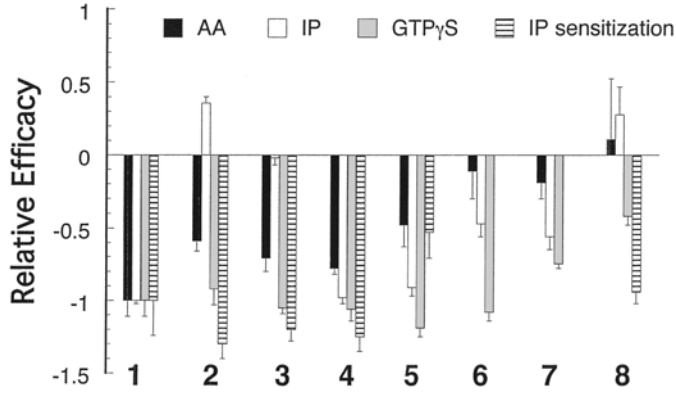

Fig. 7. Relative efficacy of 5-HT_{2C} ligands measured from different effector systems illustrates that constitutive receptor activity is response dependent. Relative efficacy for inverse agonists was calculated with SB 206553 (1) as the reference ligand for PLC, PLA2, and GTP[γ^{35}S] binding responses in CHO-1C7 cells (high levels of 5-HT_{2C} receptor expression, optimized to detect inverse agonism). For IP sensitization, CHO-1C19 cells (low levels of receptor expression) were treated for 24 h with the indicated ligands and washed to remove the inverse agonist; the subsequent enhanced response to the agonist DOI was measured. Concentrations of drugs were used to produce maximal receptor occupancy (>100K_d). Some of these data appear in refs. *22* and *54*. 1 = SB 206553; 2 = SB 242084; 3 = SB 243213; 4 = clozapine; 5 = mianserin; 6 = mesulergine; 7 = ketanserin; 8 = 5-methoxygramine.

agonist at 5-HT_{2C}-PLA_2-AA and GTP[γ^{35}S] binding. It is important to note that the PLC-PI and PLA_2-AA responses to SB 242084 were obtained from the same cells (same multiwell), simultaneously. Thus, SB 242084 acts as an agonist and an inverse agonist at the same receptor, at the same time, depending on the response measured. The existence of protean ligands underscores the need to reference the receptor system, the response, and the conditions under which the response was measured when labeling a ligand as an agonist, antagonist or inverse agonist.

2.2. 5-HT_{2A} Receptors

The 5-HT_{2A} receptor is highly homologous to the 5-HT_{2C} receptor. Both receptors share about 50% overall sequence homology and about 80% within the transmembrane-spanning domains. Therefore, it is not surprising that the pharmacological characteristics of these receptors are also quite similar, with relatively few selective ligands available. Although both of these receptors couple to the PLC-PI and PLA_2-AA signal transduction pathways *(21,64)*, there are also important differences in cellular signaling *(21,65–68)*.

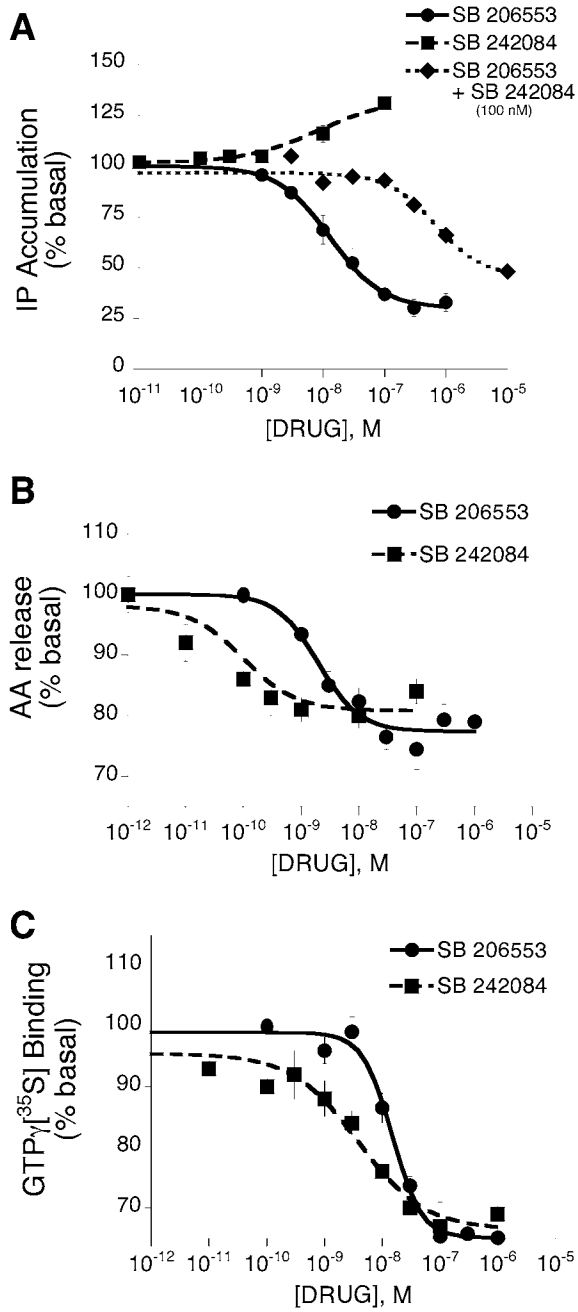

Fig. 8. SB 242084 is a agonist for PLC-PI and an inverse agonist for PLA_2-AA and GTP[γ^{35}S] binding to $G_{\alpha i}$ (i.e., SB 242084 is a protean ligand) for the 5-HT_{2C} receptor. Accumulation of IP and release of AA were measured in CHO-1C7 cells, which express a high level of the human 5-HT_{2C} receptor (10–20 pmol/mg protein, optimized to detect

2.2.1. Agonist-Directed Trafficking of 5-HT$_{2A}$ Receptor Stimulus

Like 5-HT$_{2C}$ receptor ligands, 5-HT$_{2A}$ receptor ligands also traffic receptor stimuli differentially to PLC-PI and PLA$_2$-AA. Figure 9 shows the relative efficacies of a series of 5-HT$_{2A}$ ligands for stimulation of the PLC-PI and PLA$_2$-AA pathways (shown to be independent responses and measured simultaneously from the same cells) in CHO cells expressing a low density of the human 5-HT$_{2A}$ receptor (absence of receptor reserve for 5-HT). In addition, the relative efficacies of the ligands to elicit increases in intracellular calcium levels, measured with FURA-2 spectrofluorimetry, was also assessed. There were marked differences in relative efficacy of some ligands between the PLC-PI and the PLA$_2$-AA responses, with most ligands favoring the PLA$_2$-AA pathway. For example, tryptamine was a very strong agonist for the PLA$_2$-AA cascade (relative efficacy of 1.3 with respect to 5-HT), but it was much weaker than 5-HT for the PLC-PI response (relative efficacy of 0.6). On the other hand, quipazine was an equally efficacious partial agonist for both the PLC-PI and PLA$_2$-AA responses (relative efficacy of 0.4 for both responses). As mentioned above, traditional receptor theory requires that the relative efficacies of the ligands be the same between responses. Differences in relative efficacy indicate that some 5-HT$_{2A}$ ligands are functionally selective. There was no difference in the relative efficacy of ligands between the PLC-PI and the calcium responses. Because the calcium response is presumably derived from the generation of inositol trisphosphate from the PLC-PI pathway, it would not be expected that ligands could differentially signal to the calcium response independently from the PLC-PI response. Thus, the same relative efficacy of ligands for consequential signaling pathways reinforces confidence in the differences in relative efficacy between the PLC-PI and PLA$_2$-AA responses and in the capacity of ligands to traffic receptor stimulus differentially to independent responses.

Fig. 8. *(continued)* inverse agonism), simultaneously from the same cells. Drugs were added for 25 min in the presence of LiCL (20 m*M*) and 0.1% fatty acid-free bovine serum albumin. GTP[γ^{35}S] binding was done with membranes prepared from CHO-1C7 cells. **(A)** Concentration–response curves for SB 242084 and the prototypical inverse agonist, SB 206553 (alone and in the presence of 100 n*M* SB 242084), measuring IP accumulation. SB 206553 is a strong inverse agonist, whereas SB 242084 is a weak agonist for PLC-PI. As is characteristic of a low-efficacy ligand, SB 242084 caused a rightward shift in the concentration–response curve to SB 206553. **(B)** Concentration–response curves to SB 206553 and SB 242084 for AA release. Both drugs are strong inverse agonists. **(C)** Concentration–response curves to SB 206553 and SB 242084 for activation of $G_{\alpha i}$ measured with GTP[γ^{35}S] binding. Both drugs are strong inverse agonists. Reproduced with permission from ref. *63*, copyright 2004 by the Society for Neuroscience.

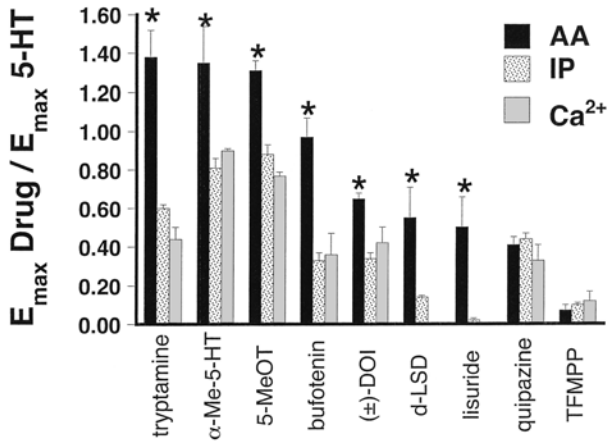

Fig. 9. Agonist-directed trafficking of 5-HT_{2A} receptor stimulus. CHO cells expressing a low level of the 5-HT_{2A} receptor (approx 200 fmol/mg protein) were incubated with maximal concentration of the indicated ligands and with 5-HT. Accumulation of IP and release of AA was measured from the same cells, simultaneously after 10 min of agonist exposure. Control experiments indicated that the PLC-PI and the PLA_2-AA responses were independent. Increases in intracellular calcium (Ca^{2+}) were measured using FURA-2 spectrofluorimetry. Bars represent the ratio of the responses to maximal concentrations of the agonists with respect to a maximal concentration of 5-HT (10 μ*M*). Agonist concentrations used were 20 × EC50 or *Ki* values as follows (in μ*M*): ±DOI, 1; *d*-LSD, 0.3; lisuride, 0.3; TFMPP, 20; quipazine, 30; bufotenin, 3; α-me-5-HT, 3; 5-MeOT, 3; tryptamine, 20. Lisuride and LSD did not elicit a measurable increase in $[Ca^{2+}]_i$. Asterisks denote significant differences in agonist relative efficacy between agonist-elicited AA vs IP accumulation ($p < 0.05$). Response-dependent differences in relative efficacy are consistent with agonist-directed trafficking of receptor stimulus, as traditional receptor theory requires that agonist relative efficacy be the same for all responses coupled to the receptor. Note that for sequential responses (PLC-PI and Ca^{2+}), which ligands cannot differentially regulate, relative efficacy is not different. Reproduced with permission from ref. *22*.

In a similar study, Kurrasch-Orbaugh et al. *(69)* also found that 5-HT_{2A} ligands could differentially activate the PLC-PI vs the PLA_2-AA responses in NIH-3T3 cells expressing about 5.5 pmol/mg protein of receptor. Although a receptor reserve was present for 5-HT, comparison of the maximal responses of clear partial agonists revealed strong evidence of stimulus trafficking for some, but not all, ligands. For example, the relative efficacy of LSD for PLC-PI differed from that for PLA_2-AA (0.22 vs 0.56, respectively), whereas that of DOB was not different (0.79 vs 0.75, respectively). The largest difference in relative efficacy between responses was for tryptamine (0.91 vs 0.41 for PLC-PI vs PLA_2-AA, respectively). This reversal of relative efficacy compared to that of

LSD strongly indicates agonist-directed trafficking of receptor stimulus. Interestingly, however, this difference in tryptamine relative efficacy was opposite that reported by Berg et al. (*see* above; *22*) for the 5-HT_{2A} receptor expressed in CHO cells, where tryptamine strongly favored the PLA_2-AA over that of the PLC-PI response. Perhaps this is one example of the impact of cell phenotype on the pharmacological properties of ligands. It is possible that the same 5-HT_{2A} receptor conformation(s) promoted by tryptamine have different activities to CHO cells vs NIH-3T3 cells, by virtue of different types and quantities of signaling molecules expressed by the cell types. Alternatively, perhaps different membrane microenvironments of the two cell types provide different restrictions on the range of conformations that the 5-HT_{2A} receptor can adopt. This difference in the pharmacological characteristics of tryptamine highlight the importance of the choice of assay system(s) for the lead optimization phase of drug discovery to optimize therapeutic relevance.

2.2.2. Agonist-Specific 5-HT_{2A} Receptor Conformations

It is generally believed that the mechanism by which ligands can differentially traffic receptor stimulus to different effector pathways is based on their capacity to promote ligand-specific receptor conformations. Using site-directed mutagenesis and molecular modeling techniques, Shapiro et al. *(70)* reported that even small changes in the structure of a ligand or receptor can lead to dramatic and unpredictable changes in 5-HT_{2A} receptor conformation. Experiments in which the capacity of a series of ligands to compete for the binding of agonist ([^{125}I]DOI and [^{3}H]DOB) radiolabeled human 5-HT_{2A} receptor in the human brain and expressed in CHO cells revealed biphasic competition curves for some prototypical antagonist ligands (ketanserin and MDL100,907), but not all (SB 242084 and mesulergine) *(71)*. The biphasic binding curves of ketanserin and MDL100,907 were not altered by high concentrations of Gpp(NH)p to uncouple the receptor from G protein. These results suggest that DOI and DOB promote multiple 5-HT_{2A} receptor conformations that could be discriminated by certain ligands. In functional assays measuring the antagonism of 5-HT- and DOI-mediated PI hydrolysis, there was no evidence of biphasic antagonism by ketanserin or MDL100,907. All four antagonists produced parallel and surmountable shifts in the concentration–response curves to 5-HT and DOI, with similar pA_2 values for each agonist.

Recently, Brea et al. *(72)* extended the previous work of López-Giménez et al. *(71)* to include antagonism of 5-HT_{2A}-mediated PLA_2-AA as well as PLC-PI in CHO cells expressing low levels of the human 5-HT_{2A} receptor. As previously, antagonist curves for inhibition of 5-HT-mediated PLC-PI were monophasic. However, when tested against 5-HT-stimulated PLA_2-AA signaling, curves for ketanserin, clozapine, and MDL100,907 were biphasic, whereas

curves for mesulergine and haloperidol were monophasic. It is important to note that the PLC-PI and PLA_2-AA responses were measured from the same cells, simultaneously. In binding studies, competition curves for clozapine against [^{3}H]-DOB were biphasic. Thus, compounds that exhibited biphasic competition curves against radiolabeled agonist binding also displayed biphasic antagonist curves against 5-HT-mediated AA release and the pIC_{50} values for the biphasic inhibitions were very close to the pK_i values obtained for binding experiments. Compounds that were monophasic in binding were also monophasic for AA release. These data suggest that 5-HT activates the PLA_2-AA cascade via two distinct 5-HT_{2A} receptor conformations (or groups of conformations) that can be distinguished by certain ligands (clozapine, MDL100,907, and ketanserin).

2.3. 5-HT_{1A} Receptors

The 5-HT_{1A} receptor is a member of the 5-HT_1 family of serotonin receptors best known for their capacity to inhibit adenylyl cyclase activity via pertussis toxin-sensitive G proteins. These receptors also couple to K^+ channels, Ca^{2+} channels, PLC, intracellular calcium, mitogen-activated protein kinase cascade, and Na^+/K^+-ATPase *(73,74)*. This receptor is an important target for the management of anxiety, depression, and schizophrenia *(75–78)*.

2.3.1. Agonist-Directed Trafficking of 5-HT_{1A} Receptor Stimulus

The first evidence that 5-HT_{1A} ligands might provoke differential signaling came from the work of Gettys et al. *(79)*. Using a coimmunoprecipitation strategy with a photoreactive radiolabeled GTP analog in CHO cells expressing the human 5-HT_{1A} receptor, the efficacy of ipsapirone to promote coupling of the 5-HT_{1A} receptor to $G_{i\alpha 3}$ was greater than that for $G_{\alpha i2}$, whereas the full agonist 5-HT and the partial agonist rauwolscine did not distinguish between the two G proteins. Newman-Tancredi et al. *(80)* also examined agonist-mediated coupling of the human 5-HT_{1A} receptor expressed in CHO cells to $G_{\alpha i3}$ using antibody-capture and a scintillation proximity assay. Some ligands reported to have high efficacy, such as 5-HT and 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), produced bell-shaped concentration–response curves for GTP[γ^{35}S] binding to $G_{\alpha i3}$, whereas curves for weaker agonists (e.g., pindolol) were monophasic. Although these differences in ligand efficacy to activate $G_{\alpha i3}$ could be evidence of stimulus trafficking, more experiments are necessary to rule out "strength of stimulus" mechanisms, as the authors suggest.

Pauwels and Colpaert *(81)* investigated the action of a variety of 5-HT_{1A} ligands on 5-HT_{1A}-mediated increases in intracellular calcium and GTP[γ^{35}S] binding in CHO cells and in C6-glial cells. Both responses were sensitive to prior treatment with pertussis toxin, suggesting that both were mediated by

$G_{\alpha i/o}$ proteins. The relative efficacy of some ligands varied with the response measured. Many ligands were not able to mobilize intracellular calcium even though their relative efficacy to stimulate GTP[γ^{35}S] binding (with respect to 5-HT) was reasonably high. The pharmacological profiles for F 14679 and flesinoxan were especially interesting. Although both drugs had similar relative efficacy for GTP[γ^{35}S] binding (0.93 vs 0.81, respectively), the relative efficacy to promote intracellular calcium release was markedly different (0.87 vs 0.05, respectively). These data suggest that some drugs can strongly differentiate between two responses coupled to the 5-HT_{1A} receptor.

3. Concluding Remarks

There are many reports in the literature of agonist action that cannot be explained by traditional receptor theory, but that are consistent with agonist-directed trafficking of receptor stimulus (for reviews, *see* refs. *7,8,10,14,56,* and *82*). Evidence obtained from experiments with a large variety of receptor systems now strongly suggests that ligands, in addition to having differential affinity for different receptor subtypes, have selectivity with respect to the signaling systems activated from a single receptor subtype. Intrinsic efficacy, previously defined as a single-drug property (and a constant) that reflected the ability of a drug to activate a receptor (i.e., produce a conformational change), now must reflect the different meanings of the conformational change(s) to the cell. Rather than having one intrinsic efficacy, ligands might have several that are dependent on the cellular signal transduction machinery. Moreover, these intrinsic efficacies themselves are not constants, but will vary with cell phenotype (e.g., the quality and quantity of signaling molecules available) as well with time in a single cell type. Differential changes in responsiveness (e.g., desensitization, sensitization) of signaling pathways via homologous or heterologous (crosstalk) receptor activation would be expected to alter stimulus trafficking. Consequently, it is clear that a drug should not be labeled as an agonist (partial or full), inverse agonist (partial or full), or antagonist, without reference to the receptor system (receptor and signaling pathway/response) and experimental conditions used.

The importance of functional selectivity by ligands is difficult to overestimate. The impact of ligands eliciting multiple effects from a single receptor subtype extends from the theoretical (receptor theory), to the lab bench (experimental design), to the clinic (pharmacotherapy). Although it now appears that the pharmacological actions of drugs are considerably more complex that previously thought, with this complexity also might come the opportunity for better drug development and improved therapeutics. For example, it has been proposed that the novel atypical antipsychotic drug, aripiprazole, owes its favorable therapeutic

profile to its functionally selective actions at dopamine D_2 receptors *(10,83,84)*. To take advantage of this opportunity, we need to develop a greater understanding of the dynamics of receptor systems and signaling mechanisms and to develop appropriate assay systems that will be predictive of therapeutic drug action. The challenge now for pharmacologists is to learn to exploit, for therapeutic advantage, the newly discovered diversity that makes up the treasure chest of drugs.

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7

Identification of 5-HT$_2$ and 5-HT$_4$ Receptor-Interacting Proteins

A Proteomic Approach

Joël Bockaert, Carine Bécamel, Lara Joubert, Sophie Gavarini, Aline Dumuis, and Philippe Marin

Summary

G protein–coupled receptors (GPCRs) not only interact with heterotrimeric G proteins but also with accessory proteins, called GPCR-interacting proteins (GIPs). GIPs are implicated in GPCR targeting to specific cellular compartments, in their assembling into large functional complexes called "receptosomes," in their trafficking to and from the plasma membrane, as well as in the fine-tuning of their signaling properties. Here, we describe "receptosomes" associated with the C-terminal tails of 5-hydroxytryptamines 5-HT$_{2A}$, 5-HT$_{2C}$, as well as 5-HT$_{4a}$ and 5-HT$_{4e}$ receptors. The three last residues of these receptor C-termini are canonical PDZ ligands interacting with type I PDZ domain-containing proteins (5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_{4A}$ tails) and type II (5-HT$_{4e}$). The entire C-terminal tails fused to glutathione-*S*-transferase or synthetic peptides encompassing the last 14 C-terminal residues of the receptors were used as baits to fish out GIPs from mouse brain. Controls were made with mutant bait (mutated in the PDZ ligand). Proteins, which were specifically retained on native PDZ ligand-containing peptides, were separated on two-dimensional gels and identified by MALDI-TOF mass spectrometry or immunoblotting. Ten and seven PDZ domain-containing proteins were found to bind to the 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors, respectively. The sequences of the C-terminal PDZ ligands of 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors are very similar (SSV and SCV, respectively). If some of the PDZ domain-containing proteins associated with these receptors were identical (ARIP-1/MAGI-2, SAP97, PSD-95,

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Dlgh3/MPP3), others were clearly different. The 5-HT_{2C} but not the 5-HT_{2A} receptor interacted with the SAP102 and Veli3/CASK/Mint1 ternary complex, whereas the 5-HT_{2A} but not the 5-HT_{2C} receptors interacted with CIPP. MUPP1, which was found to interact with the 5-HT_{2C} receptor in a two-hybrid screen, was also fished out by the 5-HT_{2C} and 5-HT_{2A} C-termini. A few other non-PDZ domain-containing proteins were found in these "receptosomes." Electron microscopy (EM) studies of 5-HT_{2A} and 5-HT_{2C} receptors and some of their interacting proteins led to the proposition of a presynaptic and postsynaptic localization of 5-HT_{2C} receptors and a preferential postsynaptic localization of 5-HT_{2A} receptors. In a similar manner, we identified 10 and 3 proteins that interacted specifically with the 5-HT_{4a} and 5-HT_{4e} receptor splice variants, respectively. Most of them are PDZ proteins. Among them, NHERF recruited 5-HT_{4a} receptors in microvilli, where they localized with activated ezrin, consistent with a role of 5-HT_{4a} receptors in cytoskeleton remodeling. The same variant interacted with both the constitutive and the inducible (upon methamphetamine treatment) forms of SNX27 (SNX27a and SNX27b, respectively). SNX27a redirected part of the 5-HT_{4a} receptors to early endosomes.

Key Words: GPCRs; 5-HT; 5-HT receptors; PDZ proteins; proteomics.

1. Introduction

All researchers interested in 5-hydroxytryptamine (5-HT) receptors have read the seminal paper of Gaddum and Picarelli *(1)* with some amused feelings. At that time, all of the physiological effects of 5-HT were attributed to two major receptors. Almost 50 yr later, the situation has dramatically changed with the existence, in mammals, of 14 different 5-HT receptor subtypes, each encoded by a different gene *(2)*. Alternative splicing and other posttranslational modifications, such as RNA editing, further increased the number of 5-HT receptor proteins that recognize 5-HT as a physiological ligand. Indeed, more than 30 receptor proteins have been described to date. Three important steps have led to the discovery and then to the accumulation of great knowledge on most of these proteins. The first one was the possibility of analyzing the pharmacological characteristics of 5-HT receptors by measuring a proximal response, such as second-messenger production, mainly cAMP, Ca^{2+}, and inositol phosphates. The second was the rapid development of sophisticated pharmacological tools, which offer fantastic possibilities to activate or inhibit specifically most of these receptors. Finally, the cloning and genomic sequencing gave the final touch to the 5-HT receptor family portrait.

This extreme diversity of 5-HT receptor proteins has, without doubt, a physiological meaning and one would expect that each of them has a distinct signaling pathway pattern. However, if one considers the six 5-HT receptor families that are G protein–coupled receptors (GPCRs) (5-HT_1, 5-HT_2, 5-HT_4,

5-HT_5, 5-HT_6, and 5-HT_7 families), only three main types of primary coupling to G proteins have been described *(2)*. The 5-HT_1 receptors activate the G_i/G_o proteins, the 5-HT_2 receptors activate G_q/G_{11}, and the 5-HT_4, 5-HT_6, and 5-HT_7 receptors activate G_s; the coupling of the 5-HT_5 receptor is uncertain. Although these primary couplings result in a greater variety of activation or inhibition of signaling cascades depending on the cell in which the receptors are expressed, the redundancy of couplings among different receptor subtypes remains relatively high *(2)*. Therefore, one question is to know how each of the different 5-HT receptors and their splice variants generate an accurate and specific physiological response. One solution could be that the specificity of 5-HT receptor signaling is, in part, reached through their organization into functional and distinct units, which have already been described for other receptors, such as presynaptic and postsynaptic glutamate receptors, the *Drosophila* rhodopsin-receptor–INAD (inactivation-no-after-potential D) complex and called "receptosomes" *(3,4)*. These are complex architectures of membrane-bound receptors associated with multidomain-containing proteins called GIPs (GPCR-interacting proteins) when associated with GPCRs. These GIPs have been shown to control the pharmacology of GPCRs, their targeting to a specific subcellular compartment such as the postsynaptic or presynaptic neuronal compartments or the epithelia apical or baso-lateral compartments, their trafficking to or from the plasma membrane, as well as the fine-tuning of their signaling pathways.

Many recent reviews have analyzed the nature and functions of these GIPs in detail *(3–6)*. Here, we will focus on the proteomic approach that we have used to establish the molecular composition of the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 splice variant "receptosomes" *(7–9)*.

2. Toward a Global Analysis of "Receptosomes"

Most GIPs, including the first GIP described that interacts with the 5-HT_{2C} receptor, the MUPP-1/MPDZ (multi-PDZ-domain protein), have been found using the yeast two-hybrid system *(10,11)*. This system was developed from the modular structure of the yeast transcription factor GAL4, which includes a DNA-binding domain and a transcription activation domain. One of the proteins of interest, which could be, for example, a cytosolic domain of one GPCR (either the C-terminus or an intracellular loop), is expressed as a fusion protein with the GAL4 DNA-binding domain, whereas the other (a cell library) is fused to the transactivation domain. The interaction of the two proteins within the yeast cell reconstitutes a functional GAL4 transcription factor and induces transcription of reporter genes integrated in the region downstream from the GAL4 DNA-binding site. The system is sensitive and allows the discovery of new interacting proteins without handling any protein molecules.

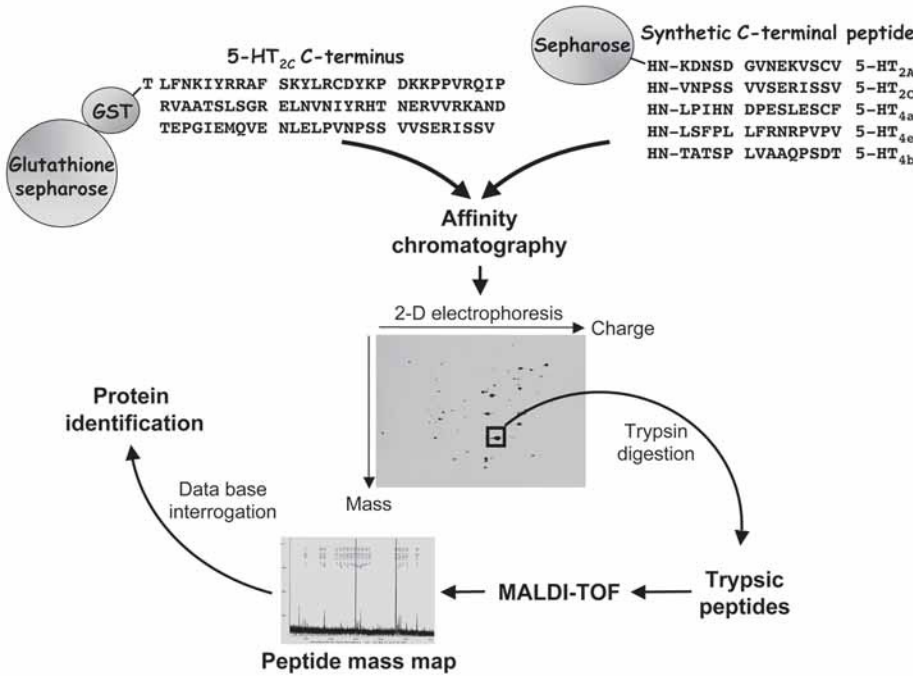


Fig. 1. Schematic representation of the different steps of the proteomic screen used to identify 5-HT "receptosomes." The sequence of the entire 5-HT_{2C} receptor C-terminus that was fused to glutathione-*S*-transferase (GST) and those of the C-terminal peptides of the 5-HT_{2A}, 5-HT_{2C}, 5-HT_{4a}, 5-HT_{4b}, and 5-HT_{4e} receptors used as bait to fish out GIPs associated with these receptors are depicted. Peptides bearing mutation or truncation within the last three residues (PDZ ligand) were also used (not shown). These baits were incubated with mouse brain protein extracts and proteins retained by affinity, separated onto 2D gels, and detected by silver staining. Those specifically retained were excised and digested in gel using trypsin and identified from their peptide mass fingerprint determined by MALDI-TOF mass spectrometry. For more details, *see* refs. *8* and *29*.

However, this system has several limitations: (1) It enables the detection of binary interactions but not global characterization of multiprotein networks; (2) it is not optimal to detect protein–protein interactions that depend on post-translational modifications and interactions with membrane proteins; (3) it can generate many false positives or false negatives.

Proteomic approaches aimed at providing a global characterization of protein networks have already been used to identify proteins associated with receptors such as NMDA receptors *(12)*, P2X7 ATP receptors *(13)*, and, more recently by our group, with 5-HT receptors *(7,8)*. The general principle (*see* Fig. 1) is to

use an affinity chromatography step, using part or the totality of a cytosolic domain of a given receptor immobilized on beads as bait. Although not yet used to analyze the 5-HT "receptosomes," GIPs that interact with a receptor can also be isolated by coimmunoprecipitation *(14)*. The affinity-purified or immunoprecipitated complexes are then separated onto one-dimensional (1D) or two-dimensional (2D) gels and subsequently identified "in gel" trypsin digestion by either MALDI-TOF mass spectrometry or tandem mass spectrometry interfaced with a nanoscale liquid chromatography system (referred to as LC-MS/MS). The first approach is generally used to identify relatively simple protein samples, such as those separated by 2D electrophoresis, which is still the most resolving technology to separate proteins. However, 2D electrophoresis remains subject to technical and analytical limitations, the most significant of these being that certain classes of proteins, such as membrane proteins and proteins with extreme isoelectric points, are not efficiently represented on 2D gels. The second one, which combines resolutive peptide separation by reverse-phase liquid chromatography and sensitive MS/MS peptide sequencing, is generally used to characterize complex protein mixtures such as those separated by 1D electrophoresis. This approach offers automated and sensitive analyses but is probably less suitable for differential analyses that are required to discriminate specific from nonspecific binding in the affinity chromatography step.

2.1. An Example of "Receptosome" Established Using the Entire C-Terminal Domain of the 5-HT$_{2C}$ Receptor as Bait **(8)**

Proteins from mice brain detergent-solubilized extracts that interact with the C-terminal domain of the 5-HT$_{2C}$ receptor were isolated by affinity chromatography. The entire C-terminus (90 amino acids) fused to glutathione-*S*-transferase (GST–5-HT90SSV), the C-terminus bearing a mutation on the C-terminal residue (GST–5-HT90SSA), and GST were immobilized on glutathione–Sepharose beads. Affinity-purified proteins were separated by 2D electrophoresis and revealed by silver staining (Fig. 2A). Some proteins bound to the GST–5-HT90SSV bait in a nonspecific manner (Fig. 2A). These include the GST fusion protein and its degradation products and many "bacterial" contaminating proteins. Comparing the 2D protein patterns retained on GST–5-HT90SSV and GST–5-HT90SSA (arrows, Fig. 2A) provided a way to selectively identify a first set of proteins that bind to the receptor through a PDZ-based mechanism. Indeed, the three C-terminal residues (SSV) of the 5-HT$_{2C}$ receptor constitute a typical motif capable of interacting with class I PDZ domain(s) (-S/T-X-Φ, where Φ represents a hydrophobic residue). PDZ domains are 90-amino-acid-long sequences first identified in the postsynaptic density protein *P*SD-95, the *Drosophila* tumor suppressor Disc-large and the epithelial tight junction protein Zonulla occludens-1 *(15)*. The proteins recruited

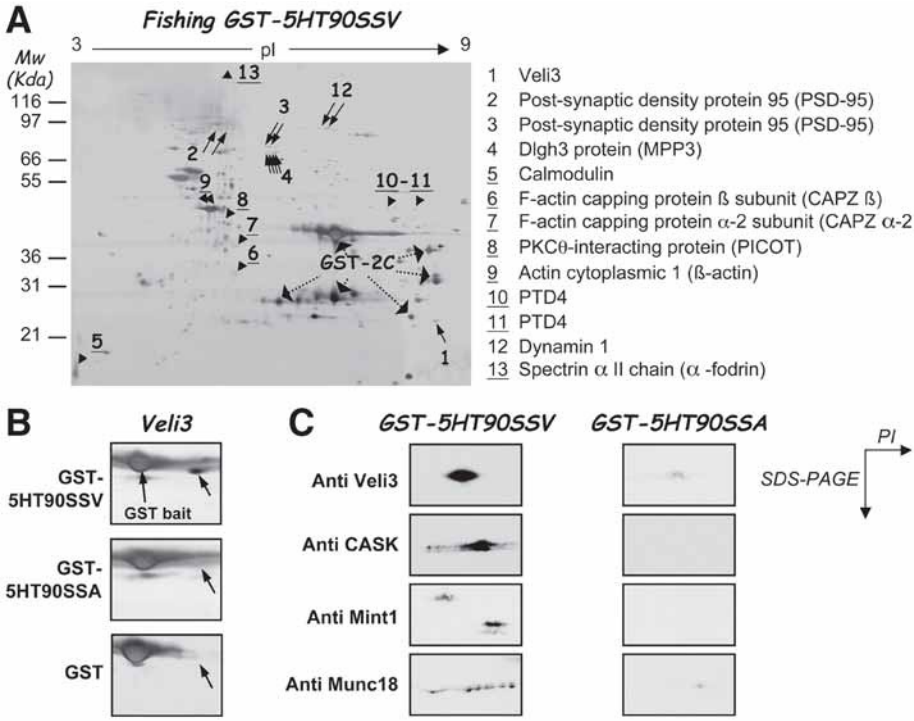


Fig. 2. Two-dimensional analysis of the 5-HT_{2C} "receptosome." **(A)** A typical 2D gel obtained after a pull-down experiment using GST–5-HT90SSV as bait is illustrated. Proteins that interact specifically with the PDZ ligand of the receptor (arrows) were detected by comparing protein patterns obtained with GST–5-HT90SSV and a mutant bait in which the last residue was replaced by an alanine (GST–5-HT90SSA). Arrowheads indicate proteins that interact equally with both wild-type and mutated baits but not, or less, represented in a purification using GST as bait. The name of the proteins are provided on the right panel. **(B)** A region of the 2D gels including the spot corresponding to Veli3 from experiments using GST–5-HT90SSV, GST–5-HT90SSA, and GST. **(C)** Representative 2D immunoblots showing the interaction of the 5-HT_{2C} receptor C-terminus with the tripartite complex Veli3/CASK/Mint1 and Munc18 are illustrated; modified from ref. *8*.

by the 5-HT_{2C} receptor C-terminus through a PDZ-based mechanism were unambiguously identified by MALDI-TOF mass spectrometry after trypsin digestion. An illustration of MALDI-TOF peptide mass maps is provided in Fig. 4B,C. They include three known PDZ-containing proteins, Veli3, one of the vertebrate homologs of the *Caenorhabditis elegans* protein LIN-7 (*see* the detailed regions of 2D gels in Fig. 2B, in which the specificity of the Veli3 binding to the GST–5-HTSSV vs the GST–5-HTSSA bait is shown), PSD-95

and Dlgh3/MPP-3, two modular proteins dubbed membrane-associated guanylate kinases (MAGUKs). A schematic representation of all PDZ domain-containing proteins described is provided in Fig. 7.

The last protein found to be associated with the 5-HT_{2C} receptor C-terminus in a PDZ-dependent manner is Dynamin 1. This protein does not contain a PDZ domain but is known to interact with PDZ proteins, such as CASK, a Veli3-interacting protein. An immunoblotting screen was then performed to identify additional 5-HT_{2C} receptor-associated proteins that were not recruited in sufficient amounts to be detected by silver staining of 2D gels. Veli proteins form stable tripartite complexes with two other PDZ multidomain proteins: the CASK and Mints proteins, which are the vertebrate orthologs of the *Caenorhabditis elegans* proteins LIN-2 and LIN-10. These ternary complexes are involved in the proliferation and differentiation of vulval cells *(16)*. In vertebrates, they might be implicated in synaptic transmission *(17)*. Immunoblotting experiments revealed that the entire ternary complex Veli3/CASK/Mint1 binds to the 5-HT_{2C} receptor C-terminus in a PDZ-dependent manner (Fig. 2C). Mint1 is known to bind to Munc18, a presynaptic protein that is essential for exocytosis of synaptic vesicles. Immunoblotting also indicated that Munc18 was present in the "receptosome" complex fished out in a PDZ-dependent manner by the 5-HT_{2C} receptor C-terminus (Fig. 2C). These experiments illustrate one of the advantages of this "proteomic" approach, which can fish out not only the primary interacting proteins but also the secondary and possibly the ternary partners of the receptors.

Although Veli3, CASK, and Mint1 all contain PDZ domains (*see* Fig. 7), we propose that the primary partner of the 5-HT_{2C} receptor is Veli3. The only experimental argument that we have so far is that silver staining indicated that the amount of Veli3 retained by affinity chromatography was much greater than the amount of fished CASK or Mint1. However, this should be verified with specifically designed experiments.

As seen in Figures 2–4, several isoforms of PSD-95 that migrate at two very different positions on 2D gels were fished out with the 5-HT_{2C} receptor C-terminus. Although these forms showed considerable differences in isoelectric points (0.6) and molecular masses (15 kDa), they generated identical peptide mass fingerprints (31 peptides representing 40% overall sequence coverage; *see* Fig. 4B–D). Two alternatively spliced isoforms of PSD-95 have been recently identified in rodent and human; a short isoform containing a pair of N-terminal cysteines that can be palmitoylated and a form containing a longer N-terminus *(18)*. We found that the N-terminus of the putatively palmitoylated form was present in the two major isoforms isolated. Thus, they did not represent alternative splice variants. Furthermore, we detected no peptides bearing palmitate, because the palmitoylated peptide is insoluble in the nonionic deter-

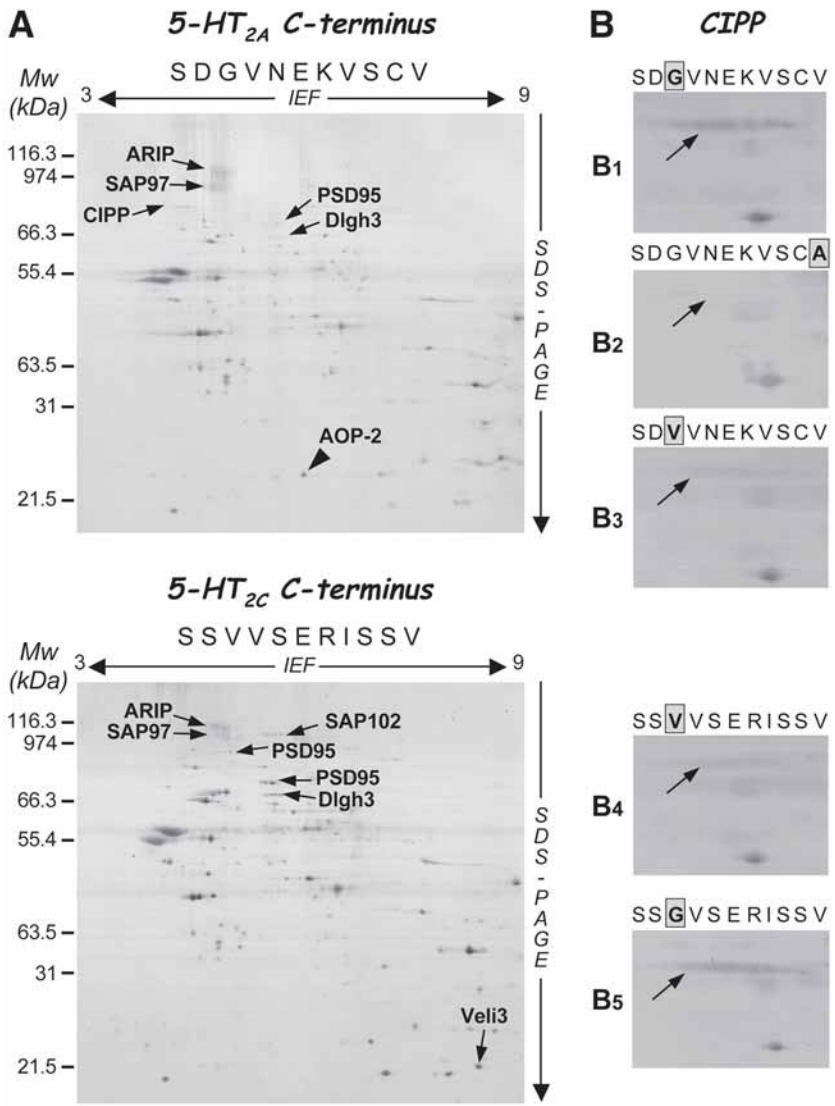


Fig. 3. Two-dimensional analysis of PDZ proteins interacting with the 5-HT_{2A} and the 5-HT_{2C} receptors C-termini. **(A)** Proteins from mice brain that bind to the C-terminus of the last 14 residues of the receptors were separated on 2D gels and stained with silver. Proteins that interact specifically (directly or indirectly) with the PDZ ligand of the receptor (arrows) were detected comparing protein patterns obtained with the native peptides (*see* Fig. 1) and mutant peptides in which the last residue was replaced by alanine. The position of one protein retained in a PDZ-independent manner by the 5-HT_{2A} receptor C-terminus is also indicated (arrowhead). **(B)** Molecular determinants in the C-terminus of 5-HT_{2A} receptor involved in its preferential interaction with CIPP.

gent used. The two isoforms certainly differ in other regions that were not covered by the peptides detected in our mass spectrometry analyses. More work is obviously required to solve this interesting problem.

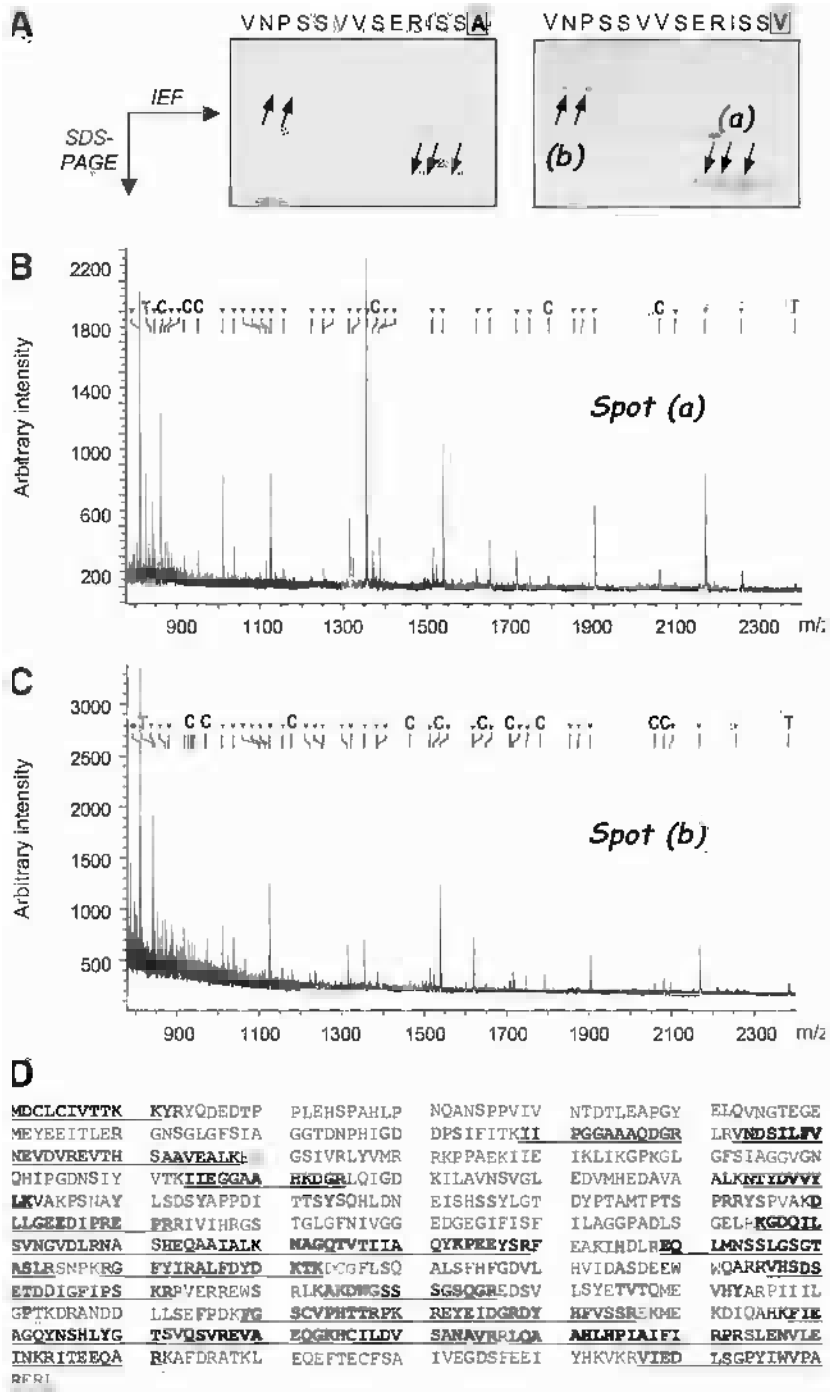
Comparing the 2D protein patterns retained on GST–5-HT90SSV and GST revealed that other GIPs interacted with motifs upstream of the PDZ ligand in the 5-HT_{2C} receptor C-terminus. (Fig. 2A, arrowheads) *(8)*. These included calmodulin, PICOT, a protein that was identified as a binding partner of protein kinase C (PKC) θ *(19)*, PTD4 a putative GTP-binding protein, and cytoskeleton proteins, such as α-fodrin and both α- and β-chains of CAPZ. CAPZ is a capping protein that binds as a dimer to the barbed end of actin filaments and inhibits the growth of actin microfilaments. The association of the 5-HT_{2C} receptors with the 4.1 proteins, a family of peripheral membrane proteins involved in the attachment of actin to the plasma membrane, confirms the relationship of this receptor with the cytoskeleton.

In order to make certain that the GIPs fished out during the proteomic screen, which is based on an in vitro binding assay, interact with the entire 5-HT_{2C} receptor, coimmunoprecipitation between the receptor and the GIPs was performed in brain extracts when the antibodies were available *(8)*. Coimmunolocalization in cell lines transfected with the receptor and the GIPs of interest was also performed. These experiments generally confirmed the interactions detected by the proteomic assay.

2.2. *Proteomic Identification of GIPs Associated With 5-HT_{2C} and 5-HT_{2A} Using Short C-Terminal Peptides as Bait*

One of the difficulties we had with the GIP fishing using the GST-fusion proteins was a relatively high background including the GST fusion proteins and proteins from mice or bacteria that we were unable to remove from the column despite preclearing and severe washing. Therefore, we decided to prepare affinity columns using synthetic peptides, taking advantage of the fact that PDZ-based interactions involve a well-defined region around the extreme C-terminus of the receptors. The last 14 amino acids of the 5-HT_{2A} and 5-HT_{2C}

Fig. 3. *(continued)* Proteins from mice brain were incubated with different peptides derived from the 5-HT_{2A} C-terminus (B1, B2, B3) and from the 5-HT_{2C} C-terminus (B4, B5). B1 and B4 are native peptides; B2 is a 5-HT_{2A} peptide bearing a valine to alanine mutation at the last position; B3 is a 5-HT_{2A} peptide in which the residue at the –8-position (glycine) was replaced by the corresponding residue in the 5-HT_{2C} receptor (valine); B5 is a 5-HT_{2C} peptide in which the residue at the –8-position (valine) was replaced by the corresponding residue in the 5-HT_{2A} receptor (glycine). Regions of representative 2D gels including the CIPP protein spots, revealed by silver staining, are illustrated. Modified from ref. *7*.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receptors (Fig. 1) as well as the corresponding peptides in which the C-terminal valine was mutated into an alanine were coupled via their N-terminal residue to Sepharose beads. This procedure considerably reduced the nonspecific binding of proteins (compare Figs. 2 and 3) *(7)*. This provided a way of detecting three additional PDZ-domain-containing proteins associated with the 5-HT_{2C} receptor C-terminus (*see* Figs. 2 and 3). These include activin receptor-interacting protein 1 (ARIP-1 also called MAGI-2 [membrane-associated guanylate kinase with inverted domain 2]) and two proteins of the postsynaptic density (SAP97 and SAP102, synaptic-associated proteins).

3. Molecular Determinants Associated With the Specificity of Interaction of PDZ-Domain-Containing Proteins With the 5-HT_{2A} or 5-HT_{2C} Receptor C-Termini

It was interesting to compare GIPs associated with the 5-HT_{2C} receptor C-terminus and those associated with the 5-HT_{2A} receptor C-terminus because both receptors express very similar PDZ ligands (-SSV for the 5-HT_{2C} receptor and -SCV for 5-HT_{2A} receptor). Five proteins containing at least one PDZ domain were found to interact with the C-terminus of the 5-HT_{2A} receptor. They include ARIP-1/MAGI-2, SAP97, PSD-95, Dlgh-3/MPP-3, and CIPP (channel-interacting PDZ protein). Moreover, the C-terminus of the 5-HT_{2A} receptor binds to the antioxidant protein 2 (a thioredoxin peroxidase) in a PDZ-independent manner. Several of these interactions were verified using coimmunoprecipitation *(7)*.

These experiments indicate that each 5-HT_2 receptor interacts with specific sets of PDZ proteins. We found that the specific association of Veli3 with the 5-HT_{2C} receptor was based mostly on the presence of the serine at the −1-position

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Fig. 4 *(opposite page)* MALDI-TOF analysis of PSD-95 isoforms recruited by the 5-HT_{2C} receptor C-terminus. **(A)** Regions of representative 2D gels obtained after affinity purification using a native 5-HT_{2C} peptide (right panel) or a mutant peptide in which the C-terminal residue (valine) was replaced by an alanine (left panel) are illustrated. The position of spots corresponding to PSD-95 is indicated by arrows. Note the marked difference in the positions of groups of spots (a) and (b). **(B,C)** MALDI-TOF peptide mass maps obtained from spots in positions (a) and (b). Ion signals with measured masses that matched calculated masses of protonated trypsic peptides from mouse PSD-95 are indicated by arrowheads. T indicates the ion signals corresponding to the autolysis products of trypsin and C indicates the ion signals of peptides contained in most spectra (contaminants). **(D)** The sequence of the short PSD-95 variant (PSD-95α) that contains a pair of N-terminal cysteines that can be palmitoylated is depicted. Peptides detected in our MALDI-TOF analyses are underscored. Note that they include the N-terminal peptide, which is specific of PSD-95α.

in this receptor. A peptide corresponding to the 5-HT_{2A} receptor C-terminus in which the cysteine at the −1-position was replaced with a serine (the residue at the −1-position in the 5-HT_{2C} receptor) strongly interacted with Veli3. This mutant also recruits CASK and Mint1. Similarly, the 5-HT_{2C} C-terminus was a stronger partner than the 5-HT_{2A} C-terminus for PSD-95, SAP102, and Dlgh3/MPP3. The single substitution of cysteine at the −1-position did not modify the situation. Only a peptide carrying a double substitution at the −1- and −6-positions generated a robust interaction of the mutated 5-HT_{2A} C-terminus peptide with all the three proteins *(7)*.

In contrast, CIPP, already identified as a partner of the potassium channel Kir4.0 family, the NMDA receptor NR2 subunits, neurexin, neuroligins, and acid-sensing ionic channel 3 *(20,21)*, interacted much more strongly with the 5-HT_{2A} receptor C-terminus than with the 5-HT_{2C} receptor C-terminus. Interestingly, we found that when a single substitution at the −8-position (glycine for valine) was operated within the 5-HT_{2A} C-terminal peptide, little interaction with CIPP was left. This indicates that this residue is critical for the interaction. In agreement with this proposal, the reciprocal substitution, on the 5-HT_{2C} receptor C-terminus, at the −8-position (valine for glycine) generated a robust binding to CIPP, comparable to that found with the 5-HT_{2A} receptor C-terminus (Fig. 3B). This finding indicates that whereas residues of a PDZ ligand localized at positions 0 and −2 are necessary for its interaction with any PDZ domain, the specificity of interaction with a given PDZ domain might be related to residues localized far from the last three residues. For example, tyrosine at position −7 in the tyrosine kinase receptor ErbB2 C-terminus is involved in the binding to the Erbin PDZ domain *(22)*.

4. Presynaptic Versus Postsynaptic Localization of 5-HT_{2A} and 5-HT_{2c} Receptors and Associated Receptosomes

Most of the proteins identified as binding partners of 5-HT_{2A} and 5-HT_{2C} receptors are recognized for their role in organizing synaptic protein networks. Electron microscopy (EM) experiments (Fig. 5) indicated that, in neurons, the 5-HT_{2C} receptor is highly concentrated at postsynaptic and presynaptic thickenings of axo-dendritic synapses, consistent with its interaction with both proteins of the postsynaptic density such as PSD-95, SAP97, and SAP102 and the Veli3-CASK-Mint1 ternary complex, which is known to interact with proteins of the presynaptic termini. Veli3 was, indeed, found at both the presynaptic and the postsynaptic level in EM observations (Fig. 5). In contrast, the 5-HT_{2A} receptor, which does not interact with this complex, was mainly detected in postsynaptic processes *(23)*. Altogether, these results indicate that the 5-HT_{2A}

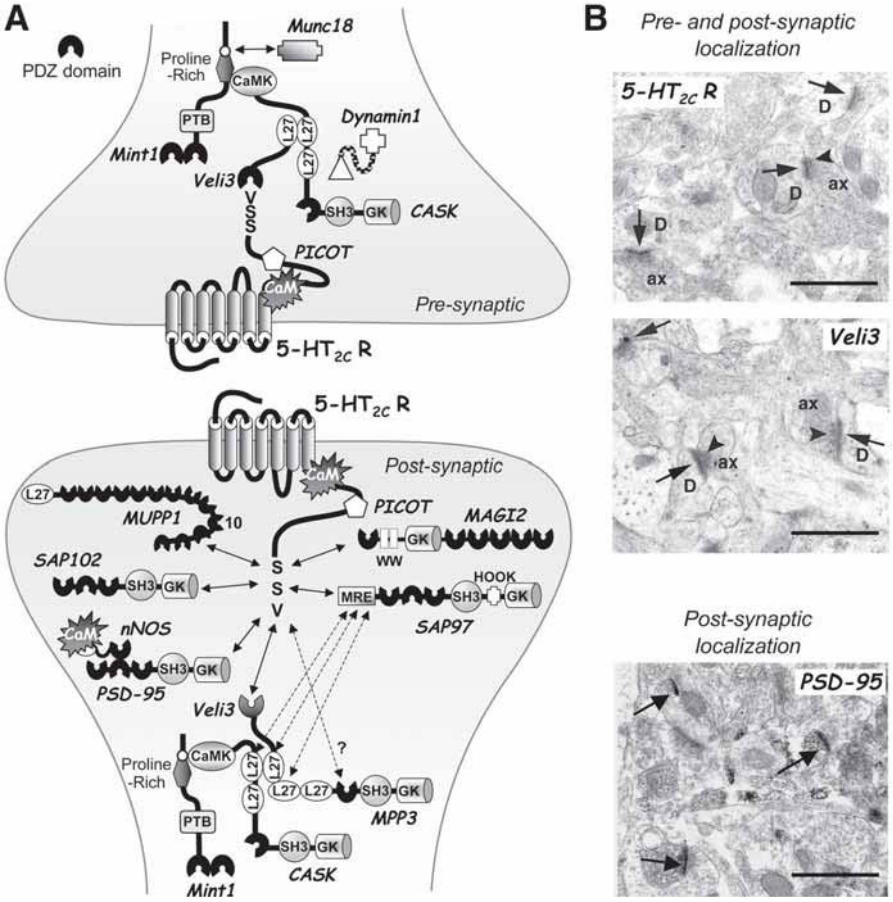

Fig. 5. Ultrastructural distributions of the 5-HT_{2C} receptor, Veli3, and PSD-95. **(A)** Schematic representation of the putative presynaptic and postsynaptic 5-HT_{2C} "receptosome." This schema is based on the data discussed in this review. **(B)** Comparison among the subcellular distribution of the 5-HT_{2C} receptor, Veli3, and PSD-95. Postsynaptic and presynaptic staining are indicated by arrows and arrowheads, respectively. Modified from refs. *7* and *8*.

and 5-HT_{2C} "receptosomes" are not identical and show distinct ultrastructural distribution in the mammalian central nervous system.

5. GIPs Associated With Some 5-HT_4 Splice Variants

The 5-HT_4 receptor is certainly one of the GPCRs for which alternative splicing generates the greatest number of variants *(24)*. To date, eight variants have been cloned in humans, four in mice, and three in rats. All these variants

differ in their C-termini after a single position (L358). Some differences in the signal transduction of these variants have been reported mainly in heterologously transfected systems *(24)*. However, the involvement of each splice variant in specific physiological processes remains to be elucidated. Among the four mouse 5-HT_4 receptor splice variants, three express a canonical recognition motif for PDZ domains at their C-termini. The PDZ ligand of the 5-HT_{4a} receptor (-SCF) belongs to ligands known to interact with class I PDZ domains, whereas that of the 5-HT_{4e} and 5-HT_{4f} receptor variants (-VPV) might interact with PDZ domains belonging to class II (-ΦXΦ).

We used a proteomic approach to define the composition of "receptosomes" associated with 5-HT_{4a} and 5-HT_{4e} receptor variants *(9)*. We have also tried to identify a possible "receptosome" associated with the C-terminus of the 5-HT_{4b} receptor variant, which does not contain a terminal PDZ ligand consensus sequence.

Synthetic peptides corresponding to the last 14 C-terminal residues of the 5-HT_{4a}, 5-HT_{4e}, and 5-HT_{4b} receptors (*see* Fig. 1), immobilized on Sepharose beads, were used as bait to fish out proteins of mice brain and primary cultured neurons that interact with the C-termini of these receptors. Truncated peptides lacking the three C-terminal residues were used as negative controls to fish for PDZ-containing proteins. As described for the fishing of 5-HT_{2A} and 5-HT_{2C} "receptosomes," retained proteins were separated on 2D gels, stained with silver and the proteins that specifically bind to wild type, vs truncated peptides, were identified by MALDI-TOF mass spectrometry.

We detected 10 spots or groups of spots in gels obtained with the C-terminal peptide of the 5-HT_{4a} receptor variant, which were absent in gels obtained with the truncated (ΔSCF) peptide. Among them, seven encompass one or several PDZ domains (Fig. 6): ARIP-1/MAGI-2, Dlgh3/MMP3, SNX27a (Sortin Nexin 27a), NHERF (Na^+/H^+ exchanger regulatory factor), Veli1, Veli2, and Veli3. The most original one was SNX27 (also called Mrt1). This protein contains one PDZ domain and one Phox-homology domain (PX domain). SNX27 belongs to the huge sorting nexin protein family implicated in the regulation of membrane-protein trafficking. Two isoforms of SNX27 generated by alternative splicing (SNX27a and SNX27b, respectively, also referred to as Mrt1a and Mrt1b), which differ in the length of their C-termini, have been identified *(25)*. SNX27a is constitutively expressed in adult brain and testis, whereas SNX27b is specifically induced in adult brain by methamphetamine treatment *(25)*. We examined the interaction of the 5-HT_{4a} receptor with both SNX27a and SNX27b in HEK293 cells expressing each SNX27 isoform together with the 5-HT_{4a} receptor *(9)*. Coimmunoprecipitation of the receptor with SNX27a and SNX27b was obtained. ΔSCF mutated receptors, as well as the deletion of the PDZ domain of SNX27, but not that of the PX domain, suppressed

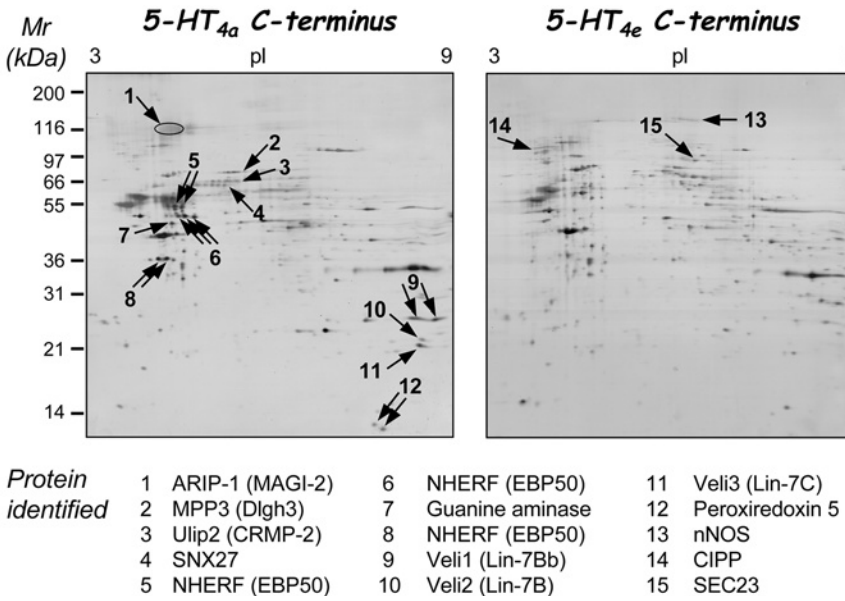

Fig. 6. Two-dimensional analysis of proteins interacting with the 5-HT_{4a} and the 5-HT_{4e}-receptor C-termini in a PDZ-dependent manner. Proteins from mice brains that bind to the C-terminus (last 14 residues) of the receptors (*see* Fig. 1) were separated on 2D gels and stained with silver. Proteins that interact specifically (directly or indirectly) with the PDZ ligand of the receptor (arrows) were detected by comparing protein patterns obtained with the native peptides (*see* Fig. 1 for sequences) and mutant peptides in which the last three residues (PDZ ligand) were deleted. Modified from ref. *9*.

coimmunoprecipitation. Moreover, we have shown that, like most members of the SNX protein family, SNX27a and SNX27b are mainly localized in early endosomes, as assessed by their colocalization with the early endosome antigen 1 (EEA1). SNX27 was also able to redirect part of the 5-HT_{4a} receptors to early endosomes *(9)*. The physiological role of SNX27-5-HT_{4a} receptor interaction remains to be found. However, there is some evidence to suggest that 5-HT_4 receptors could play a role in drug sensitization *(26)*.

The other interesting protein associated with 5-HT_{4a} receptors was NHERF *(9)*. In NIH-3T3 cells, 5-HT_{4a} receptors were not localized in microvilli. However, when cotransfected with NHERF, a strong colocalization of the two proteins was observed in microvilli *(9)*. NHERF interacts with the four-point-one/ezrin/radixin/moesin (FERM) domain of activated ezrin. Ezrin is homogenously distributed throughout the cytoplasm of NIH-3T3 cells. This distribution remains in cells transfected with the 5-HT_{4a} receptor. In contrast, in cells cotransfected with the 5-HT_{4a} receptor and NHERF, erzrin was redistributed within microvilli *(9)*.

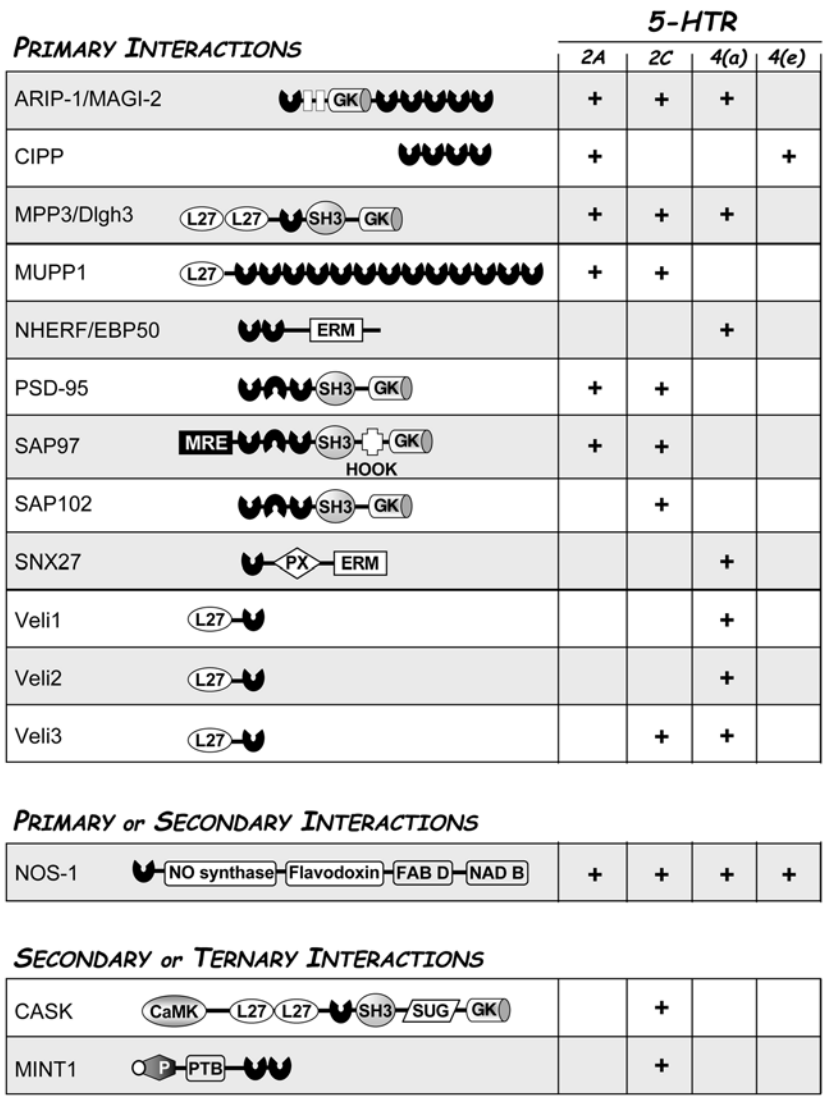

Fig. 7. Domain structures of the PDZ-containing proteins associated with 5-HT_{2A}, 5-HT_{2C}, 5-HT_{4a}, and 5-HT_{4e} receptors.

This indicates that ezrin might constitute a link between the 5-HT_{4a} receptor–NHERF complex and the cytoskeleton in order to control cell adhesion and motility.

In addition to PDZ-domain-containing proteins, the C-terminus of 5-HT_{4a} receptors recruited non-PDZ-domain-containing proteins, such as guanine

aminase and peroxiredoxin 5 *(9)*. However, the C-termini of these proteins can be considered to be PDZ ligands (SSV and SQL respectively), suggesting that these proteins might be secondary partners of 5-HT_{4a} receptors and recruited via one of the PDZ proteins associated with the receptor. The last protein recruited in a PDZ-dependent manner by the 5-HT_{4a} receptor is Ulip2. First described as collapsin-response-mediator protein 2 (CRMP-2), this protein is involved in the regulation of axonal growth *(27)*. The mechanism by which the 5-HT_{4a} receptor C-terminus binds to Ulip2 remains to be elucidated because the sequence of this protein lacks any obvious PDZ domain and/or PDZ ligand.

Proteins associated with the PDZ ligand of the 5-HT_{4e} receptor C-terminus are completely different from those that bind to the 5-HT_{4a} receptors. They include the neuronal isoform of nitric oxide synthase (nNOS) and CIPP, two PDZ-domain-containing proteins, and Sec 23, which lacks any obvious PDZ domain *(9)*. Sec 23 is a member of a protein complex designated as COPII, which is involved in the budding of vesicles from the endoplasmic reticulum (ER) and ER-to-Golgi transport of proteins.

Finally, the C-terminal peptide of the 5-HT_{4b} receptors was unable to retain any specific protein, consistent with the lack of any identified interaction motif in its sequence.

6. Conclusion

In this review, we have focused our interest on the identification of GIPs associated with 5-HT_{2A}, 5-HT_{2C}, 5-HT_{4a}, and 5-HT_{4e} receptors using a proteomic approach. We have not discussed the available data concerning the functions of some of these GIPs. They are discussed in Chapter 8 by Xia et al.

It is likely that additional GIPs comprising the already described "receptosomes" will be found. It is especially likely that their nature will be shown to be dependent on the cell in which receptors are expressed. However, it is clear that more challenging issues remain, such as the fine description of the kinetics of interactions and their regulations, as well as the understanding of their roles in physiological/pathological states.

In this context, it is interesting to note that some GIPs associated with 5-HT receptors might be implicated in addiction. Metamphetamine treatment repeated daily for 5 d induced a persistent increase in SNX27b (or Mrt1b) in the adult rat neocortex *(25)*. This effect, which was blocked by D_1 antagonists, is reminiscent of stimulant-induced behavioral sensitization. Genetic studies indicate that *mpdz* gene (coding for MUPP-1/MPDZ protein) is an addiction quantitative trait gene for drug-withdrawal severity seizures *(28)*. Roles for GIPs in pathologies is just an emerging area. In parallel, drugs targeted to the GPCR–GIPs interaction will probably be proposed *(6)*.

Acknowledgments

This work was supported by grants from the Centre National de la Recherche Scientifique, the French Ministère de la Recherche et des Nouvelles Technologies (ACI-JC no. 5075), the Génopole de Montpellier Languedoc–Roussillon, the European Community's 6th framework program (grant LSHB-CT-2003-503337), and Pfizer Japan.

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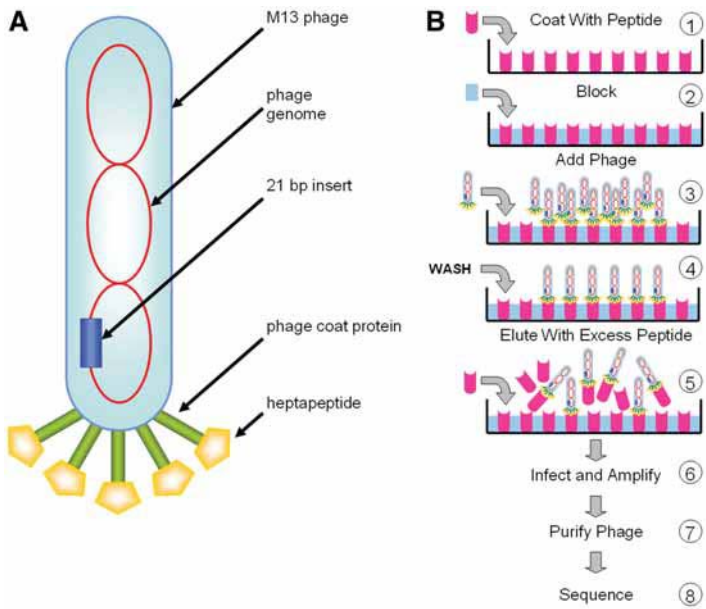
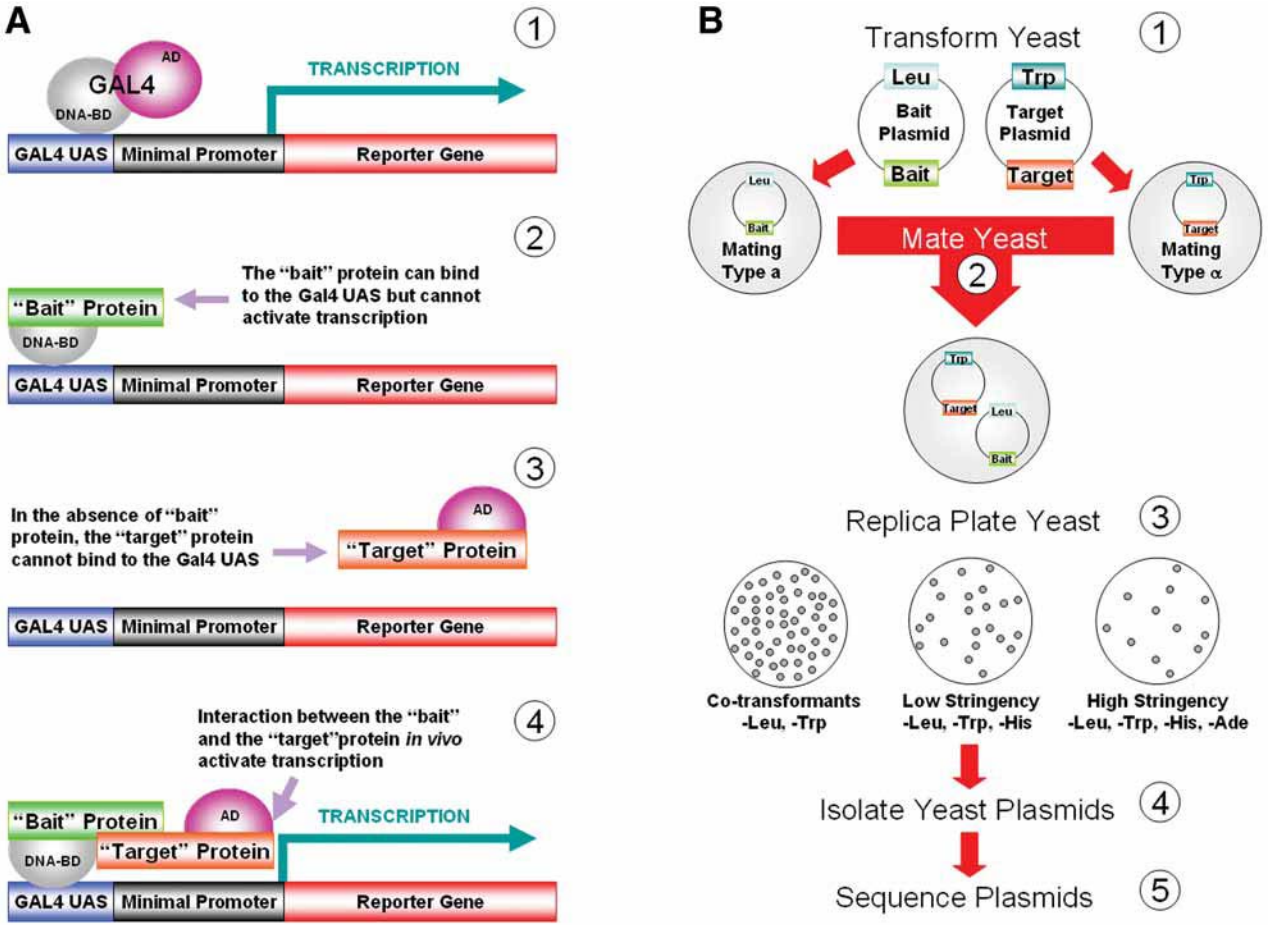
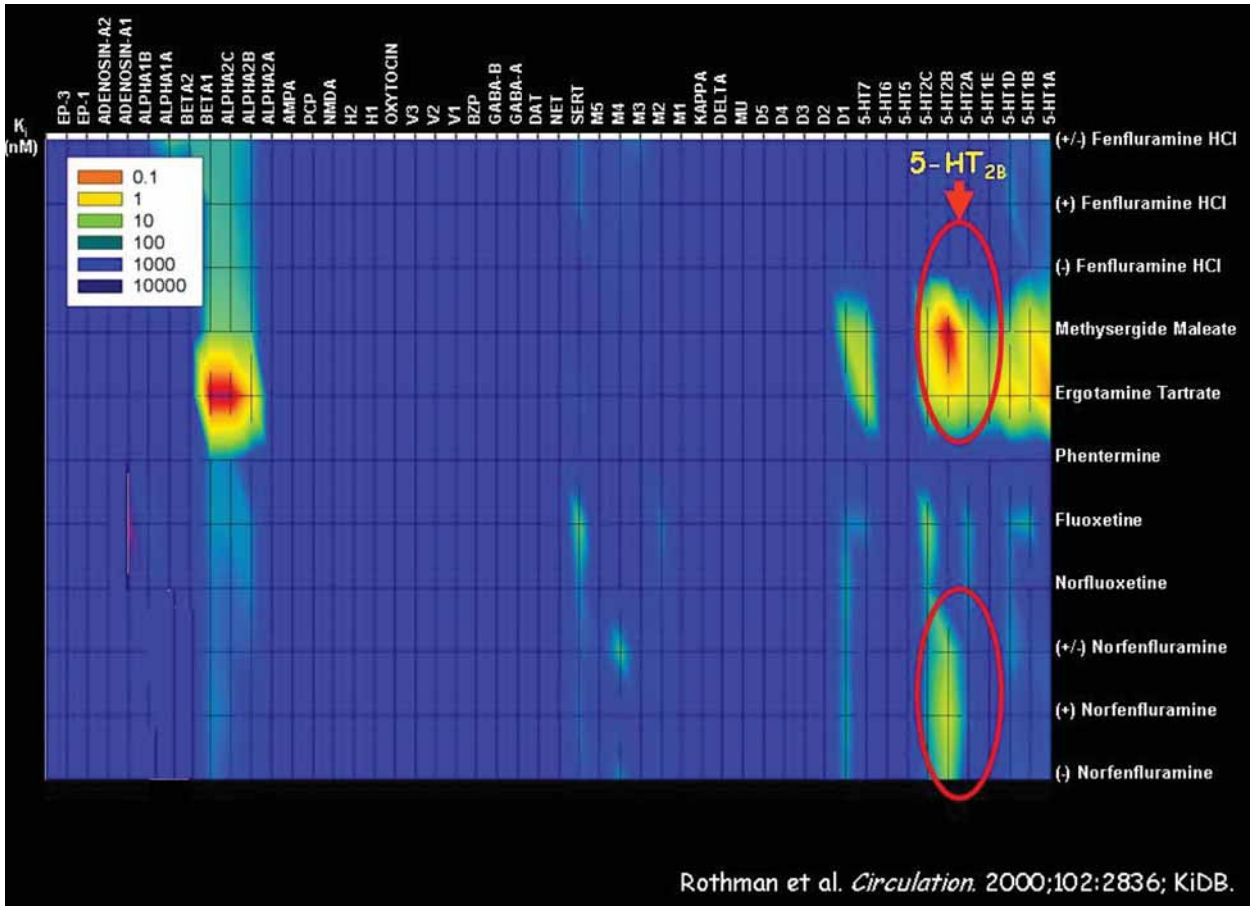
Chapter 2, Fig. 2, p. 46: A 5-HT_{2A}-receptor model constructed from the rhodopsin crystal structure.



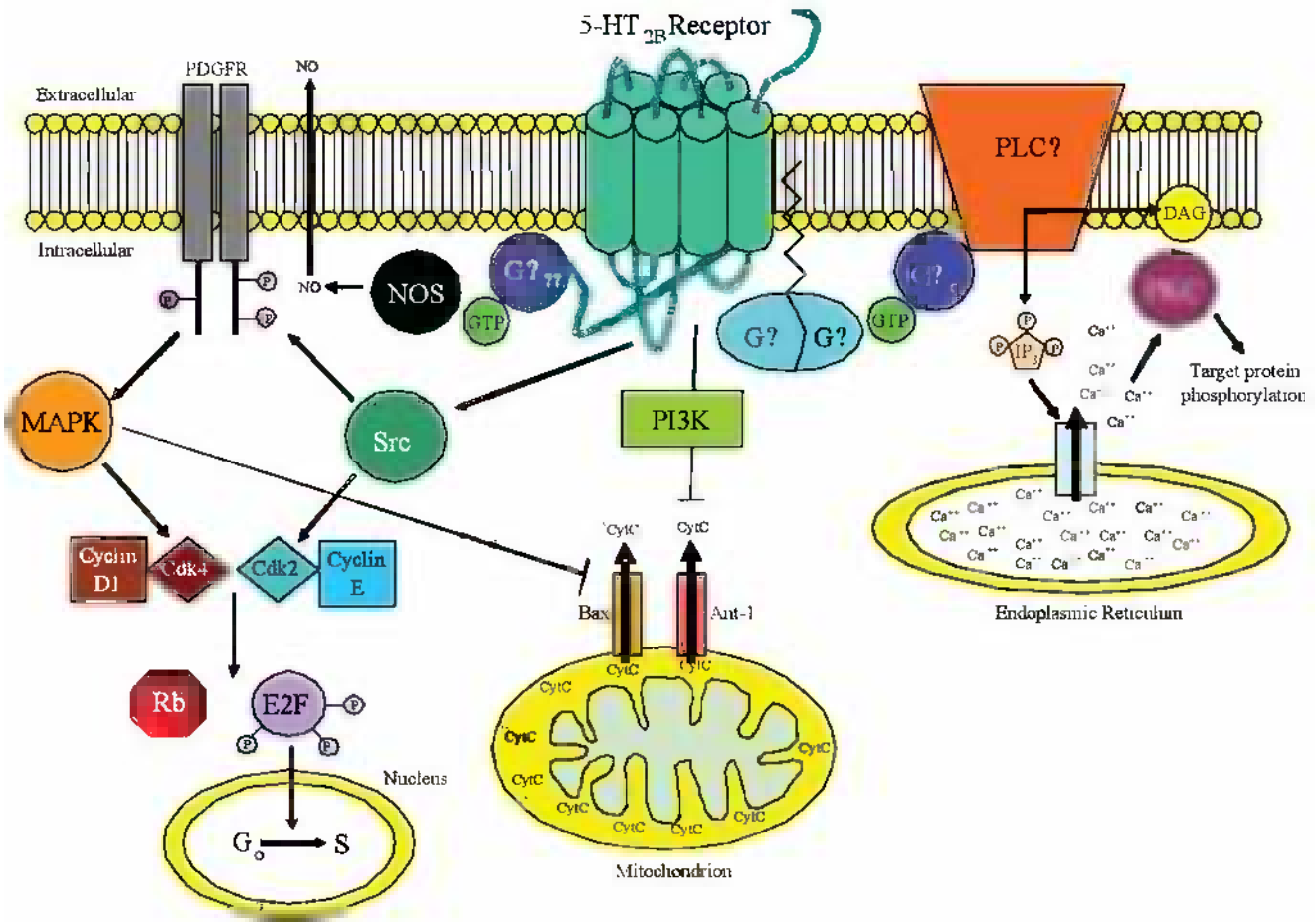
Chapter 8, Fig. 2, p. 261: An overview of phage display as a technique to identify FRAPs.



Chapter 8, Fig. 1, p. 260: An overview of yeast two-hybrid screens for FRAPs.



Chapter 13, Fig. 1, p. 426: Three-dimensional representation of VHD-associated and non-VHD-associated drug affinity for a battery of GPCRs, ligand-gated ion channels, and biogenic amine transporters.



Chapter 13, Fig. 2, p. 430: Mitogenic signal transduction from 5-HT_{2B} receptors.

8

5-HT Receptor-Associated Proteins (FRAPs)

Relevance for Targeting, Trafficking, and Signal Transduction

Zongqi Xia, Douglas J. Sheffler, and Bryan L. Roth

Summary

Dysfunction in the serotonergic system contributes to the etiology and pathophysiology of a variety of neuropsychiatric and systemic disorders, and 5-hydroxytryptamine(5-HT) receptors as a group represent important therapeutic targets for many of these debilitating diseases. Encompassing a diverse group of proteins that interact with 5-HT receptors, 5-HT receptor-associated proteins (FRAPs) coordinate receptor targeting to the appropriate subcellular compartments, organization of networks of signaling and structural proteins (known as receptosomes), and calibration of receptor-mediated signal transduction, including desensitization, resensitization, and crosstalk with other signaling pathways. The study of FRAPs opens a decidedly wide arena for novel pharmaceutical research approaches. We will provide an overview of the burgeoning field of FRAPs, with a focus on their role in modulating signal transduction.

Key Words: Serotonin; 5-HT receptor; 5-HT receptor-associated protein; receptosome; trafficking; targeting; signaling.

1. Introduction

Serotonin or 5-hydroxytryptamine (5-HT) receptors with 7 subtypes and at least 15 distinct members mediate a diverse array of physiological functions both in the central nervous system and in the periphery *(1)*. Their varied cellular distribution and complex signal transduction in part account

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Edited by: B. L. Roth © Humana Press Inc., Totowa, NJ

for the observation that 5-HT receptors play pivotal roles in appetite, attention, cognition, mood, perception, and sleep, as well as cardiovascular development, vasculature tone, gastrointestinal and platelet function, cellular proliferation, and neuroendocrine interactions *(2,3)*. Not surprisingly, dysfunction in the serotonergic system contributes to the etiology and pathophysiology of a plethora of neuropsychiatric and systemic disorders that afflict a large portion of the human population, ranging from anxiety, mood disorders, schizophrenia, and obsessive–compulsive disorder to migraine, obesity and progressive neurological disorders *(1,4,5)*.

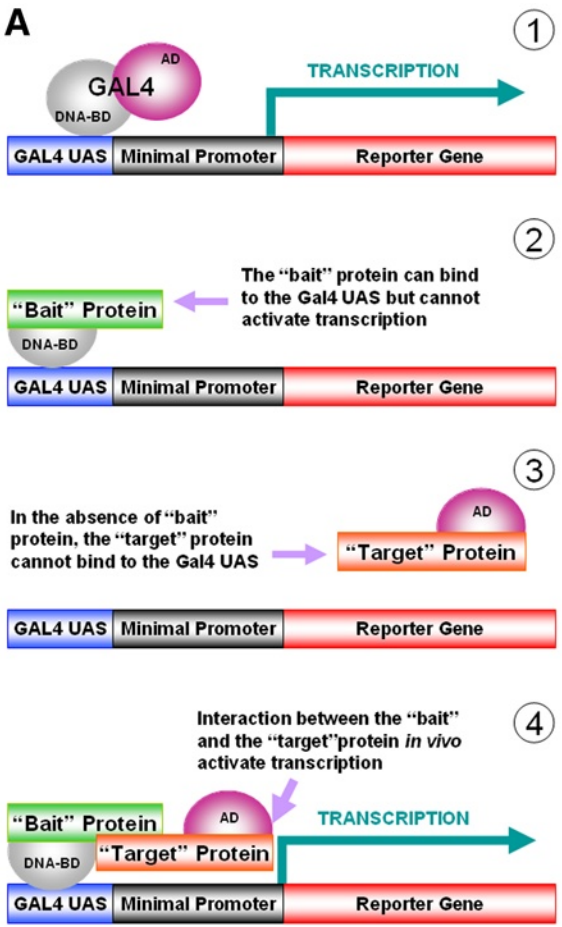
Attempts to understand the regulation of 5-HT receptor function have led to better appreciation for the critical involvement of the 5-HT receptor-associated proteins (FRAPs), specifically in targeting receptors to the appropriate subcellular compartments, assembling multimolecule complexes, and modulating receptor-coupled signaling pathways. We will review the recent progress in the field of FRAPs, with emphasis on their regulation of the 5-HT receptor targeting, trafficking, and signaling.

2. Discovering 5-HT Receptor-Associated Proteins and Receptosomes

The majority of the 5-HT receptor subtypes are heptahelical G protein–coupled receptors (GPCRs) with the noted exception of 5-HT_3 receptors, which form ligand-gated ion channels *(6,7)*. Thus, GPCR-interacting proteins, either soluble or transmembrane, stood out as some of the best characterized FRAPs. Together, 5-HT receptors and their FRAPs constitute a structural and functional network of proteins known as the "receptosome" *(8)*.

For years, it was thought that the cytosolic carboxyl termini as well as various intracellular loops of the 5-HT receptors bind to FRAPs. Efforts from our lab to identify FRAPs utilized yeast two-hybrid screens (*see* Fig. 1 for an overview), phage display (*see* Fig. 2 for an overview), and direct biochemical approaches *(8–13)*. These studies led to the discovery of many 5-HT_{2A} receptor-interacting proteins, including caveolin-1 (Cav-1), arrestin-2 (Arr-2), arrestin-3 (Arr-3), microtubule-associated protein-1A (MAP-1A), and postsynaptic density protein-95 (PSD-95) (*see* Table 1).

Combining affinity chromatography, two-dimensional electrophoresis, and mass spectroscopy, Bécamel and colleagues pioneered a global proteomic approach to 5-HT receptosomes (Chapter 7). These groundbreaking studies employed either the entire or partial carboxyl terminus of the 5-HT_2 receptors as bait and heralded the discovery of multiple FRAPs *(14,15)*. Since then, the proteomic strategy has been successfully applied to other 5-HT receptor subtypes *(16,17)*.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GAL4 UAS
Minimal Promoter
Reporter Gene
3
In the absence of "bait" protein, the "target" protein cannot bind to the Gal4 UAS
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"Target" Protein
GAL4 UAS
Minimal Promoter
Reporter Gene
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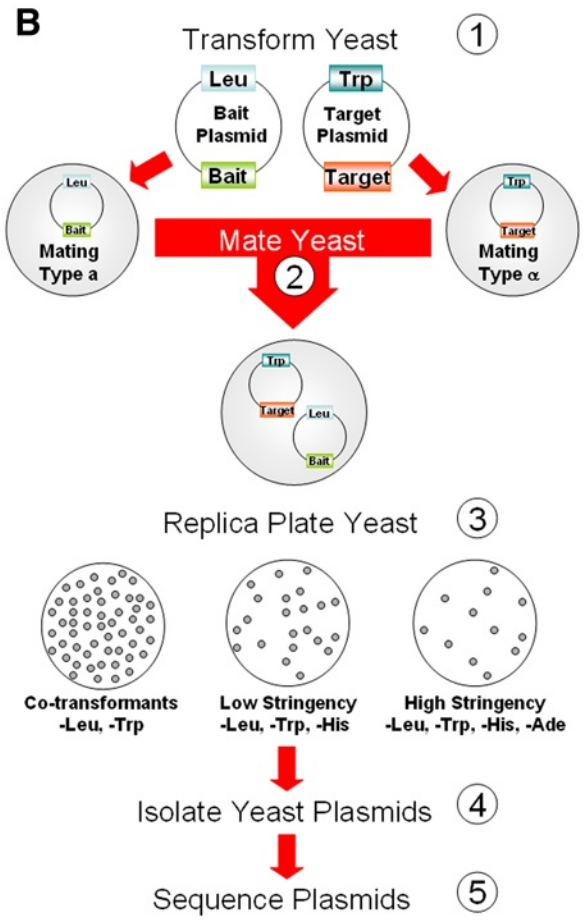
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Co-transformants -Leu, -Trp
Low Stringency -Leu, -Trp, -His
High Stringency -Leu, -Trp, -His, -Ade
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2.1. PDZ Proteins

Multi-PDZ domain protein 1 (MUPP1) proved to be the first PDZ domain-containing protein (i.e., PDZ protein) that directly interacts with any 5-HT receptor—in this case, the 5-HT_{2C} receptor in vivo as well as with the 5-HT_{2A} and 5-HT_{2B} receptor in vitro *(14,18)*. Considering that the carboxyl termini of 5-HT_2 receptor subtypes contain the binding motif for the PDZ domain, it is not surprising that PDZ proteins, which were also among the first FRAPs identified by the proteomic strategy *(15)*, have emerged as a prominent component of the 5-HT receptosome (*see* Table 1). The PDZ domain is an evolutionarily conserved protein–protein interaction motif named after the three prototypic proteins where it was first described: the postsynaptic protein (PSD) protein 95 (PSD-95), its *Drosophila* homolog (discs large), and the tight junction protein (zonula occludens, ZO-1) *(19–21)*. Recent genomic analysis estimates that the human genome contains at least over 300 PDZ proteins *(17)*.

Fig. 1. *(previous page)* An overview of yeast two-hybrid screens for FRAPs. **(A)** A schematic showing the molecular basis of a yeast two-hybrid screen. In classical yeast two-hybrid screens, a transcription factor (illustrated here by the Gal4 transcriptional activator) (1) can modulate transcription by binding to an upstream activating sequence (UAS) through a DNA-binding domain (DNA-BD) and activate transcription through a transcriptional-activation domain (AD). These transcription factor domains can be separately fused to a "bait" peptide sequence (e.g., a GPCR c-terminal domain) to create a DNA-BD-"bait" fusion protein, which (2) cannot activate transcription alone, as it lacks a AD. Through the use of cDNA libraries, a "target" peptide can be fused to the AD to create a peptide library of "target"-AD fusion proteins. These "target"-AD fusion proteins (3) are unable to activate transcription, as they lack a DNA-BD. If the DNA-BD-"bait" and "target"-AD fusion proteins are both present (4) and the "bait" and "target" can interact with one another, the DNA-BD and AD of the transcription factor are brought within close proximity and the activity of the transcription factor is reconstituted. **(B)** A general yeast two-hybrid protocol. Plasmids containing DNA-BD-"bait" and "target"-AD constructs are prepared and (1) transformed into opposite yeast mating strains. These plasmids also contain genes that allow for growth on nutrient-deficient media; in this example, the "bait" plasmid contains a gene required for leucine biosynthesis (Leu) and the "target" plasmid contains a gene required for tryptophan biosynthesis (Trp). Following transformation into opposing yeast mating types, the yeast are (2) mated to produce yeast that contain both "bait" and "target" plasmids and then (3) replica plated onto nutrient deficient media. In this example, plating the yeast on media lacking leucine and tryptophan is a confirmation of the presence of both the "bait" and "target" plasmids. In addition, the yeast strain transformed by the "bait" and "target" plasmids also contains genes required for histidine (His) and adenine (Ade) biosynthesis. Therefore, growth of the yeast on media lacking leucine, tryptophan, histidine, and adenine implies that the "bait" and "target" proteins interact. Plasmid isolation (4) and sequencing (5) from these surviving colonies provides a list of potential FRAPs. (Illustration appears in color in insert that follows p. 240.)

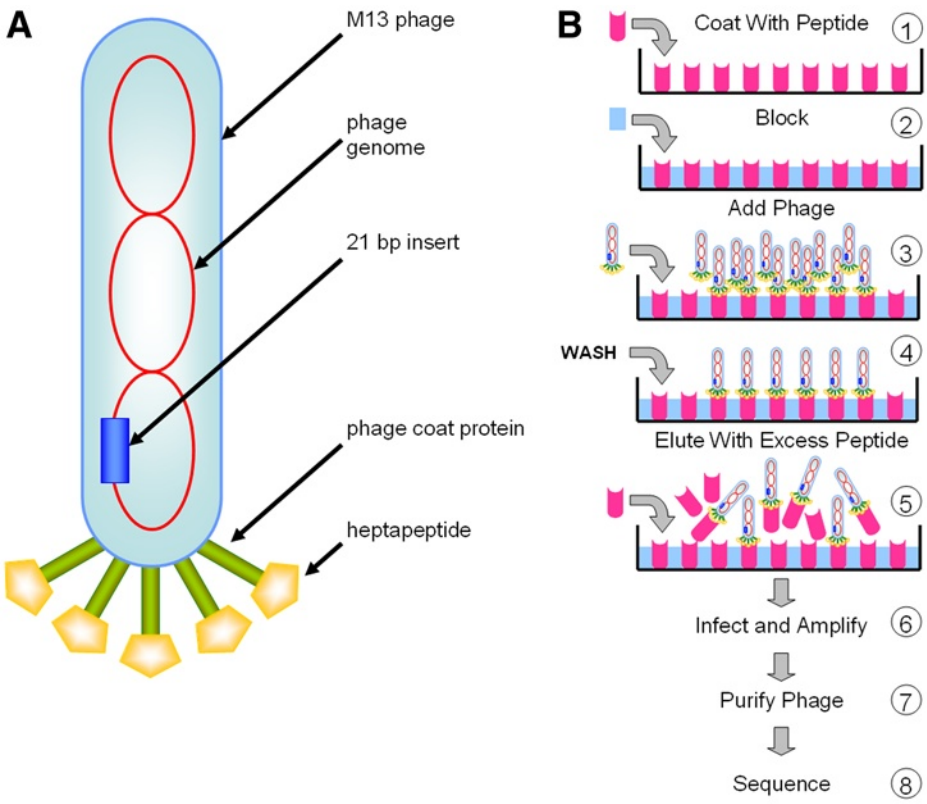

Fig. 2. An overview of phage display as a technique to identify FRAPs. **(A)** Schematic of a peptide-displaying phage. For phage display, a 21-basepair random DNA sequence is inserted into the phage genome, which is then expressed as a heptapeptide on the surface of the M13 phage. This provides a randomized peptide library for screening for FRAPs. **(B)** An overview of the phage-display protocol. To identify FRAPs, plates are (1) coated with a peptide of interest (e.g., the third intracellular loop of a GPCR), (2) blocked for non-specific interaction, (3) incubated with a randomized M13 phage library, and (4) washed extensively to remove excess phage. A specifically bound M13 phage is eluted (5) with an excess of the peptide that was originally used to coat the plate. These specifically bound phages are then used to (6) infect *Escherichia coli* in order to amplify their genomes, after which the phages are purified (7) and sequenced (8) in order to determine the heptapeptide sequences. These sequences can then be aligned to known peptide sequences to identify FRAPs. (Illustration appears in color in insert that follows p. 240.)

As PDZ proteins typically contain multiple PDZ domains as well as additional types of protein-interacting motifs such as the SH3 and Veli-binding domain, they are the ideal FRAPs for assembling 5-HT receptosomes *(17,22)*. As such, not all PDZ proteins in the receptosomes are required to interact with 5-HT

Table 1
Summary of Known FRAPs and Their Associated 5-HT Receptors

FRAP	5-HT Receptors
Arrestin-2, Arrestin-3	5-HT_{2A}
ARIP-2 = MAG12	5-HT_{2A}, 5-HT_{2C}, $5\text{-HT}_{4(a)}$
ARF-1	5-HT_{2A}
Calmodulin	5-HT_{1A}, 5-HT_{2C}
Calveolin-1	5-HT_{2A}
CASK	5-HT_{2C}
CIPP	5-HT_{2A}, $5\text{-ht}_{4(e)}$
Dlgh3 = MPP3	5-HT_{2A}, 5-HT_{2C}, $5\text{-HT}_{4(a)}$
JAK	5-HT_{2A}
MAG12 = ARIP-2	5-HT_{2A}, 5-HT_{2C}, $5\text{-HT}_{4(a)}$
MAP1A	5-HT_{2A}
MPP3 = Dlgh3	5-HT_{2A}, 5-HT_{2C}, $5\text{-HT}_{4(a)}$
MUPP1	5-HT_{2A}, 5-HT_{2C}
Mint-1	5-HT_{2C}
NH ERF	$5\text{-HT}_{4(a)}$
NOS1	5-HT_{2C}, $5\text{-HT}_{4(e)}$
PSD-95	5-HT_{2A}, 5-HT_{2C}
PICOT	5-HT_{2C}
RSK-2	5-HT_{2A}
SAP97	5-HT_{2A}, 5-HT_{2C}
SAP102	5-HT_{2C}
SNX-27	$5\text{-HT}_{4(a)}$
Ulip2/CRMP2	$5\text{-HT}_{4(a)}$
Veli-1	$5\text{-HT}_{4(a)}$
Veli-2	$5\text{-HT}_{4(a)}$
Veli-3	5-HT_{2C}, $5\text{-HT}_{4(a)}$

Note: ARIP1 = activin receptor interacting protein 1; ARF-1 = ADP-ribosylation factor-1; CASK = vertebrate homolog of the *Caenorabditis elegans* PDZ protein Lin2; CIPP = channel-interacting PDZ protein; CRMP2 = collapsing response mediator protein 2; Dlgh3 = *Drosophila* discs large; JAK = Jun-activated kinase; MAGI2 = MAGUK with inverted domain structure 2; MAP1A = microtubule-associated protein 1A; Mint-1 = vertebrate homolog of the *C. elegans* PDZ protein Lin10; MPP3 = membrane protein palmitoylated 3 = MAGUK p55 subfamily membrane protein; MUPP1 = multi-PDZ domain protein 1; NH ERF = Na/H exchanger regulatory factor; NOS1 = nitric oxide synthase 1; PICOT = PKCθ-interacting protein); PSD-95 = postsynaptic density protein 95; RSK-2 = ribosomal S6 kinase-2; SAP = synapse-associated protein; SNX27 = sorting nexin 27; Ulip = Unc-33-like phosphoprotein; Veli = vertebrate homolog of the *C. elegans* PDZ protein Lin7.

receptors directly. For example, it is thought that Veli-3 (the vertebrate homolog of the *Caenorabditis elegans* PDZ protein Lin-7) provides the necessary scaffold for the recruitment of CASK (the vertebrate homolog of the *C. elegans* PDZ protein Lin-2) and Mint-1 by 5-HT_{2C} (the vertebrate homolog of the *C. elegans* PDZ protein Lin-10) *(15)*.

In general, the last three amino acid residues in the distal carboxyl terminus of many 5-HT receptors constitute the PDZ ligand, or the minimal sequence necessary for binding to PDZ proteins. Of note, evidence also exists for an internally located PDZ ligand *(23)*. The residue at the second to the last (or −2) position typically determines the major specificity of the PDZ ligands, dividing them into three classes: I (-S/T-x-Φ), II (-Φ-x-Φ), and III (-ψ-x-Φ), where S is serine, T is threonine, x is any residue, Φ is hydrophobic residue, and ψ is acidic residue *(19)*. Residues at the −1-position or those upstream of the canonical PDZ ligand in 5-HT receptors further fine-tune the binding specificity and affinity of interaction with the associated PDZ proteins *(19,24)*. Thus, 5-HT_{2A}, 5-HT_{2C}, and 5-$HT_{4(a)}$ receptors, which share the class I PDZ ligand, recruit common but also distinct set of FRAPs *(17,24)* (*see* Table 1). The Veli-3/CASK/Mint-1 ternary complex, for example, is a component of the 5-HT_{2C}, but not the 5-HT_{2A}, receptorsome *(15)*. Furthermore, although both interact with PSD-95, 5-HT_{2C} receptors bind to PSD-95 with greater affinity than 5-HT_{2A} receptors *(13,25)*.

It is worth noting that recognition by FRAPs might require more than the presentation of a simple linear sequence on the cytosolic side of 5-HT receptors. Other alternative scenarios could plausibly exist in vivo: (1) Multiple, and potentially competing, binding motifs might be present within the same protein; (2) noncontiguous amino acid sequences might constitute a binding motif; (3) essential binding motifs might require "unmasking" (e.g., phosphorylation, palmitoylation) before appropriate recognition. Thus, phosphorylation of the serine residue at the −2 position of the PDZ ligand following agonist exposure could reversibly inhibit 5-HT_{2C} receptor interaction with MUPP1 *(26)*. MUPP1 interaction with the unphosphorylated 5-HT_{2C} receptor is thought to keep the receptor in a conformation state that masks the receptor from some of its downstream signaling partners. Receptor phosphorylation at the PDZ ligand releases MUPP1 from the receptor, causes change in receptor conformation, and reveals binding sites for interaction with additional FRAPs in the 5-HT_{2C} receptorsome *(26)*.

2.2. Non-PDZ Proteins That Interact With the Carboxyl Termini of 5-HT Receptors

The carboxyl termini of the 5-HT receptors also contain protein-interacting motifs other than the PDZ ligand. The NPxxY motif located at the junction

between the seventh transmembrane and the carboxyl terminus constitutes perhaps the best known PDZ-independent binding domain in GPCRs *(27)*. Glutathione-*S*-transferase (GST) pull-down and coimmunoprecipitation studies have demonstrated that the NPxxY motif of the 5-HT_{2A} receptor binds to the ADP-ribosylation factor-1 (ARF-1), an interaction that leads to alternative signaling by the receptor *(28)* (*see* below).

Proteomic studies have revealed additional FRAPs that are not PDZ proteins *(17,24,29)*. Examples of these FRAPs include components of the actin–spectrin cytoskeleton (e.g., beta-actin, spectrin alpha II chain) and the intracellular signaling apparatus (e.g., calmodulin, and protein kinase C [PKC]θ-interacting protein).

The PDZ proteins might function in recruiting some of these non-PDZ proteins to the 5-HT receptosomes. CASK, a PDZ protein, recruits dynamin I (a GTPase involved in endocytosis) to 5-HT_{2C} receptors *(15)*. Likewise, the Veli-3/CASK/Mint-1 complex mediates the association between Munc-18 (a protein of the exocytotic apparatus) and the 5-HT_{2C} receptor *(15)*. Evidence points to PSD-95 as the FRAP that provides scaffold for the neuronal form of nitric oxide synthase (NOS1) in the 5-HT_{2C} and possibly the 5-HT_{2A}, receptosome *(15)*.

2.3. Proteins That Interact With the Third Intracellular Loop of the 5-HT Receptors

Similar to other GPCRs, the third intracellular loop (or i3) of the 5-HT receptors couples to the heterotrimeric G proteins. Although the i3 loop is likely to mediate other important protein–protein interactions, relatively few FRAPs have been described to bind to this region. Those interactions that have been described appear to have important functional significance. Examples of FRAPs that bind to the i3 loop of 5-HT receptors include Arr-2, Arr-3 *(30)*, calmodulin *(31)*, and MAP-1A *(9)*. That the i3 loop of the 5-HT_{2A} receptors binds to MAP1A is particularly intriguing because MAP-1A colocalizes in the large apical dendrites with 5-HT_{2A} receptors and is thought to play an important role in receptor targeting in neurons *(9,32–35)*.

3. FRAPs Mediate the Subcellular Localization of 5-HT Receptors

The subcellular distribution of the 5-HT receptors likely contributes to their distinct functions. In polarized cells, several lines of evidence support the notion that receptor interaction with FRAPs targets and anchors 5-HT receptors to the appropriate subcellular compartments and modulates receptor internalization. We should also keep in mind that although most studies described in this section capture only a static image, we have only recently begun to appreciate the

dynamic process of intracellular receptor targeting and trafficking. Thus, PKC can move 5-HT_{3A} receptors in and out of the cell membrane through actin-dependent pathways, thereby dynamically regulating the cell surface expression of 5-HT_{3A} receptors and receptor-mediated currents *(36)*.

3.1. FRAPs Target 5-HT Receptors to Distinct Subcellular Regions

Protein-interacting motifs in 5-HT receptors that are necessary for binding to FRAPs are also essential in targeting receptors to the appropriate subcellular regions. Such a targeting signal-dependent mechanism has long been proposed to operate in both polarized epithelial cells and neurons *(37)*. The 5-HT_4 receptor with its eight carboxyl-terminal splice isoforms, of which only a subset contain any PDZ ligand at the distal end, illustrates this point. Na^+/H^+ exchanger regulatory factor (NHE-RF), which was the first PDZ protein reported to modulate the function of any GPCR *(38)*, binds to the 5-$HT_{4(a)}$ isoform that contains a class I PDZ ligand, but to neither the 5-$HT_{4(e)}$ isoform that contains a class II PDZ ligand nor the 5-$HT_{4(b)}$ isoform that lacks any PDZ ligand *(16)*. When expressed in NIH 3T3 fibroblasts, NHE-RF targets 5-$HT_{4(a)}$ receptors from the cytoplasm to the microvilli, where the 5-$HT_{4(a)}$ receptosome colocalizes with activated ezrin, a NHE-RF-interacting protein and a component of the cytoskeleton, whereas the other 5-HT_4 splice variants remained in the cytoplasm *(16)*.

Ample evidence also points to the cytosolic carboxyl terminus in preferentially targeting the axon- and dendrite-selectively bound proteins *(39–45)*. Although the PDZ ligand is well known to mediate polarized targeting *(46,47)*, its essential role in the dendrite-selective transport of any GPCR, and certainly any 5-HT receptor, was first described with the 5-HT_{2A} receptor *(48)*. Disrupting the PDZ ligand greatly diminished the targeting of 5-HT_{2A} receptors from the soma to the postsynaptic processes *(48)*. Because the PDZ ligand of the 5-HT_{2A} receptor directly binds to PSD-95 *(13,25)*, it is conceivable that PSD-95 is one of a series of FRAPs that chaperone and then anchor 5-HT_{2A} receptors to appropriate subcellular regions *(35)*. It is evident that additional FRAPs would have to be involved in the process because disrupting the PDZ ligand failed to completely abolish the dendrite-selective transport of 5-HT_{2A} receptors *(48)*. In this regard, components of the cytoskeletal network such as MAP-1A appear as likely candidates (*see* above).

3.2. FRAPs Colocalize With 5-HT Receptors

A subset of FRAPs and 5-HT receptors share ultrastructural similarities, suggesting their close proximities in specialized subcellular regions. Confocal and electron microscopy demonstrated a concentration of 5-HT_{2C} receptors in the microvilli bordering the apical surface of the epithelial cells in the choroids plexus where they colocalize with Veli-3 and MUPP1, both of which have been

shown to interact with the 5-HT_{2C} receptor by proteomic studies *(15)*. At the axo-dendritic synapses of the olfactory nucleus, electron microscopy has revealed that 5-HT_{2C} receptors are enriched at both presynaptic and postsynaptic thickenings where colocalization with both proteins of the presynaptic terminal (e.g., Veli-3/CASK/Mint1 ternary complex and Munc-18) and the postsynaptic density (e.g., PSD-95) have been described *(15,25)*. In contrast, 5-HT_{2A} receptors are only found in the postsynaptic side of the axo-dendritic synapses in pyramidal neurons of the frontal cortex, where their distribution overlaps with PSD-95 in both the cytosol of the dendritic shaft and the plasma membrane of the postsynaptic density (*34,49,50*, and Xia and Roth, unpublished data), but not with proteins of the presynaptic terminal *(25)*. Furthermore, Arr-2 and Arr-3 colocalize with 5-HT_{2A} receptors in the cell bodies of cortical pyramidal neurons while MAP-1A colocalizes with 5-HT_{2A} receptors in apical dendrites. Taken together, evidences of close proximity between 5-HT receptors and PDZ as well as non-PDZ proteins support the scenario in which these FRAPs organize 5-HT receptosomes in the appropriate subcellular regions.

Certain FRAPs that are known to colocalize with 5-HT receptors seem to form clusters with receptors. PSD-95 and MUPP1 promote the clustering of 5-HT_{2A} and 5-HT_{2C} receptors on the cell surface, respectively *(13,14)*. In both cases, receptor interaction with FRAP induces subcellular distribution patterns for both proteins (i.e., colocalized on the cell surface) that are distinct from those when either protein (i.e., 5-HT receptor or FRAP) is present alone (i.e., diffuse in the cytoplasm). The "coclustering" observation, which has been previously described with PSD-95 and β_1-adrenergic receptor (β_1-AR) *(51)*, likely reflects the assembly of receptosomes on the cell surface that either inhibit the internalization of the components of these multimolecule complexes or impair the interaction with FRAPs that would otherwise facilitate internalization. That PSD-95 inhibits agonist-induced internalization of 5-HT_{2A} receptors in heterologous cells lends credence to the former mechanism *(13)*.

Curiously, coclustering is not observed when β_1-AR is coexpressed with another interacting PDZ protein, membrane-associated guanylate kinase inverted-2 (MAGI-2) *(52)*. In contrast to the membrane-bound PSD-95, MAGI-2 is loosely associated with the plasma membrane and thus lacks the capacity to assemble multiprotein complexes on the cell surface. Whereas PSD-95 inhibits *(51)*, MAGI-2 facilitates the internalization of β_1-AR *(52)*. Differences in lipid modification of these two PDZ proteins might account for their differential regulation of the β_1-AR *(52)*. Specifically, PSD-95 undergoes palmitoylation and becomes membrane bound *(53)*, whereas MAGI-2 does not undergo lipid modification and dissociates from the membrane much more

facilely than PSD-95 *(52)*. Although similar studies have not been performed with 5-HT receptors, there is a clearly comparable level of complexity in FRAP-mediated regulation of intracellular receptor trafficking.

3.3. FRAPs Modulate 5-HT Receptor Internalization

Interaction with FRAPs controls the equilibrium in the competing processes of receptor internalization vs receptor retention at the plasma membrane. The 5-HT_{2A} receptor provides a case study of how FRAPs could regulate receptor internalization. PSD-95, a FRAP well known for its ability to organize receptosomes and scaffold proteins at the cell surface, inhibits agonist-induced 5-HT_{2A} receptor internalization in HEK cells *(13)*. PSD-95 could prevent receptor internalization by competing for the same binding motif with regulatory molecules that facilitate 5-HT_{2A} internalization. G protein–coupled receptor kinases (GRK)-mediated phosphorylation of the serine or threonine residue at the −2 position of the PDZ ligand and the resulting decrease in affinity for PDZ proteins were thought to be the regulatory mechanism with which PSD-95 competes *(38,51,54,55)*. Because the internalization of the 5-HT_{2A} receptor involves neither GRK2 nor GRK5 *(12)*, the specific counterregulatory mechanism that rivals PSD-95 remains unidentified.

Although the i3 loop of the 5-HT_{2A} receptor has been shown to interact with several different arrestins in vitro and cortical 5-HT_{2A} receptors colocalize with arrestins in intracellular vesicles in neuronal cell bodies in vivo *(30)*, arrestins do not appear to play a role in either agonist or antagonist-mediated internalization of 5-HT_{2A} receptors when expressed in HEK cells *(56)*. The lack of evidence for receptor phosphorylation *(12)* might partially explain the arrestin-independent mode of 5-HT_{2A} receptor internalization, as the association of arrestin with GPCRs is typically contingent on GPCR phosphorylation by G protein–coupled receptor kinases (GRKs) *(57,58)*. It is likely that arrestins are not involved with the internalization of 5-HT_{2A} receptors in neurons but might, instead, play a role in anchoring 5-HT_{2A} receptors in the cytoplasm, particularly within the apical dendrites *(11,35)*. Given that 5-HT_{2A} receptors in cortical pyramidal neurons are predominantly intracellular *(34,59)*, it is thought that constitutively maintaining a large intracellular pool of 5-HT_{2A} receptors at baseline serves to sequester receptors from the active zone of synaptic activity. Hence, FRAPs could anchor 5-HT receptosomes not only on the cell surface but also in the cytoplasm, and intracellular sequestration is likely to be an important regulatory mechanism of 5-HT receptor signaling.

In contrast, GRK and arrestin both modulate the internalization of 5-HT_{2C} receptors. Posttranscriptional RNA modification involving adenosine-to-inosine editing in the second intracellular loop generates 14 different isoforms of the

5-HT_{2C} receptor in the human brain such that the nonedited isoforms are fully active at baseline, whereas the edited isoforms demonstrate lesser constitutive activity *(60,61)*. The constitutive activity of the 5-HT_{2C} isoforms is correlated with the degree of their spontaneous internalization in the absence of agonist stimulation in that the nonedited and fully constitutively active isoforms display the least cell surface expression *(62)*. GRK2-mediated stabilization of 5-HT_{2C} interaction with β-arrestin 2 in the endosome compartment is thought to be responsible for the increased internalization of the nonedited isoform *(62)*.

Receptor internalization is classically associated with regulation of receptor signaling, but a recent study has uncovered an entirely novel role for 5-HT receptor internalization. Pharmacological and monoclonal antibody studies revealed that 5-HT receptors, particularly the 5-HT_{2A} receptors, function as coreceptors on the cell surface for the JC virus, which causes a fatal demyelinating disease known as progressive multifocal leukoencephalopathy (PML), particularly in the immunocompromised patient population *(5)*. The observation of colocalization between the JC virus and the 5-HT_{2A} receptors in the endosomes following 5-HT_{2A} internalization raises the intriguing possibility that JC virus infects glial cells of the human central nervous system by gaining entry into the 5-HT_2 receptosomes, which are also widely present in glia cells *(5)*. How JC virus interacts with 5-HT receptors is unknown, but the virus might have conceivably evolved molecular mimicry to express proteins that resemble FRAPs that normally bind to 5-HT receptors.

4. FRAPs Modulate 5-HT Receptor Signaling

4.1. FRAPs Regulate 5-HT Receptor Signaling

5-Hydroxytryptamine receptor-associated proteins, particularly the PDZ proteins, can facilitate 5-HT receptor-coupled signal transduction by assembling functional receptosomes. The 5-HT_{2A} receptosome provides some well-studied examples. Direct interaction with PSD-95 potentiates 5-HT_{2A}-mediated activation of phospholipase C (PLC) without altering basal receptor activity, agonist potency, or kinetics of receptor desensitization *(13)*. PSD-95 also had no effect on the ability of a constitutively active $G\alpha_q$ mutant to activate PLC, suggesting that PSD-95 augments receptor signaling at the level of receptor–effector coupling and not by acting on downstream effectors. Given that PSD-95 could interact with at least 50 distinct signaling proteins *(19)*, it likely provides scaffold for G_q and PLC and thereby facilitates 5-HT_{2A} interaction with these downstream signaling molecules. The coclustering phenomenon that occurs in cells coexpressing PSD-95 and 5-HT_{2A} receptors further supports such a mechanism (see above). Similarly, caveolin-1, an integral membrane protein enriched in caveolae that associates with lipid-modified signaling apparatus, potentiates

5-HT_{2A} receptor-mediated calcium mobilization and extracellular regulated kinase (ERK) activation by facilitating receptor interaction with $G\alpha_q$ proteins *(63)*. It is reasonable to speculate that there would be a net increase in 5-HT_{2A}-mediated signaling in subcellular regions where 5-HT_{2A} receptors and these FRAPs are colocalized. Hence, the receptosome milieu could easily function to enhance the accuracy, efficiency, and specificity of 5-HT receptor-mediated signal transduction in vivo *(64)*.

The implication that PDZ proteins such as PSD-95 regulate 5-HT_{2A} targeting and signaling in neurons is profound because these FRAPs might be the missing link between the serotonergic and glutamatergic systems of neurotransmission. Reduced glutamatergic function in the prefrontal cortex and perturbation in 5-HT_{2A} receptor-mediated functions are both thought to contribute to the etiology and/or pathophysiology of schizophrenia *(65–70)*. Although it remains controversial whether the expression of PSD-95 or other related PDZ protein in schizophrenia is altered *(71,72)*, *N*-methyl-D-aspartic acid subtype of glutamate receptor (NMDA-R)-deficient mice exhibit impaired social interaction that can be rescued by administration of atypical antipsychotic drugs that block the 5-HT_{2A} receptor *(73)*.

4.2. FRAPs Regulate 5-HT Receptor Desensitization and Resensitization

Given that FRAPs play a role in 5-HT receptor internalization (*see* above), it is not surprising that they can also regulate receptor desensitization and resensitization, as these processes are all classically linked. Calmodulin, a ubiquitous Ca sensor that interacts with 5-HT_{1A} receptors in living cells, regulates receptor coupling to G proteins and receptor desensitization *(31)*. In what appears to be a recurring theme, calmodulin binding competes with PKC-mediated phosphorylation at the same residues in the juxtamembrane regions of the i3 loop of the 5-HT_{1A} receptor *(31)*. Furthermore, SNX27, a member of the sorting nexin family, redirects the 5-$HT_{4(a)}$ receptor from the plasma membrane to the early endosome and has been implicated in the rapid desensitization of the 5-HT_4-mediated cAMP accumulation in cultured neurons *(16)*. Interestingly, PDZ proteins do not appear to regulate 5-HT receptor desensitization because disruption of the PDZ ligand prevents neither 5-HT_{2A} nor 5-HT_{2C} receptors from undergoing desensitization *(13,74)*. On the other hand, an intact PDZ ligand is essential for the resensitization of 5-HT_{2C} receptors, suggesting that PDZ proteins might modulate this process *(74)*.

4.3. FRAPs Regulate Alternative Signaling and Crosstalk

Interaction with different FRAPs could couple 5-HT receptors to alternative signaling pathways. As an example, the 5-HT_{2A} receptor is classically thought to

activate PLC via $G\alpha_q$. In fetal myoblasts, the carboxyl terminus of the 5-HT_{2A} receptor recruits Jun-activated kinase (JAK) following agonist-dependent tyrosine phosphorylation, and the 5-HT_{2A} receptosome subsequently activates the JAK-STAT signaling pathway *(75)*. Interaction with ARF-1 allows 5-HT_{2A} receptors in COS cells to activate phospholipase D in a G_q-independent manner *(28)*. Finally, 5-HT_{2A} receptors can also couple to phospholipase A2-mediated signaling pathway in NIH-3T3 cells following ERK or p38 kinase activation *(76,77)*, but the identity of the FRAP in that case is unknown.

Similar versatility in receptor-coupled signaling has also been observed in other members of the 5-HT_2 family. Like the 5-HT_{2A} receptor, 5-HT_{2B} and 5-HT_{2C} are both G_q-coupled receptors. Interaction with PDZ protein is likely to be important for 5-HT_{2B} receptor-mediated activation of nitric oxide synthase (NOS) *(78)*. When expressed in fibroblasts, 5-HT_{2B} receptors mediate 5-HT-induced mitogenesis by recruiting c-Src for cell cycle progression via the mitogen-activated protein kinase (MAPK) pathway *(79)*. Crosstalk with signaling pathways that result in cell proliferation holds great interest because 5-HT_{2B} activation contributes to cardiac valve hyperplasia *(80,81)*, lung vascular proliferation, and pulmonary hypertension *(82)* and is likely to be important in disorders or drugs associated with increased 5-HT plasma levels such as carcinoid tumors or 3,4-methylenedioxymethamphetamine (Ecstasy) consumption *(83)*. Finally, interaction with MUPP1 provides the basis for 5-HT_{2C} receptor-mediated crosstalks between different second-messenger pathways such PLC and NOS signaling *(14)*.

5. Conclusion

The heterogeneous group of proteins, collectively known as the FRAPs, regulate a diverse array of 5-HT receptor activities, including subcellular localization, endocytic process, receptosome organization, and signal transduction. Given that dysfunction in the serotonergic system has been implicated in the pathogenesis of a number of human diseases, failure of FRAPs to interact with 5-HT receptors and appropriately carry out their functions could likely account for some of the underlying mechanisms. Increasingly clear is that interaction with the same FRAP produces different outcomes depending on the individual 5-HT receptor and the unique cellular milieu involved. Thus, FRAPs present as novel targets for pharmacological intervention, as they potentially allow for more specific modulation of 5-HT receptors that tailor to the affected cellular context. In this regard, the goal for serotonin research in the next phase is to establish the role of interactions between FRAPs and 5-HT receptors in vivo.

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9

Cellular and Subcellular Localization of Serotonin Receptors in the Central Nervous System

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Summary

Immunocytochemistry allows for a precise localization of neurotransmitter receptors in tissues and cells. This review summarizes much of the available data on the cellular and subcellular distribution of serotonin (5-hydroxytryptamine [5-HT]) receptors in the mammalian central nervous system. Among fourteen 5-HT receptor types, all cloned and sequenced, only a few have yet been amenable to detailed immunocytochemical visualization, not only at the light microscopic but particularly at the electron microscopic level. The 5-HT_{1A} and 5-HT_{2A} receptors have been the most thoroughly investigated and provide a meaningful demonstration of the wealth of information to be gained from this methodological approach, not only in terms of anatomical and cytological localization, and thus physiological role and eventual implication in health and disease, but also of functional properties and drug effects.

Key Words: 5-Hydroxytryptamine; immunocytochemistry; anatomical distribution; cellular and subcellular localization; internalization; colocalization.

1. Introduction

The first of at least 14 serotonin (5-hydroxytryptamine [5-HT]) receptor types currently known to exist in the mammalian central nervous system (CNS) was pharmacologically defined more than 25 yr ago *(1,2)*. Yet, it is only within the last 15 yr, with the cloning and sequencing of these receptors, that it has become possible to visualize their distribution at cellular and subcellular

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levels, by means of light and electron microscopic immunocytochemistry, respectively. All 5-HT receptor types, except one (5-HT_3), belong to the superfamily of seven-transmembrane-domain, G protein–coupled receptors, and their amino acid sequences display a relatively high degree of homology within their transmembrane domains *(3)*. In their terminal portions (N- and C-terminal) and intracellular loops, however, there are sufficient differences between receptor types to allow for the production of specific antibodies raised against synthetic peptides corresponding to segments of these particular domains or fusion proteins in which the entire amino acid sequences of these domains are included.

For many years, ligand-binding autoradiography of frozen (and dried) sections was the only method for localizing transmitter receptors in CNS tissue. Although providing unique information on the regional distribution of binding sites for the different 5-HT receptor subtypes throughout the brain (*see* Chapter 10), this method is of limited resolution for their visualization at both cellular and subcellular levels (but *see* ref. *4*). Moreover, it is hardly applicable to 5-HT receptor radioligands, which would wash out of tissue during the chemical fixation, dehydration, and embedding procedures required for light or electron microscopic autoradiography. *In situ* hybridization autoradiography or histochemistry has allowed one to localize and measure receptor mRNAs at regional and even at cellular levels (*see* Chapter 10), but only sites of synthesis are then identified (i.e., neuronal cell bodies and proximal dendrites), as opposed to other eventual sites of functional activation. In this regard, immunocytochemistry is much more powerful, as, in principle, it permits the selective detection of any molecularly defined receptor type in its sites of utilization as well as synthesis and is applicable for electron as well as light microscopy.

However, to obtain reliable results from the immunocytochemical study of transmitter receptor localization at the light microscopic level, and especially at the electron microscopic level, several methodological requirements must be satisfied. First and foremost, the specificity of any immunolabeling, for selective as it may look, must be formally established. Thus, it is not sufficient to demonstrate that adsorption of the primary antibodies with the expected antigen (synthetic peptide) suppresses the immunostaining. This merely verifies the immunological selectivity of the antibodies. What needs to be established is the specificity of the observed immunolabeling. Indeed, the tissue might contain unknown proteins with similar amino acid sequences as the antigen to be visualized or that become immunoreactive after the chemical fixation and/or further processing of the tissue. It is important to point out that the specificity controls provided by companies that produce anti-receptor antibodies are often limited to Western blots, using denaturating conditions that are quite distinct from those of tissue immunocytochemistry.

Artifactual labeling might also result from the presence in antisera of γ-globulins directed against other epitopes than those of the receptors to be detected. When polyclonal antibodies are produced against synthetic peptides, carrier proteins (e.g., BSA, KLH) are often coupled to these peptides to make them more antigenic. It is then essential to submit the resulting antisera to immunopurification procedures in order to avoid unwanted immunolabeling.

To demonstrate immunolabeling specificity, the observed localizations must be compared to results obtained from other experimental approaches, such as radioligand-binding autoradiography, and/or reproduced with other primary antibodies directed against different domains of the receptor sequence. Heterologous cell lines transfected with the gene of interest might also be used as a positive control. The ideal control, however, is simultaneously processed tissue from knockout animals for the receptors under investigation. Unfortunately, until now, this is only applicable in mice, and not always available commercially.

At the ultrastructural level, the electron-dense markers bound to secondary antibodies used to locate the complexes formed by antigens and primary antibodies impose their own limitations *(5)*. When using the immunoperoxidase technique with diaminobenzidine (DAB) as the chromogen, preferential deposition of the fine precipitate along the plasma membrane of neuronal cell processes, postsynaptic densities, microtubules, and/or the outer membrane of other intracellular organelles (e.g., synaptic vesicles, mitochondria) should not necessarily be considered of biological significance, because this label is diffusible *(6)* and tends to aggregate on filamentous or membranous constituents of nerve cells. After pre-embedding immunogold labeling, assigning localizing value to single particles might also be misleading, because these scattered particles might merely represent background labeling near the surface of sections, within the few microns of tissue penetrated by this immunoreagent.

Care should also be taken not to overinterpret negative results. A lack of immunolabeling does not mean the absence of the corresponding antigen. The affinity of antibodies for their antigens is extremely variable. Chemical fixation of the tissue, required for its morphological preservation, often weakens or totally suppresses this affinity, especially when strong fixatives, such as glutaraldehyde or acrolein, are used, instead of paraformaldehyde. The electron-dense markers also vary in their efficacy. For example, the pre-embedding immunogold technique is known to be much less sensitive than the immunoperoxidase technique and, therefore, does not provide reliable quantitative information regarding the frequency of labeled neuronal elements in a given region. Moreover, it has been claimed that the pre-embedding method might generally fail to detect some receptors located in the main body of synapses (i.e., at junctional complexes) because of restricted access of immunoreagents to the synaptic cleft, as demonstrated for AMPA receptor subunits using antipeptide

antibodies *(7)*. Post-embedding immunogold techniques do not suffer from these limitations and are consequently better adapted for purposes of quantitative cytological studies. Unfortunately, however, there has not yet been a single successful demonstration of any of the 5-HT receptor types by means of this approach. It should also be emphasized that all of the above precautions and limitations become even more critical in double immunocytochemical studies aimed at visualizing a receptor together with another bioactive molecule (e.g., transmitter, receptor, transporter, enzyme) or when combining receptor immunocytochemistry with another neuroanatomical technique, such as anterograde or retrograde axonal tracing.

Because these caveats have not always been taken into account in the immunocytochemical studies thus far published on central 5-HT receptors, the present review will critically assess the available data and concentrate on those studies in which at least some of the criteria of specificity were met. Only results obtained from the mammalian CNS will be considered; therefore, results obtained mostly from rodents and exceptionally from primates will be presented. For the sake of clarity and accuracy, a descriptive terminology different from the traditional pharmacological division into presynaptic and postsynaptic receptors will be used. A first distinction will always be made between *autoreceptors* and *heteroreceptors*, depending on whether the neuronal elements bearing the receptors under study also contain (and presumably release) the corresponding transmitter (i.e., 5-HT in the present case). In terms of distribution, the receptors will be described as *somatic*, *dendritic*, *axonal*, or *axo-terminal*, depending on which part of neurons they are found. For lack of a better word, the term "axon terminal" will be used as a synonym for axon varicosity or axonal bouton, without implying that it is the physical end of an axon. The word "synapse" will be restricted to the designation of morphologically specialized contacts characterized by a junctional complex between neuronal elements. Throughout, whenever possible, the functional significance of the observed localizations will be emphasized.

2. The 5-HT$_1$ Receptor Family

The 5-HT$_1$ family is now known to comprise five receptor types: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, and 5-HT$_{1F}$, which share the property of inhibiting adenylate cyclase upon activation. A large number of electrophysiological and pharmacological studies have established that, both as autoreceptors and heteroreceptors, somato-dendritic 5-HT$_{1A}$ receptors mediate a 5-HT control on neuronal firing and, hence, release of neurotransmitters, whereas axo-terminal 5-HT$_{1B/1D}$ receptors mediate a 5-HT control on transmitter release. In this family, only 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors have thus far been amenable to immunocytochemical study at both light and electron microscopic levels.

2.1. 5-HT$_{1A}$ Receptors

The 5-HT$_{1A}$ receptors were the first 5-HT receptor type to be cloned, in human *(8)* and then in rat *(9)*. Because of the availability of highly selective agonists such as 8-OH-DPAT *(10,11)* and of antagonists such as WAY 100635 *(12,13)* and, more recently, of specific anti-5-HT$_{1A}$ antibodies *(14,15)*, they are now well characterized, anatomically, electrophysiologically, and pharmacologically.

Several groups have produced and characterized anti-5-HT$_{1A}$ antibodies intended for immunocytochemistry, raised against synthetic peptides *(14–19)* or fusion proteins *(20,21)* corresponding to portions of the sequence of the human or rat 5-HT receptors. Until now, however, only the polyclonal antibodies produced and characterized in Michel Hamon's laboratory *(14,15)* have fully demonstrated their specificity and usefulness for immunocytochemistry at both the light and electron microscopic levels.

The synthetic peptide used for generating these polyclonal antibodies in rabbit consisted of a 26-amino-acid sequence (Gly^{243}–Glu^{268}) corresponding to part of the third intracellular loop of rat 5-HT$_{1A}$ receptors, the region showing the lesser homology with any other sequence of G protein–coupled receptors *(22)*. The resulting antibodies, purified on an affinity column bearing the synthetic peptide, were both immunologically and immunocytochemically specific *(14,15)*. They immunoprecipitated [^{3}H]8-OH-DPAT binding sites from a soluble extract of rat hippocampal membranes, recognized a single protein with an apparent molecular weight of 63 kDa corresponding to that of 5-HT$_{1A}$ receptors *(23,24)*, and displayed an anatomical distribution in rat brain sections that completely matched that obtained by autoradiography with the specific ligand [^{3}H]8-OH-DPAT and by immunoautoradiography *(15)*.

The strongest 5-HT$_{1A}$ immunolabeling was then detected in the mesencephalic raphe nuclei, hippocampus, and septum, whereas extrapyramindal nuclei (caudate-putamen, substantia nigra) and the cerebellum did not show any labeling *(15,25,26)*. No labeling was ever observed with these antibodies after incubation in preimmune serum of the same rabbit, in the absence of primary antibodies in the incubating medium, or after preadsorption with the antigenic peptide. These antibodies are so specific that they do not recognize the mouse 5-HT$_{1A}$ receptor, in spite of the strong homology between the 5-HT$_{1A}$ sequences of rat and mouse, which differ by only two amino acids (7%) in the amino acid domain corresponding to the antigenic peptide. None of the commercially available anti-5-HT$_{1A}$ antibodies have yet satisfied the above criteria of immunolabeling as well as of immunological specificity.

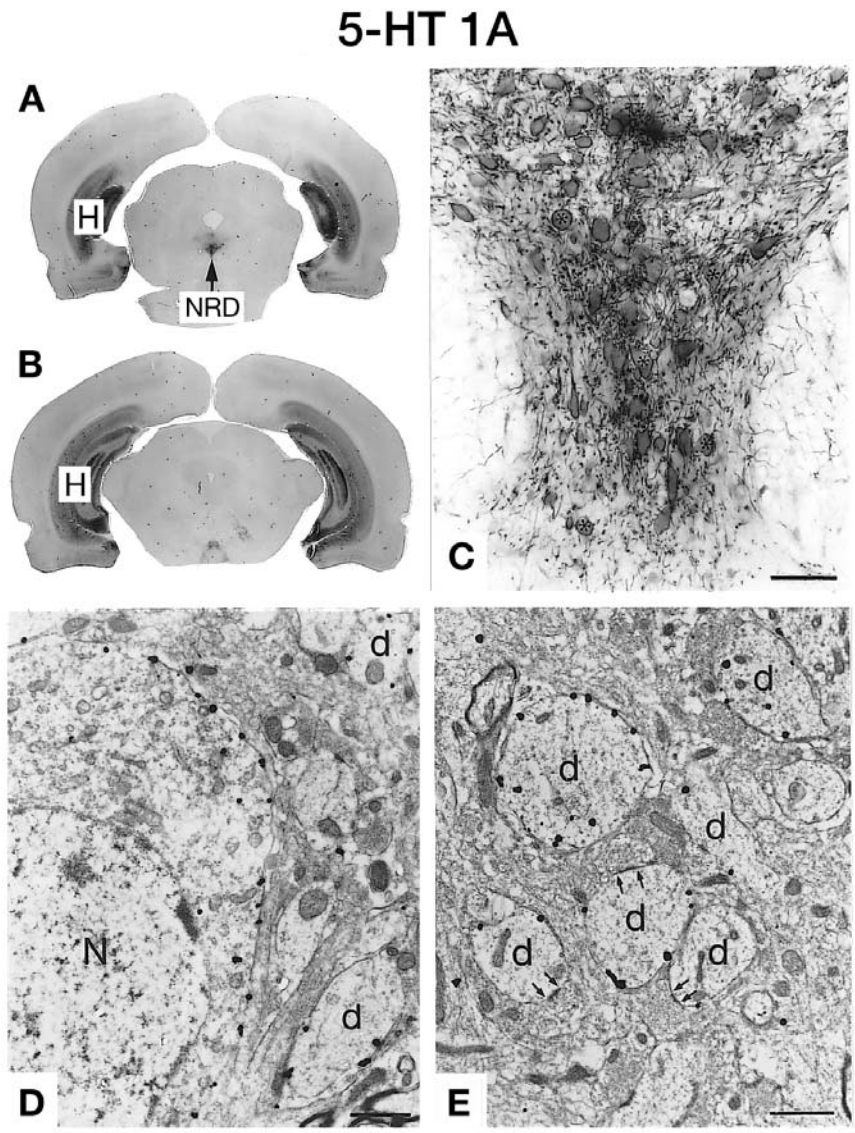
Hamon's anti-5-HT$_{1A}$ antibodies have been used successfully by several laboratories to immunolocalize 5-HT$_{1A}$ receptors at the light microscopic level in the rat CNS *(14,15,25–30)*. One of these studies has definitely proven that 5-HT$_{1A}$ receptors are autoreceptors by showing their presence on cell bodies

and dendrites of nucleus raphe dorsalis (NRD) neurons that were simultaneously immunostained for 5-HT itself *(27)*. Moreover, 5-HT_{1A} immunolabeling was no longer found in rat NRD after a selective destruction of its 5-HT neurons by 5,7-dihydroxytryptamine *(29)*.

These same 5-HT_{1A} antibodies have been used in several electron microscopic studies *(25,31–34)*. The first of these, carried out with an immunoperoxidase technique, confirmed the localization of 5-HT_{1A} receptors to the soma-dendrites of NRD neurons (autoreceptors) and spinous dendrites of the hippocampus (heteroreceptors). Subsequently, both the NRD and CA3 region of the hippocampus were examined after 5-HT_{1A} immunolabeling with a pre-embedding immunogold technique *(25)*. As shown in Fig. 1, it could then be conclusively demonstrated that 5-HT receptors are somato-dendritic and mainly located on extrasynaptic portions of the neuronal plasma membrane, whether as autoreceptors on 5-HT neurons in the NRD or as heteroreceptors on pyramidal and granule cells in the hippocampus (not illustrated). Glial labeling has not been observed with these antibodies (but *see* ref. *18*).

Importantly, because 5-HT_{1A} receptors could then be visualized with a non-diffusible and particulate label, it became possible to investigate their eventual redistribution upon pharmacological treatment. 5-HT_{1A} autoreceptors are known to be subject to a rapid desensitization upon activation, whereas 5-HT heteroreceptors do not desensitize *(35–38)*. Accordingly, 15 min and 1 h after the acute administration of 8-OH-DPAT (0.5 mg/kg, iv), a significant decrease (>30%) in the density of membrane immunogold labeling, associated with a concomitant increase in their cytoplasmic labeling, could be demonstrated in NRD soma-dendrites, with a return to baseline level within 24 h *(32,34)*. This internalization of 5-HT_{1A} autoreceptors was blocked by prior administration of the selective antagonist WAY 100635, which, by itself, had no apparent effect on the subcellular distribution of 5-HT_{1A} receptors. In CA3, there were no changes in the distribution of 5-HT_{1A} heteroreceptors at any time after 8-OH-DPAT administration. These findings strongly suggested that the desensitization was the result of the internalization.

Fig. 1. *(opposite page)* Light microscopic visualization of 5-HT_{1A} receptors in transverse histological sections across the mesencephalon **(A**,**C)** and hippocampal formation **(B)** of the adult rat brain and subcellular distribution of these receptors in nucleus raphe dorsalis (NRD) after immunogold labeling for electron microscopy **(D**,**E)**. In **(A)**, the immunoperoxidase– diaminobenzidine labeling fits the configuration of the 5-HT neuron population in the NRD (autoreceptors), whereas in both **(A)** and **(B)**, the layered pattern in the hippocampus (H) corresponds to the selective somato-dendritic labeling of pyramidal and granular neurons (heteroreceptors). **(C)** illustrates the peripheral distribution of this labeling in the NRD neuronal somata (e.g., cell bodies designated by asterisks).



The immunoreactive processes intermingled in the neuropil represent dendrites. Scale bar: 50 µm. In **(D)**, the immunogold labeling of part of an NRD immunoreactive neuronal cell body and of nearby dendrites (d) predominates on their plasma membrane. **(E)** Immunogold labeling of 5-HT_{1A} immunoreactive dendrites (d) in NRD. The silver-intensified immunogold particles are mostly associated with the plasma membrane of these processes. Note the absence of particles in areas of synaptic differentiation on these dendrites (between small arrows). Scale bars in **(D)** and **(E)**: 1 µm. (Reproduced with permission from ref. *25*.)

The subcellular distribution of 5-HT_{1A} receptors in dendrites from NRD (autoreceptors) and hippocampal neurons (heteroreceptors) was also examined after acute treatment with the specific serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine *(33)*. Indeed, because 5-HT_{1A} autoreceptors normally mediate an inhibition of 5-HT firing and release, their desensitization is essential for obtaining an enhancement of 5-HT transmission upon treatment with SSRIs. One hour after fluoxetine (10 mg/kg ip), there was a 36% decrease in the immunogold labeling of the plasma membrane of NRD dendrites and a concomitant increase in their cytoplasmic labeling, without any change in hippocampal dendrites, as previously observed after 8-OH-DPAT administration. Moreover, in parallel experiments carried out in treated and untreated rats stereotaxically implanted with β-microprobes, the in vivo binding of the 5-HT_{1A} selective PET radioligand [^{18}F]MPPF was shown to be reduced by 35% in the NRD and unchanged in the hippocampus 1 h after the single dose of fluoxetine. Interestingly, in brain sections, the regional density of [^{18}F]MPPF in NRD and the hippocampus, as measured by autoradiography, did not differ between treated and untreated rats. This allowed one to conclude that the in vivo reduction of [^{18}F]MPPF binding in NRD was a consequence of the internalization of 5-HT_{1A} autoreceptors and not of a decrease in their total number in this region. It might be expected from these results that this measurable internalization, which accounts for the desensitization of 5-HT_{1A} autoreceptors and thus conditions the therapeutic efficacy of antidepressants, might be amenable to brain imaging in human, providing a means to determine the eventual usefulness of an antidepressant treatment soon after its onset *(33,34)*.

2.2. 5-HT_{1B} Receptors

The 5-HT_{1B} receptors have been cloned in the rat *(39)* and mouse *(40)*, as well as their homolog in man *(41,42)*. In all three species, another receptor of the same type, 5-HT_{1D}, is much less abundant than 5-HT_{1B}. There is only a 77% sequence homology between the transmembrane domains of 5-HT_{1D} and 5-HT_{1B} receptors, but their pharmacological properties are identical. Conversely, the homology between human and rodent 5-HT_{1B} receptors is about 96%, whereas their pharmacology is relatively distinct *(43,44)*. Since 1996, according to the "Serotonin Club Nomenclature Committee," the 5-HT_{1B} receptor of rodents (rat, mouse, hamster) should be designated as r5-HT_{1B} and its homolog in man as h5-HT_{1B} *(45)*. In this chapter, however, only r5-HT_{1B} receptors are considered and are therefore called 5-HT_{1B} receptors.

At variance with 5-HT_{1A} receptors, the regional distribution of 5-HT_{1B} receptor-binding sites in the rat brain does not correspond to that of their mRNA. Thus, in autoradiographs, the highest densities of 5-HT_{1B} binding sites are detected in the basal ganglia, notably in the globus pallidus and substantia nigra *(46–48)*, whereas *in situ* hybridization displays abundant mRNA in raphe nuclei,

striatum, cerebellum, hippocampus, cerebral cortex, and nucleus accumbens *(39,40,49)*. Such a segregation suggests that the receptors are dispatched away from their sites of synthesis, to projection sites, and therefore located in axon terminals. Several studies have indeed shown decreases in the number of 5-HT_{1B} binding sites in the substantia nigra after cytotoxic lesions of the striatum or of the raphe nuclei *(50,51)*. These studies support the notion of a transport of the 5-HT_{1B} receptors to the terminal parts of axons, either as heteroreceptors in striato-nigral (GABA) or autoreceptors in raphe-nigral (5-HT) projection neurons.

In a unique study of a 5-HT receptor radioligand at the ultrastructural level, Boulenguez et al. *(52)* were able to provide the first demonstration of the existence of 5-HT_{1B} receptors in axon terminals of the rat superior colliculus. However, the limited resolution of the autoradiographic technique did not allow one to localize the receptors to the plasma membrane as opposed to the axoplasm of these nerve endings. Such a plasma membrane localization of 5-HT_{1B} receptors was formally demonstrated in our laboratory *(25)*, by pre-embedding immunogold immunocytochemistry with polyclonal anti-5-HT_{1B} antibodies raised in the rabbit against a specific segment of the third intracellular loop of the predicted sequence of rat 5-HT_{1B} receptors (Val^{263}–Lys^{287}) *(53)*. The immunolabeling specificity of these antibodies was established in LLC-PK1 kidney cells transfected with the corresponding 5-HT_{1B} sequence, and in the rat CNS by demonstrating a close match between the anatomical distribution of the immunoreactivity and that of the selective radioligand [^{125}I]GTI *(53)*.

The ultrastructural distribution of 5-HT_{1B} receptors was examined in the two CNS regions displaying the densest immunoreactivity: the globus pallidus and substantia nigra (Fig. 2). Both the immunoperoxidase and the immunogold technique were used. With these same antibodies, Sari et al. *(51,54)* had already reported a localization to axon terminals in these two regions. In our study, the 5-HT_{1B} receptors appeared to be preferentially located on fine unmyelinated axons, sometimes regrouped in bundles, but not in axon terminals proper. After immunogold labeling, there was a clear association with the plasma membrane of these axons as opposed to their axoplasm, accounting for more than 80% of the labeling as documented by quantitative analysis of the distribution of the immunogold particles.

In both the substantia nigra and globus pallidus, our study also revealed the existence of 5-HT_{1B} receptors located in endothelial cells of CNS microvessels, as previously demonstrated in human surgical specimens as well as rat tissue *(55)*. It is noteworthy that, in this location, the immunogold-labeled receptors were not found on the plasma membrane, but in the cytoplasm. This was consistent with biochemical data suggesting that they might then mediate a nitric oxide–dependent vasodilation induced by circulating 5-HT and distinct from its vasoconstricting effects when centrally released *(56)*. In human microvessels, the 5-HT_{1B} receptors

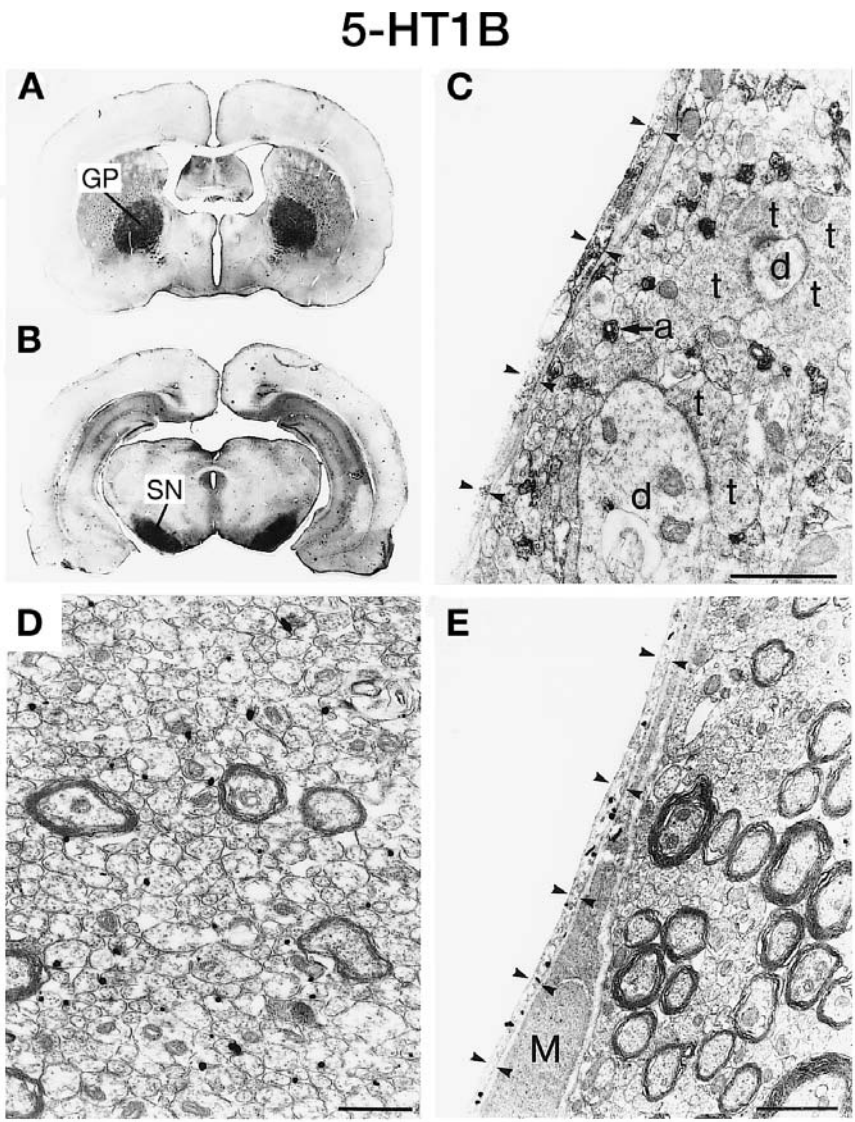


Fig. 2. (A,B) Light microscopic visualization of 5-HT_{1B} receptors in the globus pallidus (GP in **A**) and substantia nigra (SN in **B**) of adult rat brain after immunoperoxidase–diaminobenzidine labeling. In **(A)**, note the presence of labeled axon fascicles coursing underneath the medioventral aspect of the GP. **(C)** Electron micrograph illustrating the distribution of this 5-HT_{1B} labeling in the vicinity of a microvessel in the SN (the endothelial lining is located between arrowheads). In the parenchyma, the immunoprecipitate is essentially found in small unmyelinated axonal profiles, as shown at arrow (a). All axon terminals (t) and dendrites (d) are immunonegative. Also note the endothelial immunoreactivity. Scale bar: 0.5 μm. **(D)** Silver-intensified

had also been found within myocytes surrounding the microvessels, which could account for their role in the mediation of vasoconstrictive effects of antimigrainous agents of the triptane family that act as 5-HT_{1B} agonists *(57)*.

A largely preterminal axonal localization was further documented by Pickard et al. *(58)* on retinal and other afferent axons of mouse suprachiasmatic nucleus (SCN), after immunoperoxidase–diaminobenzidine labeling with other antipeptide antibodies directed against a C-terminal sequence of 5-HT_{1B} receptors. These authors also described neuronal somata and dendrites and some axon terminals displaying 5-HT_{1B} immunoreactivity in the SCN, as well as a 5-HT_{1B} immunolabeling of the endothelium of small blood vessels.

In keeping with the results of numerous microdialysis studies, these immunocytochemical data support a role of 5-HT_{1B} receptors as autoreceptors or heteroreceptors mediating a regulation of the release of various neurotransmitters: 5-HT *(59–61)*, acetylcholine *(62,63)*, dopamine *(64)*, and GABA *(65,66)*. The predilection of these receptors for the membrane of preterminal axons as opposed to axon terminals proper suggests that they might mediate such local effects through an action on axonal conduction (ionic conductance) rather than transmitter release mechanisms themselves.

3. The 5-HT_2 Receptor Family

The 5-HT_2 family comprises three receptor types: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, which show a high sequence homology, the similarity between their transmembrane domains being above 70%. These receptors are positively coupled to phospholipase C and phospholipase A_2 through the G_q-protein and their activation leads to cell excitation (*see* Chapters 12 and 13 for details).

3.1. 5-HT_{2A} Receptors

Seven different antibodies have been produced thus far against N-terminal *(67–72)* or C-terminal portions of the rat or human 5-HT_{2A} receptor sequence *(73,74)*. Whether used for immunocytochemical studies in the rat, mouse, monkey, or man, these antibodies have generally yielded concordant results, at least at the light microscopic level.

Following early studies in the rat *(70,75–78)* and monkey brain *(79)*, a thorough immunocytochemical mapping of the anatomical distribution of 5-HT_{2A}

Fig. 2. *(continued)* 5-HT_{1B} immunogold labeling of unmyelinated axons in the neuropil of the GP. Most of the immunogold particles are located on the axolemma rather than axoplasm of these fine processes. In **(E)**, a small microvessel in the GP displays 5-HT_{1B} immunogold labeling. The immunogold particles are mostly located in the cytoplasm rather than on the plasma membrane of the endothelial cell. No immunoreactivity is detected in an underlying myocyte (M in nucleus). Scale bars in **(D)** and **(E)**: 1 µm. (Reproduced with permission from ref. *25*.)

receptors throughout the rat brain and spinal cord was published by our laboratory *(80)*, followed by numerous reports on particular regions of the CNS: rat spinal cord and cerebellum *(81–84)*; medulla oblongata and pons *(85)*; olfactory bulb, neocortex, neostriatum, hippocampus, and amygdala *(86)*; suprachiasmatic nucleus *(87)*; periaqueductal gray *(88)*; midbrain tegmentum *(89)*; nucleus of solitary tract *(90)*; olfactory bulb *(91)*; and septum and hippocampus *(92)*. The primate cerebral cortex was further examined *(93)*, as was the mouse barrel field cortex *(94)* and several human brain regions: midbrain *(95)*; cerebellum *(96)*; and prefrontal cortex, hippocampus, and nucleus accumbens *(97)*.

In several of these studies, another immunohistochemical or neurochemical technique was combined with the light microscopic visualization of 5-HT_{2A} receptors to establish the transmitter identity of the neurons bearing these receptors. In the rat, 5-HT_{2A} receptors were thus demonstrated in GABA neurons of the periaqueductal gray *(88)*; parvalbumin-containing interneurons of the striatum *(98)*; dopamine neurons in the ventral tegmental area *(89)*; catecholaminergic neurons in the nucleus of the solitary tract *(90)*; mitral cells endowed with β1-adrenoceptors in the olfactory bulb *(91)*; and septal cholinergic neurons, GABA neurons in the medial septum/diagonal band of Broca, and hippocampal interneurons identified by the presence of glutamic acid decarboxylase, parvalbumin, calbindin, somatostatin or neuropeptide Y *(92)*. In the rat and primate cerebral cortex, 5-HT_{2A} receptors were shown to be present in parvalbumin and calbindin-containing GABA interneurons, as well as in pyramidal neurons *(93,99)*. 5-HT_{2A} receptor immunohistochemistry was also combined with retrograde axonal tracing to demonstrate the existence of 5-HT_{2A} receptors on striatal afferent neurons in the rat cerebral cortex and globus pallidus *(98)*.

Other light microscopic studies emphasized the mismatches between the regional or laminar distribution of 5-HT_{2A} heteroreceptors and the local density of innervation by 5-HT neurons *(100–102)* (*see also* ref. *80*). Such mismatches indicate that 5-HT_{2A} receptors are located at a distance from sites of 5-HT release and are consistent with a diffuse mode of transmission for 5-HT, as originally proposed by Descarries and Beaudet almost 30 yr ago *(103–105)*. Two recent studies have also been carried out on the patterns of 5-HT_{2A} receptor expression in the rat brain during development *(84,106)*.

In keeping with earlier radioligand-binding *(107–109)* and *in situ* hybridization data on the rat brain *(110–112)*, most CNS regions were shown to contain a variable number of neuronal cell bodies and dendrites displaying 5-HT_{2A} receptors. 5-HT_{2A} immunoreactivity was also detected in myelinated axons issued from many of the anatomical regions exhibiting these neurons *(80)*.

At the electron microscopic level, the cellular and subcellular localization of 5-HT_{2A} receptors has been examined in many regions of rat CNS: cerebral cortex *(70,74,80,113,114)*; caudate-putamen and accumbens nuclei *(115)*;

ventral tegmental area *(116,117)*; nucleus of the solitary tract *(118)*; dorsal nucleus of the vagus *(119)*; and spinal cord *(114)*. Extensive observations have also been made on the prefrontal cortex of the monkey *(79,93)*.

Some of these electron microscopic studies combined the immunocytochemical visualization of 5-HT_{2A} receptors with that of another marker of neurotransmission. Thus, the study of Doherty and Pickel *(116)* provided the first demonstration of the presence of 5-HT_{2A} receptors in dopamine (tyrosine hydroxylase–immunoreactive) as well as nondopamine neurons of the rat ventral tegmental area. In the rat nucleus of the solitary tract, Huang and Pickel *(118)* combined visualization of 5-HT_{2A} receptors and of the plasma membrane 5-HT transporter (5-HTT) to show that axon terminals endowed with the transporter formed symmetric or asymmetric synapses on dendrites without detectable 5-HT_{2A} immunoreactivity. The distribution of 5-HT_{2A} receptors was also investigated in relation with that of *N*-methyl-D-aspartate (NMDA) receptors in rat motor and limbic striatum *(115)*, ventral tegmental area *(117)*, and dorsal motor nucleus of the vagus *(119)*. In the striatum, the 5-HT_{2A} and NMDA receptors were colocalized in medium-sized spiny neurons, but differentially targeted to their dendritic branches and spines, the latter being often labeled for NMDA but very rarely for 5-HT_{2A} receptors. Co-localization of the two receptors was also demonstrated in soma/dendrites of the ventral tegmental area and of the dorsal motor nucleus of the vagus. In all of these studies, some 5-HT_{2A} labeling of astrocytic processes was observed, as previously reported by Maeshima et al. *(81)* in the white matter of the rat spinal cord.

As illustrated in Fig. 3, a predominant if not exclusive localization of 5-HT_{2A} receptors in neuronal somata and dendrites was reported in most of these studies, but results regarding the existence of 5-HT_{2A} in dendritic spines varied. In the monkey prefrontal cortex and the rat fronto-parietal cortex, for example, no immunoperoxidase or immunogold labeling of dendritic spines had been observed using the monoclonal 5-HT_{2A} antibody raised against the whole N-terminal sequence (Met^{1}–Trp^{76}) of human 5-HT_{2A} receptors, in spite of strong 5-HT_{2A} labeling of a majority of pyamidal cell dendrites *(74,79,80)*. In contrast, another study performed on the rat prefrontal cortex with a polyclonal antibody against a peptide (Gln^{428}–Cys^{443}) from the C-terminal portion of the rat receptor sequence described some 5-HT_{2A} immunoperoxidase labeling of dendritic spines in as much as 25% of labeled cellular profiles in this region, compared to 7% when the monoclonal N-terminal antibody was used *(113)*. In the striatum, where most, if not all, medium spiny neurons were shown to be 5-HT_{2A} immunoreactive, dendritic spines were estimated to represent approx 6% of all cellular profiles labeled with the monoclonal N-terminal antibody *(115)*.

Discrepancies were also reported regarding the 5-HT_{2A} labeling of axon terminals. In general, a low frequency of 5-HT_{2A} immunoreactivity in axon

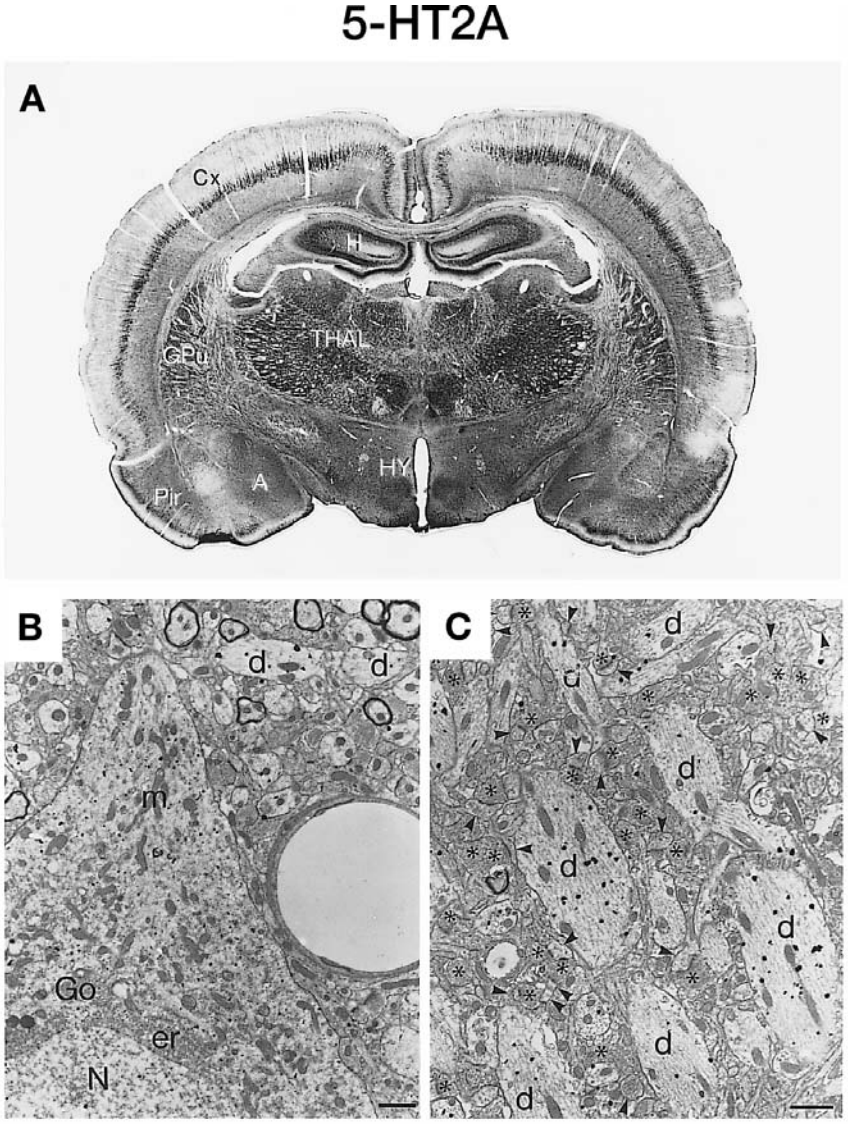


Fig. 3. (A) Transverse histological section across adult rat brain displaying 5-HT_{2A} immunoreactive neurons in the cerebral cortex (Cx), hippocampus (H), several thalamic nuclei (THAL), caudate-putamen (CPu), piriform cortex (Pir), amygdala (A) and hypothalamus (HY). In each region, more or less dense somato-dendritic immunoperoxidase–diaminobenzidine labeling characterizes subpopulations of the neurons. **(B)** Electron micrograph illustrating the 5-HT_{2A} immunogold labeling of part of a pyramidal neuron (N in nucleus) and nearby dendrites (d) in the cerebral cortex. Note the prominent cytoplasmic distribution of this labeling. Within the cell body, the immunogold particles are scattered throughout the cytoplasm, but spare the mitochondria (e.g., m)

terminals was reported, at least in the rat striatum *(115)*, ventral tegmental area *(117)*, nucleus of solitary tract *(118)*, dorsal nucleus of vagus *(119)*, and spinal cord *(114)*. In the rat cortex, Cornea-Hébert et al. *(80)* had found no significant axon terminals labeling in fronto-parietal cortex, whereas Miner et al. *(113)* later described some 5-HT_{2A} labeling in intervaricose and varicose segments of putative monoamine axons in prefrontal cortex, using either N-terminal or C-terminal antibodies. In the monkey neocortex, Jakab and Goldman-Rakic *(79)* had reported "a small number of axon terminals forming asymmetric synapses" with the monoclonal N-terminal antibody.

In all of these electron microscopic studies, a predominant intracellular localization of 5-HT_{2A} receptors was described, whether in neuronal somata and dendrites or in axons and eventual axon terminals, irrespective of their anatomical location. In the study of Cornea-Hébert et al. *(80)*, it was estimated that only 10% or less of immunogold particles representing 5-HT_{2A} receptor in dendrites of the rat fronto-parietal cortex were associated with the plasma membrane of these processes, as opposed to their cytoplasm. In view of these data and of prior results demonstrating an anterograde and retrograde axonal transport of 5-HT_{2A} receptors *(120)*, as well as the eventual contribution of these receptors to the regulation of the expression of several genes (e.g., refs. *121–123)*, it was then speculated that one of the main functions of 5-HT_{2A} receptors might be to participate in intraneuronal retrograde signaling processes activated by 5-HT.

However, a more recent report has emphasized a prominent plasma membrane localization of 5-HT_{2A} receptors in cell bodies and dendrites of the rat spinal cord and fronto-parietal cortex after immunoperoxidase labeling with N-terminal 5-HT_{2A} antibodies targetting the Gly^{22}–Glu^{41} segment of the rat 5-HT_{2A} receptor sequence *(114)*. This labeling was described as confined to the extracellular side of the plasma membrane and essentially nonsynaptic. Intracellular labeling of neuronal somata and dendrites was also reported in this study.

Another feature of the subcellular localization of 5-HT_{2A} receptors is their higher density in smaller compared to larger dendritic branches, as demonstrated by Cornea-Hébert et al. *(74)* after immunogold labeling with three different 5-HT_{2A} antibodies: the monoclonal N-terminal antibody (epitope: Ser^{64}–Trp^{76}; Wu, personnal communication), a polyclonal N-terminal antibody targeting the Gly^{22}–Glu^{41} segment of the rat 5-HT_{2A} receptor sequence from

Fig. 3. *(continued)* and do not show predilection for the rough endoplasmic reticulum (er) or the Golgi apparatus (Go). In **(C)**, numerous cortical dendrites (d) display such predominantly cytoplasmic 5-HT2A immunogold labeling. Dendritic spines (arrowheads) and axon terminals (asterisks) are immunonegative. Scale bars in **(B)** and **(C)**: 1 μm. (Reproduced with permission from ref. *80*.)

B. Roth's laboratory, and a polyclonal C-terminal antibody targetting the Glu^{333}–Glu^{444} segment of the rat 5-HT_{2A} receptor sequence. This finding indicates that 5-HT_{2A} receptors are addressed to the dendritic compartment and concentrated in its distal branches. According to the results of a recent study of pyramidal neurons in culture, the PDZ-binding domain at the carboxyl terminus of 5-HT_{2A} receptors might be the necessary signal for the preferential adressing of these receptors to the dendritic compartment *(124)*. Interestingly, an increase in intracellular 5-HT_{2A} labeling of pyramidal cell bodies and decreased immunoreactivity of their apical dendrites have been described by confocal fluorescence microscopy in the medial prefrontal cortex of a rat treated for 7 d with the atypical antipsychotic clozapine *(99)*. Likewise, higher than normal levels of 5-HT_{2A} protein have been detected immunocytochemically in the soma/dendrites of pyramidal cells of cortical layer V in postmortem brain of teenage suicide victims *(97)*.

In their 2002 study, Cornea-Hébert et al. *(74)* also compared the subcellular distribution of 5-HT_{2A} receptors to that of the cytoskeletal protein MAP1A in rat cortical dendrites. Much like the density of immunogold labeling of the receptor, that of the MAP1A protein was shown to be higher in smaller dendrites than in larger dendrites. By double immunolabeling (peroxidase and gold), 5-HT_{2A} receptor and the MAP1A protein could be visualized within the same dendrites. Touri et al. *(94)* also described a co-localization of 5-HT_{2A} receptors and MAP1A protein, in large apical dendrites of the septa of the adult mouse barrel cortex by immunofluorescence confocal microscopy. In another recent immunofluorescence study by Luttgen et al. *(92)*, an increase in the number of 5-HT_{2A} immunoreactive cell bodies was documented in the septum of rats treated with colchicine, indicating that 5-HT_{2A} receptor protein does undergo microtubule-dependent anterograde transport in axons. Together with earlier yeast two-hybrid data *(125,126)*, these results suggest a physical association of 5-HT_{2A} receptors to the microtubular cytoskeleton in brain neurons, reinforcing the hypothesis of their participation in intraneuronal signaling activity.

3.2. 5-HT_{2B} Receptors

The 5-HT_{2B} receptors (initially called 5-HT_{2F}) are strongly expressed during embryogenesis, at which time they mediate trophic actions of 5-HT that appear essential for normal development *(127–130)*. In the adult, they are mainly distributed in peripheral organs *(131–134)*, but 5-HT_{2B} receptor mRNA has also been detected in the rat or human brain *(131,135,136)*, notably in the cerebral cortex, hippocampus, habenula, paraventricular nucleus, cerebellum, and several brainstem nuclei, including the locus coeruleus and the NRD of the rat *(137)*.

Only two immunocytochemical studies, both at the light microscopic level, have dealt with the cellular localization of 5-HT_{2B} receptors in the CNS: one in

the mouse *(132)* and the other in the rat *(138)*. A somato-dendritic localization was described in the cerebellum, notably in Purkinje cells of the cerebellar cortex and neurons of nucleus interpositus *(132)*, as well as in the septum, hypothalamus, and medial amygdala *(138)*. In the frontal cortex and spinal cord, only immunostained fibers were observed *(138)*. No electron microscopic studies have yet been carried out to further analyze these localizations.

3.3. 5-HT$_{2C}$ Receptors

The 5-HT$_{2C}$ receptors are considered to be restricted to the CNS *(139)*, where they are edited in many isoforms (at least 14) that appear to be differentially distributed (e.g., refs. *140–142*). In this context, antibodies specifically directed againt these isoforms could, in principle, allow one to determine their respective or common cellular and subcellular localization. However, the two antibodies available to date *(73,143)* are directed against the C-terminal portion, a region not affected by the editing.

Light microscopic immunocytochemistry in the rat has revealed abundant 5-HT2C immunolabeling in epithelial cells of the choroid plexus and a somato-dendritic neuronal labeling in numerous brain regions, such as anterior olfactory nucleus, medial and intercalated amygdaloid nuclei, hippocampal sectors CA1 to CA3, laterodorsal and lateral geniculate thalamic nuclei, caudate-putamen, and several areas of the cortex (including piriform and frontal) *(143,144)*. This closely matches the mRNA distribution previously described by *in situ* hybridization *(111)*. Immunoreactive neurons are also present in NRD, suggesting an autoreceptor function and colocalization with 5-HT$_{1A}$ autoreceptors on 5-HT neurons. The widespread somato-dendritic distribution of the 5-HT$_{2C}$ protein in most regions of the CNS suggests that, as for the heteroreceptor also, 5-HT$_{2C}$ receptors might be frequently if not always colocalized with other 5-HT receptor types in the same neurons.

The subcellular distribution of this receptor remains to be examined in detail. A first ultrastructural study, carried out on the rat spinal cord after immunogold labeling with the antibodies directed against segment Arg^{419}–Gly^{435} of the rat 5-HT2C receptor sequence *(73)*, has shown a cytoplasmic localization restricted to the *cis* cisterns of the Golgi apparatus and endoplasmic reticulum of neurons in the sacral parasympathetic nucleus, dorsal gray commissure, dorsal horn, and ventral horn, suggesting that these antibodies recognize the receptor protein at a late stage of its synthesis *(145)*. A subsequent study of mouse olfactory nucleus and choroid plexus, carried out with the antibodies raised against the segment Asn^{373}–Val^{459} of the mouse 5-HT$_{2C}$ sequence *(143)*, reported a preferential localization of 5-HT$_{2C}$ receptors at postsynaptic and, to a lesser extent, at presynaptic thickenings of axo-dendritic synapses after immunolabeling with the immunoperoxidase–diaminobenzidine technique *(146,147)*. In the choroid

plexus, the 5-HT_{2C} immunoreactivity was then described as confined to the limiting membrane of distal portions of microvilli bordering the apical surface of choroidal cells.

4. 5-HT_3 Receptors

The 5-HT_3 receptors belong to the family of ionic channels and are permeable to Na^+, K^+, Ca^{2+}, and other cations *(148)*. The first gene coding for 5-HT_3 receptors (5-HT_{3A} subunit) was cloned by Maricq et al. *(149)*. Functional 5-HT_{3A} receptors are formed by a pentameric assembly of identical subunits, each of which is constituted by four transmembrane domains and two extracellular, N-terminal and C-terminal, domains. A second subunit of 5-HT_3 receptors, 5-HT_{3B}, has been cloned *(150,151)*, but does not form functional channels, unless coassembled with 5-HT_{3A} subunits *(152)*. Several studies have shown that 5-HT_{3B} receptors are only weakly or not at all expressed in the brain *(153,154)*.

There are two splice variants of 5-HT_{3A} subunits: a long form (5-$HT_{3A\text{-}L}$) and a short form (5-$HT_{3A\text{-}S}$), with six amino acids less in its second intracellular loop *(155,156)*. The short form increases in proportion, relative to the long form, during development *(157)*. It is the most abundantly expressed in the adult CNS of all species *(158,159)*. However, no functional differences have yet been found between these two forms.

Autoradiographic studies with selective ligands have demonstrated the presence of 5-HT_3 binding sites in numerous regions of the rat brain: the cerebral cortex, amygdala, hippocampus, dorsal nucleus of the vagus, nucleus of the solitary tract (NTS), area postrema, and dorsal horn of the spinal cord *(160,161)*. In contrast, *in situ* hybridization did not show 5-HT_3 mRNA in several of these anatomical regions *(162)*, suggesting a localization to axon terminals, at least in these regions. Such a conclusion would be in keeping with abundant pharmacological data suggesting a modulation of the release of various transmitters by 5-HT_3 receptors: 5-HT *(163–165)*, dopamine *(166–168)*, glutamate *(169,170)*, acetylcholine *(171)*, and GABA *(172)*.

Several laboratories have raised and characterized anti-5-HT_3 antibodies to study the cellular and subcellular localization of these receptors. To this effect, Turton et al. *(173)* used a fusion protein corresponding to the second intracellular loop of the rat 5-HT_{3A} subunit. With the resulting antibodies, Kia et al. *(174)* described some light microscopic immunoperoxidase labeling in the superficial layers of the dorsal horn of the rat spinal cord, but not in the brain.

Morales et al. *(175,176)* produced antibodies directed against a synthetic peptide of 14 amino acids (Ser^{444}–Asp^{457}), also corresponding to a sequence in the second intracellular loop of the rat 5-HT_{3A} subunit. They then observed strong somato-dendritic immunoperoxidase neuronal labeling in the cerebral cortex,

anterior olfactory nucleus, hippocampus, amygdala, sensory and motor nuclei, nuclei of the reticular formation, and dorsal and ventral horns of the spinal cord. In addition, they reported the presence of heavily immunolabeled plexuses in the NTS and the dorsal horn of spinal cord. Electron microscopy carried out on the anterior olfactory nucleus demonstrated immunoperoxidase labeling confined to the nuclear envelope and endoplasmic reticulum of neuronal somata and within some dendrites and/or dendritic spines. From a subsequent study, combining 5-HT_3 mRNA hybridization and GABA immunocytochemistry, Morales et al. *(177)* concluded that 90% of the neurons expressing the 5-HT_3 receptor in the cerebral cortex and hippocampus were GABAergic (*see also* ref. *178*).

Antibodies directed against amino acids Gly^{23}–Gln^{36} in the N-terminal domain of 5-HT_3 receptors have also been produced by Spier et al. *(179)*. Immunoperoxidase labeling of rat brain sections with these antibodies revealed labeling of nerve cell bodies, dendrites, and varicose axons in the hippocampus, cerebral cortex, and lateral hypothalamus. In a recent electron microscopic study, Huang et al. *(180)* combined immunolabeling with these antibodies and commercially available antibodies against the plasma membrane 5-HT transporter (5-HTT) to examine the subcellular distribution of the 5-HT_{3A} receptor subunits in relation to that of the 5-HT neurons in the rat medial NTS. After both immunoperoxidase and immunogold labeling of the 5-HT_3 receptors and 5-HTT, they described a sparse distribution of either immunolabel in association with dense core vesicles and short plasmalemmal segments in fine unmyelinated axons and axon terminals, some of which were also labeled for the 5-HT transporter. Similar 5-HT_3 immunolabeling was also reported in somatic and dendritic profiles within the nucleus, as well as in small astroglial profiles.

Hamon and colleagues *(159)* have also raised polyclonal 5-HT_3 antibodies, but against the most abundant isoform in the brain: the short 5-$HT_{3A\text{-}S}$ subunit. These antibodies were raised against a 16-amino-acid peptide corresponding to the Ala^{375}–Ser^{390} sequence in the second intracellular loop of 5-$HT3_{A\text{-}S}$, lacking the six amino acids (Gly^{383}–Pro^{388}) present in the same region of the long form (Ala^{375}–Ser^{395}). These antibodies were doubly purified: first on an affinity column bearing the short peptide (Ala^{375}–Ser^{390}) and then on a column bearing the longer sequence (Ala^{375}–Ser^{395}). Their immunological and immunolabeling specificity was demonstrated in Western blots by specific radioligand binding to an immunoprecipitate from solubilized membranes of cells transfected with 5-$HT_{3A\text{-}S}$ receptors, by immunofluorescence on cells transfected with 5-$HT_{3A\text{-}S}$ receptors, and by comparison of the light microscopic immunoperoxidase labeling in rat brain sections with the autoradiographic distribution of specific 5-HT_3 binding sites.

In immunoautoradiographs of rat brain sections exposed to these antibodies and then to [^{35}S]IgG anti-rabbit IgG, the strongest immunolabeling was in the

NTS, dorsal motor nucleus of the vagus, spinal nucleus of the trigeminal nerve, and superficial layers of the dorsal horn of the spinal cord. In the forebrain, less intense labeling was observed in the cerebral cortex, hippocampus, and lateral nucleus of the amygdala. Such a regional distribution closely matched the distribution of 5-HT_3 receptor-binding sites specifically labeled with selective radioligands *(160,161,181,182)*. Moreover, a strong decrease in this immunolabeling could be observed in the ipsilateral NTS following a unilateral ablation of the nodose ganglion.

Electron microscopic examination of the NTS demonstrated peroxidase immunoreactivity in small unmyelinated axons and axon terminals *(159)*. A similar distribution of the 5-HT_3 immunoreactivity to fine axons and axon terminals was subsequently documented in the frontal and entorhinal cortex, amygdala, hippocampus, spinal nucleus of the trigeminal nerve, and dorsal horn of spinal cord *(183)*. Immunostaining within cell bodies, dendrites, and/or dendritic spines was then observed in all of these regions as well as in the NTS, but less frequently than the axonal labeling, except in the hippocampus.

In none of these studies were 5-HT_3 receptors actually demonstrated to be associated with synaptic specializations. It remains to be determined if differential addressing mechanisms exist for preferentially dispatching 5-HT_3 receptors to dendritic sites as opposed to axons and axon terminals, depending on the nature of the neurons bearing these receptors.

5. Other Families of 5-HT Receptors

5.1. 5-HT_4 Receptors

The 5-HT_4 receptors are positively coupled to adenylate cyclase *(184)*. Until now, nine different C-terminal splice variants and one internal splice variant of these receptors have been cloned in four mammalian species (human, rat, mouse, guinea pig) *(185–193)*. Detailed information on the distribution of radioligand-binding sites for these receptors is currently available *(194–196)*, as well as on the distribution of their encoding mRNA as visualized by *in situ* hybridization histochemistry *(197–199)*. In the rat CNS, 5-HT_4 receptors appear to be widely distributed and to predominate in basal ganglia, hippocampus, and olfactory tubercle. In the human brain, they are mostly expressed in the basal ganglia and limbic structures. From the comparison of these results in intact animal or after lesion studies *(200,201)*, it has been inferred that, in several projection systems and notably the striato-nigral pathway, 5-HT_4 receptors are located in both soma-dendrites and axons. However, because of the lack of specific antibodies amenable to light and electron microscopic immunocytochemistry, there has not yet been any study of their cellular and subcellular localization. This would be all the more interesting because

5-HT_4 receptors have been recently demonstrated to exert both a tonic and a phasic, positive, frequency-related control on the firing of NRD 5-HT neurons *(202)*.

5.2. 5-HT_5 Receptors

The 5-HT_5 family comprises two receptor types: 5-HT_{5A} and 5-HT_{5B}. 5-HT_{5A} receptors are negatively coupled to adenylate cyclase. They have been identified in the mouse, rat, hamster and human *(203)*. The 5-HT_{5B} receptors are also expressed in the mouse and rat *(204,205)*, but not in the human, where their coding sequence is interrupted by stop codons *(206,207)*. Both receptor types are essentially limited in distribution to the CNS, but 5-HT_{5A} receptors are also found on neurons and neuronal-like cells of the carotid body *(208)*. To date, only 5-HT_{5A} receptors have been studied by immunocytochemistry.

Within the CNS, 5-HT_{5A} receptors appear to be broadly distributed. Oliver et al. *(209)* carried out a detailed study of their anatomical distribution in the rat brain, using well-characterized, affinity-purified, polyclonal antibodies directed against the N-terminal amino acid sequence (17–34) of rat 5-HT_{5A} receptors. Immunoreactive neurons were then visualized in most CNS regions examined, and particularly numerous in anterior olfactory nucleus, several areas of the cerebral cortex, horizontal nucleus of the diagonal band of Broca, lateral septum, bed nucleus of the stria terminalis, habenula, many nuclei of the thalamus, most hypothalamic nuclei, including supraoptic and suprachiasmatic nuclei, ventral midbrain, many amygdaloid nuclei, ventral midbrain tegmentum, all raphe nuclei, interpeduncular nucleus, pontine nuclei, cerebellum, and many nuclei of the medulla oblongata, including locus coeruleus.

In all of these regions, 5-HT_{5A} messenger mRNA was also detected in a comparative *in situ* hybridization study of 5-HT_{1B}, 5-HT_{5A}, 5-HT_6, and 5-HT_7 receptor mRNAs *(210)*. From the available data, it could be surmised that 5-HT_{5A} receptors are mostly somato-dendritic and present in some acetylcholine (diagonal band nuclei), dopamine (substantia nigra and ventral tegmental area), and noradrenaline (locus coeruleus) neurons, as well as autoreceptors in 5-HT neurons (raphe nuclei). A strong neuronal immunolabeling for 5-HT_{5A} receptors was also reported in the suprachiasmatic nucleus of the Syrian hamster *(211)*, in keeping with a possible role of 5-HT_{5A} receptors in the regulation of circadian rhythms, in addition to that previously ascribed to 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, and 5-HT_7 receptors *(209–213)* (*see also* ref. *214*). Duncan et al. *(211)* also observed a co-localization of 5-HT_{5A} receptors and of 5-HT itself in the NRD of the hamster after double immunofluorescence labeling, and they concluded that some of the circadian effects of 5-HT might be mediated by 5-HT_{5A} receptors functioning as autoreceptors, as in the case of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C} (*see* above), and 5-HT_7 (*see* below) autoreceptors.

Doly et al. *(215)* have recently visualized 5-HT_{5A} receptors at the electron microscopic level as well as light microscopic level in the adult rat spinal cord, using the immunoperoxidase method. They reported dense somato-dendritic labeling of neurons in the superficial layers of the dorsal horn and in the lumbar dorsolateral nucleus (Onuf nucleus) of both male and females, as well as moderate labeling in the intermediolateral cell column. These respective locations suggested an implication of 5-HT_{5A} receptors in the spinal modulation of pain, the control of urethral sphincter muscles, and sympathetic regulation. Interestingly, in each of these regions, only cell bodies and dendrites were found to be immunolabeled, and no axon terminals. Numerous glial cell processes, identified by their irregular shape and their content in gliofilaments, were also described as being labeled. A commentary of this work emphasized the abundant pharmacological evidence suggesting control of spinal cord functions through this as well as other 5-HT receptor subtypes *(216)*.

By *in situ* hybridization histochemistry, 5-HT_{5B} receptors have been shown to be predominantly expressed in the habenular nuclei, hippocampal CA1 pyramidal cells and inferior olivary nucleus of the rat, and at weaker levels in the entorhinal cortex and NRD *(210)*. The 5-HT_{5B} receptor mRNA has recently been shown to be coexpressed with the plasma membrane 5-HT transporter in the NRD *(217)*, strongly suggesting an autoreceptor as well as heteroreceptor role for 5-HT_{5B} as well as 5-HT_{5A} receptors.

5.3. 5-HT_6 Receptors

The 5-HT_6 receptors are positively coupled to adenylate cyclase. In the absence of selective agonists and antagonists as well as radioligands, they have been initially localized in the rat brain by Northern blot analyses *(218,219)*, *in situ* hybridization histochemistry *(219–221)*, and quantitative reverse transcription followed by polymerase chain reaction *(222)* (*see* also ref. *210*). These studies have established that the receptor mRNA is abundant in extrapyramidal areas such as the striatum, as well as in limbic areas such as the nucleus accumbens, olfactory tubercle, hippocampus, and hypothalamus.

A specific antipeptide polyclonal antibody, raised in the rabbit against a synthetic peptide corresponding to the Leu^{398}–Val^{415} amino acid sequence in the C-terminal domain of rat 5-HT_6 receptors *(223,224)*, has allowed for a light microscopic immunocytochemical study of their anatomical distribution. All CNS regions previously shown to synthetize 5-HT_6 receptor mRNA were then shown to contain high levels of immunoreactivity, suggesting a somato-dendritic neuronal localization throughout brain. In keeping with this interpretation, electron microscopic images of immunoperoxidase labeled dendritic spines from the striatum and hippocampus were obtained with this antibody *(223,224)*. In this latter study, a selective localization of the receptors to the plasma membrane

of neuronal cilia in the olfactory tubercle and the island of Calleja was also demonstrated, using an immunogold pre-embedding method, and similar observations were made on neurons of the striatum and nucleus accumbens *(225)*. In view of these localizations and the reported high-affinity interactions betweeen 5-HT_6 receptors and atypical antipsychotic agents and certain tricyclic antidepressants *(226)*, it has been hypothesized that these 5-HT receptors might play a role in affective states and reward and reinforcement behaviors, as well as in the generation of volontary movement *(227,228)*.

5.4. 5-HT_7 Receptors

The 5-HT_7 receptors are also positively coupled to adenylate cyclase. Since their discovery in 1993 *(229–233)*, they have been identified in numerous mammalian species, including human. Different splice variants have been detected in the rat compared to the human, but do not seem to differ in functional properties *(234–236)*. Early and recent *in situ* hybridization studies in the guinea pig and rat have demonstrated 5-HT7 mRNAs to be most abundant in the tenia tecta and piriform cortex, hippocampus, thalamus, and hypothalamus, including the suprachiasmatic nucleus *(230,237–239)*.

Highly specific polyclonal anti-peptide antibodies have been raised in rabbit, against residues 8–23 of the N-terminal domain of rat 5-HT_7 receptors *(72,239)*. Using these antibodies, Neumaier et al. *(239)* have provided a detailed description of the light microscopic immunocytochemical distribution of the neuronal cell bodies and dendrites endowed with 5-HT_7 receptors in rat forebrain, and they demonstrated that their regional density was nearly identical to that of the receptor mRNA *in situ* hybridization signal. 5-HT_7 immunoreactivity was also detected in mouse CA1 hippocampal neurons *(240)* and rat Purkinje cells of the cerebellum *(83)*, again in keeping with earlier *in situ* hybridization results *(231)*. Finally, cytoplasmic inclusions termed "stigmoid body," particularly abundant in the hypothalamus of developing rat brain, have been described as immunopositive with these antibodies *(241)*.

In the single electron microscopic immunocytochemical study thus far carried out with these antibodies *(242)*, 5-HT_7 receptors were described as located in neuronal somata and dendrites, fine unmyelinated axons, and axon terminals of the mouse suprachiasmatic nucleus (SCN). After double immunoperoxidase (5-HT_7) and immunogold labeling, some of these SCN soma-dendrites and axon terminals could be shown to be GABA, vasoactive intestical polypeptide (VIP), or vasopressin (VP) immunoreactive. Astrocytes in the SCN, characterized by their numerous filaments, were also reported as immunopositive for 5-HT_7 receptors.

In keeping with the above anatomical locations, 5-HT_7 receptors have been implicated in thermoregulation, circadian rhythm, learning and memory, hippocampal signaling, sleep, and, more recently, mood regulation *(243)*.

6. Concluding Remarks

The immunocytochemical visualization of 5-HT receptors with specific antibodies has already yielded a wealth of meaningful information on the anatomical, cellular, and subcellular distributions of many of several 5-HT receptor types in the adult mammalian CNS. In a few instances, this has allowed one to determine the morphological and/or the transmitter phenotype of the neurons bearing the receptors. It has revealed in which neuronal parts (soma, dendrites, axons, axon terminals) the various receptor types were located, whether as autoreceptors or as heteroreceptors, and, in some instances, their presence on the plasma membrane as opposed to the cytoplasm of these neuronal parts. It has clearly demonstrated the extrasynaptic localization of all 5-HT receptor types thus far visualized at the electron microscopic level. Immunocytochemistry can also be used to investigate changes in the subcellular distribution of receptors after pharmacological treatment. In the case of 5-HT_{1A} receptors, for example, it has demonstrated a subcellular mechanism (internalization) responsible for their desensitization as autoreceptors and thus involved in the regulation and the response of 5-HT neurons to therapeutic agents.

From the localization data that are already available at the cellular level, it is clear that many of the 5-HT receptor subtypes must be co-localized within the same neurons, even though not necessarily targeted to the same parts of these cells. This is obvious from the widespread anatomical distribution of soma-dendrites endowed with different 5-HT receptor types in many of the same brain regions. The evidence is also compelling that 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, 5-HT_{5A}, and 5-HT_{5B} receptors might act as autoreceptors in the same NRD 5-HT neurons. This suggests a concomitant implication of several 5-HT receptor types in most of the physiological functions and dysfunctions in which 5-HT might be involved *(244–246)*. It also implies that heterodimerization as well as homodimerization of 5-HT receptors *(247,248)* (e.g., ref. *249*) should be envisaged as a more common mechanism than presently considered for explaining the 5-HT effects and those of serotoninergic agents.

Finally, as more and more ultrastructural data are being accrued on the subcellular distribution of the various receptor subtypes, it is becoming increasingly apparent that most functional 5-HT receptors are located at a distance from sites of 5-HT release and must therefore be activated by diffuse as well as synaptic 5-HT neurotransmission.

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10

Chemical Neuroanatomy of 5-HT Receptor Subtypes in the Mammalian Brain

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Summary

Serotonin (5-hydroxytryptamine [5-HT]) produces its effects through a variety of membrane-bound receptors, located in the central and peripheral nervous systems and in non-neuronal tissues in the gastrointestinal tract, cardiovascular system, and blood. The knowledge of the anatomical localization of the receptors is an important step forward for the understanding of their function. Several techniques allow the microscopic visualization of the sites of expression of these receptors in the tissues. In this review, we will attempt to summarize the available information on the neuroanatomical organization of each one of the 5-HT receptor subtypes.

Key Words: Serotonin; 5-HT receptor subtypes; receptor autoradiography; radioligands; mRNA; *in situ* hybridization histochemistry; immunohistochemistry.

1. Introduction

The understanding of the pharmacological, behavioral, biochemical, and electrophysiological effects of 5-hydroxytryptamine (5-HT) as well as the therapeutical and other effects of centrally acting serotonergic drugs, in different brain regions and neuronal populations, is significantly helped by the establishment of the receptor types expressed by these cells and the localization of receptors both in the neuronal circuits and on the different parts of the neurons. Three general techniques can be used to visualize receptors at the microscopic level in the brain: receptor autoradiography, immunohistochemistry, and

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in situ hybridization histochemistry. These techniques provide information on different aspects of receptor localization. Receptor autoradiography allows for the visualization of radioligand-binding sites. Furthermore, in receptor autoradiography, two types of ligand can be considered: agonist and antagonist ligands. For a given receptor, the images obtained with these two classes of ligand are not always fully comparable, depending on the possible existence of different affinity states of the receptor. The availability of antibodies permits the visualization of the receptor protein itself by immunohistochemistry. Immunohistochemical techniques might visualize not only the membrane-bound receptor that is recognized by ligands but also intracellular receptor proteins at different stages of processing. *In situ* hybridization histochemistry leads to the location of the cell bodies where the mRNA coding for a receptor is synthesized. As receptors can be transported far away from their site of original synthesis, the neuronal cell body, a lack of complete overlapping between the *in situ* hybridization histochemistry signal and receptor distribution is not surprising. However, both distributions can be reconciled when the nature of the cells expressing the receptor, its cytoarchitectural features, and the patterns of connectivity of these neurons are taken into account. One of the advantages of using autoradiographic techniques is that *in situ* hybridization and radioligand autoradiography can be carried out in consecutive sections of the same tissue, generating images whose comparison allows for the correlation between both signals.

It is worth stressing the role that the autoradiographic localization of 5-HT receptors has played in the elucidation of the complexities of 5-HT receptor families. Now we know that up to 14 different serotonergic receptors exist, belonging to 6 distinct classes of G protein–coupled receptor (GPCR) populations, namely 5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7, and a family of ligand gated ion channels, 5-HT_3 *(1,2)*. However, in the absence of this knowledge, the possibility of establishing the selective localization of a receptor linked to the ability to characterize binding sites pharmacologically at the microscopic level using more or less selective radiolabeled and unlabeled ligands opened ways to further study serotonin receptors. The initial studies on subtypes of 5-HT_1 receptors or the discovery of 5-HT_{2C} receptors are good examples of how receptor visualization contributed to understanding the role of 5-HT in brain function. The addition of the ability to visualize selectively the mRNAs coding for receptor subtypes enormously helped in understanding the complexities of these receptor families. Receptor visualization techniques can be applied to human postmortem material, allowing the study of receptor characteristics and alterations in pathological states and, again, adding significant information to the understanding of 5-HT and serotonergic drug actions in disease. Moreover, radioligand visualization of receptors and other binding sites

provides the basis for noninvasive neuroimaging of these sites in the living human using techniques such as positron emission tomography, which permits the noninvasive study of receptors in normal and disease states *(3–5)*.

Here we will review our current knowledge of the regional localization of 5-HT receptor subtypes in the brain of laboratory animals and human and nonhuman primates.

2. 5-HT$_1$ Family

The 5-HT$_1$ family consists of five different subtypes named 5-HT$_{1A}$, 5-HT$_{1B}$ (formerly also termed 5-HT$_{1D\beta}$), 5-HT$_{1D}$, (formerly 5-HT$_{1D\alpha}$), 5-ht$_{1E}$, and 5-HT$_{1F}$. The 5-ht$_{1E}$, is written in lowercase, indicating that functional endogenous receptors have not yet been found. They all are seven-transmembrane G protein–coupled receptors, encoded by intronless genes and negatively linked to adenylyl cyclase. 5-HT$_1$ receptors were first defined as those receptors exhibiting high affinity, in the nanomolar range, for serotonin *(6)*. 5-HT$_{1A}$ and 5-HT$_{1B}$ were first differentiated using [^{3}H]-5-HT and ([^{3}H]-8-hydroxy-2-[di-*N*-propylamino]tetralin) (8-OH-DPAT) and cyanopindolol *(7)*. All of these receptors, with the exception of 5-ht$_{1E}$ and 5-HT$_{1F}$ receptors, present high affinity for the agonist 5-carboxamidotryptamine (5-CT). In addition to [^{3}H]-5-HT, other labeled ligands such as ([^{3}H]-8-OH-DPAT), (–)-[^{125}I]-iodocyanopindolol ([^{125}I]-CYP) and [^{125}I]-serotonin-5-*O*-carboxymethyl-glycyl-tyrosinamide ([^{125}I]-GTI) have been, together with unlabeled ligands, useful tools for the visualization of 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1D}$ receptors. Antagonist ligands for the 5-HT$_{1A}$ receptor, such as WAY 100635 ([*O*-methyl ^{3}H]-*N*-(2-(4-(-2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl) cyclohexane carboxamide trihydrochloride) and SDZ 218082 have been made available, as well as [^{3}H]5-CT, a ligand for 5-HT$_{1A}$ and 5-HT$_{1B/1D}$ receptors, and [^{3}H]sumatriptan, another 5-HT$_{1D/1F}$ ligand.

2.1. 5-HT$_{1A}$ Receptors

The 5-HT$_{1A}$ receptors have been most frequently labeled using the selective agonist [^{3}H]8-OH-DPAT, more recently with an antagonist, such as [^{3}H]-WAY 100635, or less frequently with [^{11}C]NAD ([*R*]-3-*N,N*-dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide), all giving a qualitatively identical distribution of binding sites in the brain of several species. High densities of 5-HT$_{1A}$ receptor-binding sites are observed in the rat CA1 and CA3 fields of the hippocampus, the molecular layer of dentate gyrus, septal nuclei, entorhinal cortex, some nuclei of the amygdala, dorsal raphe nucleus, and interpeduncular nucleus *(8–11)* (*see also* Fig. 1). In the human and monkey brain *(11–17)* (*see also* Fig. 2), the anatomical distribution of 5-HT$_{1A}$ receptors is also heterogeneous and quite similar for the different radioligands used. As in the rat, the hippocampal complex is the area most

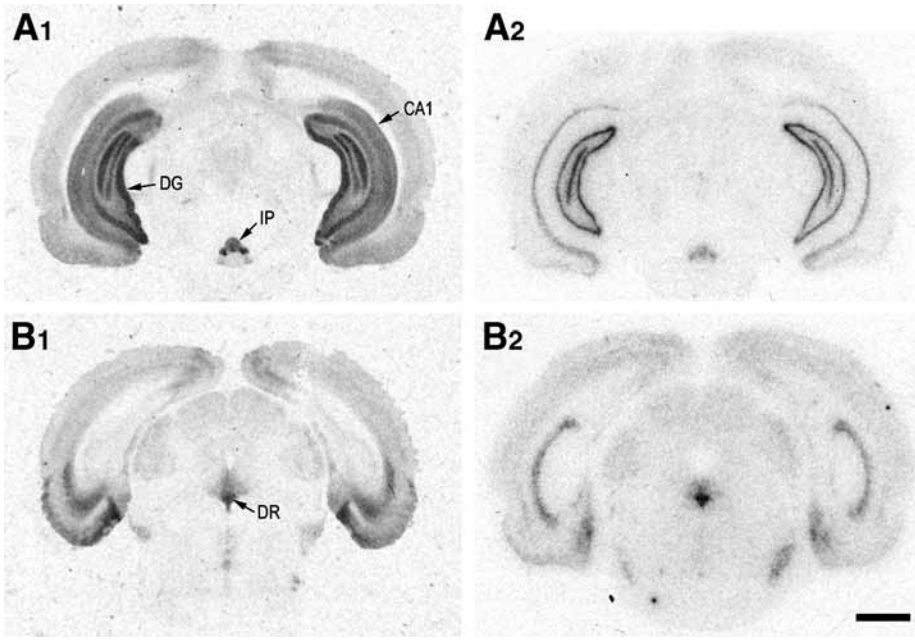


Fig. 1. 5-HT_{1A} receptors in the rat brain. **(A1,B1)** Autoradiographic images illustrate the presence of 5-HT_{1A}-binding sites as labeled with the selective 5-HT_{1A} antagonist [^{3}H]WAY 100,635 in coronal sections from the rat brain. **(A2,B2)** Autoradiograms obtained by *in situ* hybridization using a ^{32}P-labeled oligonucleotide probe complementary to the mRNA encoding 5-HT_{1A} receptors on sections consecutive to those in **(A1)** and **(B1)**. Note that most structures that contain 5-HT_{1A}-binding sites also express 5-HT_{1A} receptor mRNA; however, in the hippocampus, 5-HT_{1A} receptors and their mRNA display opposite laminar patterns of distribution. Abbreviations: DG: dentate gyrus; CA1: CA1 hippocampal field, IP: interpeduncular nucleus, DR: dorsal raphe nucleus. Scale bar: 2 mm.

enriched in 5-HT_{1A}-binding sites throughout the human and monkey brain. The CA1 field, dentate gyrus, subiculum, and claustrum present high receptor densities, as well as the external layers, particularly layer II, of different cortical areas, raphe nuclei, and a few nuclei of the thalamus and of the amygdala. In general, the basal ganglia contain low densities of these receptors, with the exception of the caudate and putamen in the monkey, where the receptor-binding sites are found distributed in form of patches, corresponding with striosomes.

Immunohistochemical localization of 5-HT_{1A} receptors has been accomplished by several groups with different antibodies *(18–24)*. The highest 5-HT_{1A} receptorlike immunoreactivity is detected in some septal nuclei, hippocampus,

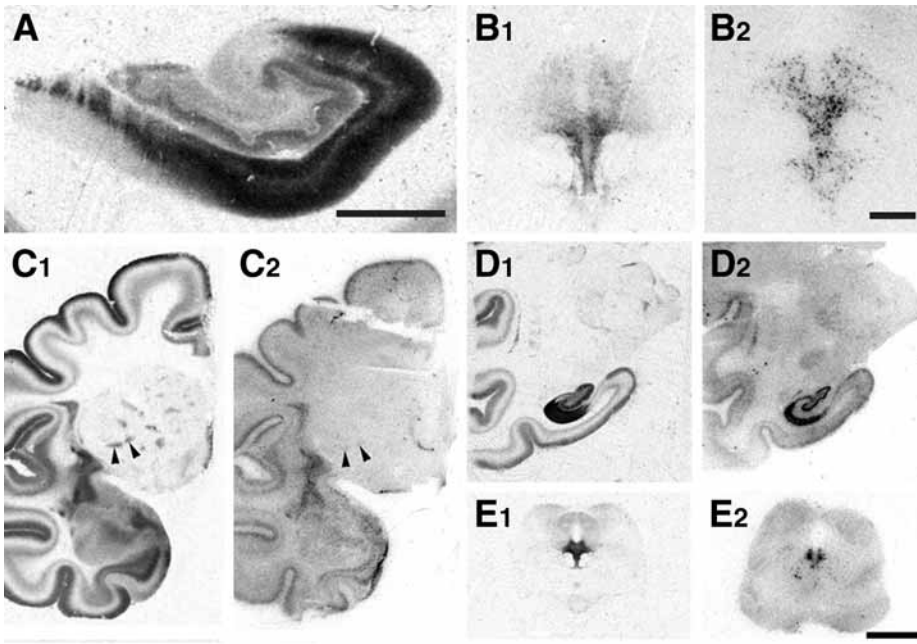


Fig. 2. 5-HT_{1A} receptors in the human and monkey brain. **(A)** Autoradiogram from a human hippocampus showing a strong labeling of 5-HT_{1A} receptors obtained with the selective 5-HT_{1A} antagonist [^{3}H]WAY 100,635. **(B)** The human dorsal raphe also contains 5-HT_{1A} receptor-binding sites as labeled with the agonist [^{3}H]DPAT **(B1)** as well as 5-HT_{1A} receptor mRNA **(B2)**. **(C–E)** Autoradiograms obtained from monkey (*Macaca fascicularis*) brain sections illustrate the presence of [^{3}H]DPAT binding **(C1–E1)** and 5-HT_{1A} mRNA **(C2–E2)** in the outer layers of the cortex, hippocampal formation, and dorsal raphe nucleus. Both 5-HT_{1A} receptor-binding sites and mRNA are present in the macaque striatum in patches that correspond to the striosomes [see arrowheads in **(C1)** and **(C2)**]. Scale bars: **A**, **B** = 2 mm; **C–E** = 3 mm.

frontal and entorhinal cortex, dorsal raphe nucleus, and interpeduncular nucleus (*see* Chapter 9 for details).

The human 5-HT_{1A} receptor gene was the first serotonin receptor to be cloned *(25)*, although it was identified as such later *(26)*. Albert and co-workers cloned the rat 5-HT_{1A} receptor gene *(27)* and the mouse gene *(28)*. The 5-HT_{1A} receptor gene exhibits an intronless open reading frame encoding a protein with seven putative transmembrane domains.

The distribution of the hybridization signal of 5-HT_{1A} mRNA-containing cells obtained by *in situ* hybridization histochemistry was studied in the rat *(9,10)* (*see also* Fig. 1), in monkey *(14)*, and human brain *(29–31)* (*see also* Fig. 2). High levels of 5-HT_{1A} mRNA are seen in areas such as the dorsal raphe

nucleus, claustrum, some septal nuclei, hippocampus, entorhinal cortex, and interpeduncular nucleus. Lower levels are visualized in other cortical areas, olfactory system, and brainstem nuclei.

In general, the distribution and abundance of the mRNA paralleled those of the radiolabeled binding sites and of the 5-HT_{1A}-like immunoreactivity, indicating that 5-HT_{1A} receptors present mainly somatodendritic localization both on serotonergic and nonserotonergic neurons.

2.2. 5-HT_{1B} and 5-HT_{1D} Receptors

The 5-HT_{1B} and 5-HT_{1D} receptor subtypes will be presented together because of their similarities in second messengers, distribution, and function. Both receptors in the nonrodent mammalian brain present a similar pharmacological profile, whereas in the rodent brain, they show different pharmacological properties, especially in what concerns their affinity for β-blockers. The presence of 5-HT_{1D} receptors in rodents was ignored for many years because of their low density in these species. Hoyer and Middlemiss *(32)* speculated that both receptors were species homologs of the same receptor. Consequently, the 5-HT_{1B} receptor was considered the receptor subtype present in rodents *(33–35)* and 5-HT_{1D} was the receptor present in other species *(36–39)*. Cloning has allowed the confirmation of the coexistence of both receptors in all the mammalian species studied so far. The first clone isolated was termed the dog RDC4 receptor *(40)*, later identified as the 5-HT_{1D} receptor *(41)*. Cloning and expression of the two receptors in the human *(42–48)* revealed a low amino acid similarity between them, with a very similar ligand-binding profile. Originally, they were named 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ *(49)*. The corresponding genes in the rat *(50–53)* were cloned. One of the rat receptors had a high homology to the human 5-$HT_{1D\beta}$ receptor sequence but encoded a receptor with the binding site properties of a 5-HT_{1B} receptor. The other rat gene was a homolog to the human 5-$HT_{1D\alpha}$ receptor gene but with a 5-$HT_{1D\alpha}$-binding site profile. Hartig et al. *(54)* proposed an unambiguous nomenclature, consistent across species. Thus, now 5-$HT_{1D\alpha}$ is called 5-HT_{1D} and 5-$HT_{1D\beta}$ is called 5-HT_{1B}, but with the indication of the species to take into account the pharmacological differences.

Rat and human 5-HT_{1B} and 5-HT_{1D} receptors have been characterized with nonspecific radioligands such as [^{3}H]5-HT *(37)*, [^{3}H]sumatriptan *(55)*, [^{3}H]dihydroergotamine *(56)*, [^{3}H]5-CT *(57)*, or [^{3}H]alniditan *(58)*. All of these radioligands have high affinity for both 5-HT_{1B} and 5-HT_{1D} receptors, but given their low selectivity, they do also bind to other receptor subtypes. More selective radioligands, such as [^{125}I]GTI *(59,60)* and [^{3}H]L694247 *(61)*, bind to both receptor subtypes, whereas other compounds such as [^{125}I]ICYP *(7)*, [^{3}H]CP93129 *(62)*, and [^{3}H]CP96501 *(63)* specifically recognize the rat 5-HT_{1B}

receptors. More selective drugs have been developed lately, GR127935 and GR125743, with high affinity for 5-HT_{1B} and 5-HT_{1D} receptors *(64,65)*, presenting partial agonist properties.

The 5-HT_{1B} receptors are one of the most abundant 5-HT receptor subtypes in the brain. In the rat, they have been selectively visualized by receptor autoradiography with [^{3}H]CP96501 *(63)*, [^{125}I]ICYP in the presence of the appropriate blocking drugs *(66)*, or with [^{3}H]GR125743 (*see* Fig. 3). By using [^{125}I]GTI and blocking conveniently its binding to the r5-HT_{1B} receptors *(66)*, r5-HT_{1D} receptors were selectively visualized in the rat brain but at lower densities in areas rich in r5-HT_{1D} receptors. Both 5-HT_{1B} and 5-HT_{1D} receptors are localized in the same structures of the rat brain: in the substantia nigra pars reticulata, entopeduncular nucleus, globus pallidus, ventral pallidum, caudate-putamen, subthalamic nucleus, ventral tegmental area, olivary pretectal nucleus, nucleus of the optic tract, superior colliculus, and frontal cortex. The localization of h5-HT_{1B} and h5-HT_{1D} receptors by receptor autoradiography in the human brain has been examined with different radioligands: [^{3}H]5-HT *(38,67)*, [^{3}H]5-HT and [^{125}I]GTI *(59)*, [^{3}H]alniditan *(68)*, [^{3}H]sumatriptan *(69,70)*, and [^{3}H]GR125743 *(71)*. In general, lower levels of h5-HT_{1D} compared to h5-HT_{1B} and receptor-binding sites are detected in the different human brain areas. The highest densities of binding sites are found in different components of the basal ganglia, ventral pallidum, substantia nigra, nucleus accumbens, globus pallidus, caudate nucleus, and putamen and in several cortical areas. This localization points to the possible involvement of these two receptors in the modulation of the input and output of the basal ganglia, known to be involved in motor functions, and of the visual and olfactory systems. Relevant densities of h5-HT_{1B} receptors are found in the pars caudalis of the spinal trigeminal nucleus and substantia gelatinosa of the cervical spinal cord, key control pain areas.

Immunohistochemical localization has been accomplished only for r5-HT_{1B} receptors with antibodies raised against synthetic peptides corresponding to residues of the third cytoplasmic loop *(22,72,73)*. The highest 5-HT_{1B} receptor immunoreactivity in the rat brain is detected in the substantia nigra pars reticulata, globus pallidus, and dorsal subiculum. In addition, moderate immunoreaction is found in the entopeduncular nucleus, superior colliculus superficial gray layer, the caudate-putamen, and the deep nuclei of the cerebellum. This distribution matched perfectly that previously described from radioligand-binding studies (*see* Chapter 9 for details).

The distribution of r5-HT_{1B} and r5-HT_{1D} receptor mRNA-containing cells has been studied by *in situ* hybridization histochemistry in the mouse *(74)*, rat *(75)* (*see also* Fig. 3), guinea pig *(76)*, monkey *(44)*, and human *(30,44,77)* brain.

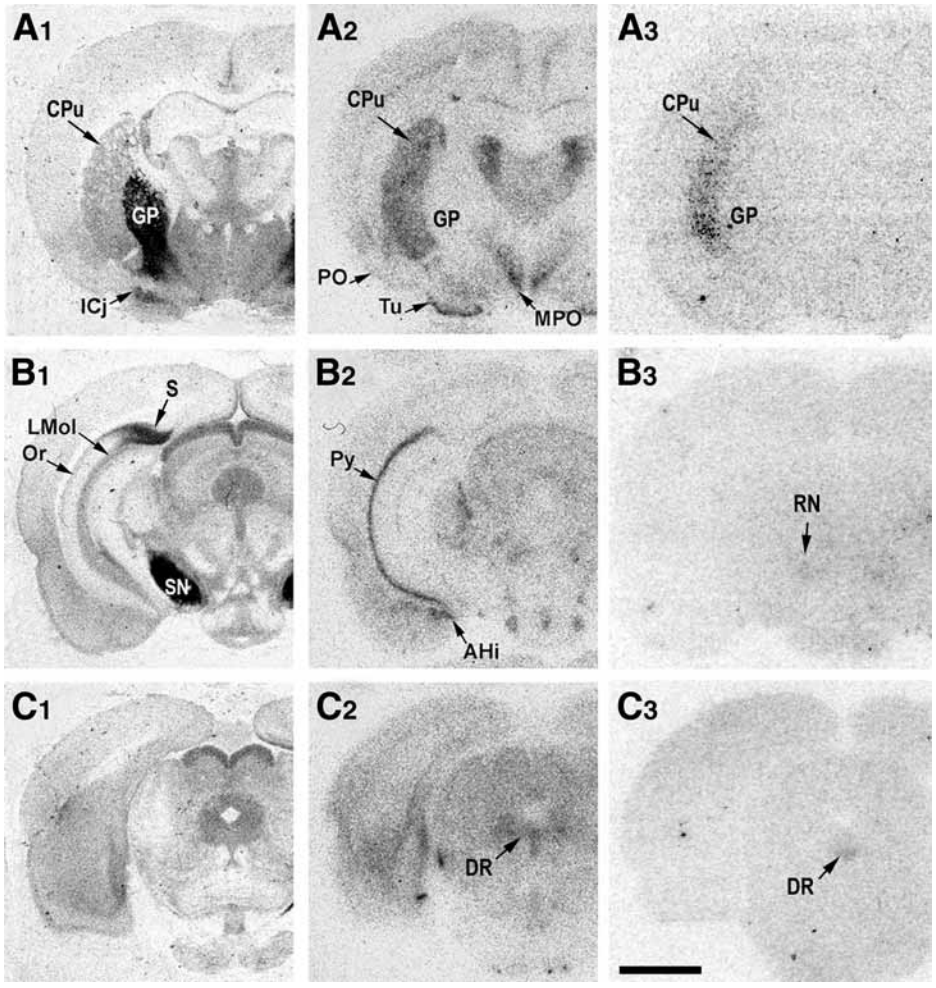


Fig. 3. 5-HT_{1B} and 5-HT_{1D} receptors in the rat brain. (**A1–C1**) Autoradiographic images obtained from rat brain sections incubated with [^{3}H]GR 125743, a selective 5-$HT_{1B/1D}$ antagonist. **(A2–C2)** Autoradiographic distribution of 5-HT_{1B} mRNA as determined by *in situ* hybridization using a ^{32}P-labeled oligonucleotide probe. **(A3–C3)** Autoradiographic distribution of 5-HT_{1D} mRNA as determined by *in situ* hybridization using a ^{32}P-labeled oligonucleotide probe. Note that there is a significant mismatch between the distribution of 5-$HT_{1B/1D}$-binding sites and 5-HT_{1B} and 5-HT_{1D} mRNAs. This is consistent with the concept that 5-HT_{1B} receptors are mainly located on nerve terminals, away from the cell bodies, which contain their mRNA. It is also remarkable that in the rat brain, 5-HT_{1D} mRNA is expressed at very low densities. Abbreviations: AHi: amygdalohippocampal area; CPu: caudate putamen; GP: globus pallidus; ICj: islands of Calleja; DR: dorsal raphe; LMol: lacunosum moleculare layer (hippocampus); MPO: medial preoptic nucleus; Or: oriens layer (hippocampus); PO: primary olfactory cortex; Py: pyramidal cell layer (hippocampus); RN: red nucleus; S: subiculum; SN: substantia nigra; Tu: olfactory tubercle. Scale bar: 3 mm.

In the rat brain, high levels of both r5-HT_{1B} and r5-HT_{1D} receptor mRNAs are found in the caudate-putamen. In contrast, no mRNA is expressed in the globus pallidus and substantia nigra, although these structures reveal the highest levels of r5-HT_{1B} receptor-binding sites. In the hippocampus, 5-HT_{1B} receptor mRNA is localized in the cell bodies of pyramidal cells of the CA1 field. In the cerebellum, this receptor is expressed in the Purkinje cells, which display no receptor-binding sites on their cell bodies. Conversely, moderate binding is found in the deep nuclei of the cerebellum, the main projection zone of the Purkinje cells. r5-HT_{1B} receptor mRNA is also detected in the dorsal raphe. Moderate hybridization levels are obtained in amygdala, hypothalamus, visual system, cortex, and pons. Concerning the sites of expression 5-HT_{1D} receptor mRNA, these are scarcely distributed in other rat brain areas and show a very weak signal. They include primary olfactory cortex, nucleus accumbens, lateral mammilary nucleus, red nucleus, dorsal raphe nucleus, and medial vestibular nucleus.

In the human brain, the highest expression levels of h5-HT_{1B} receptor mRNA are located in the caudate and putamen. Expression is also observed in layer V of the cortex, in Purkinje cells of the cerebellum. h5-HT_{1B} and h5-HT_{1D} receptor mRNAs are found in neurons within the human dorsal raphe nucleus.

2.3. 5-ht_{1E} and 5-HT_{1F} Receptors

The 5-ht_{1E} and 5-HT_{1F} receptors have been characterized for their high affinity for 5-HT and their low affinity for 5-CT, in contrast to the other receptors of this family, which display high affinity for both agonists. 5-ht_{1E} receptors were originally described in the human cortex *(78)* as the population of [^{3}H]5-HT-binding sites remaining after the blockade of 5-HT_{1A} and 5-HT_{1B} and 5-HT_{1D} receptors with 100 n*M* 5-CT and of 5-HT_{2C} receptors with 100 n*M* mesulergine. Later, several groups *(47,79–81)* independently cloned a human and guinea pig gene coding for a seven-transmembrane domain receptor that displayed a pharmacological profile similar to that described by Leonhardt and co-workers. Attempts to clone the gene coding for the rat and mouse 5-ht_{1E} receptor have been unsuccessful. In fact, a search of the mouse genome database revealed that the 5-ht_{1E} receptor gene is not present in the mouse genome *(81)*.

The gene coding for the 5-HT_{1F} receptor has been isolated and characterized in the mouse *(82)*, rat *(83)*, and human *(83,84)*. The mouse gene displays the highest sequence similarity to the human 5-ht_{1E} receptor gene, but the major difference is that whereas the human 5-ht_{1E} receptor cloned presents low affinities for 5-CT, sumatriptan, and ergotamine, the mouse and rat 5-ht_{1E}-like receptor and the human 5-HT_{1F} receptor display low affinity for 5-CT but high affinity for sumatriptan and ergotamine. The question remains as to whether those receptors are truly different or species variants of the same receptor.

The first distribution studies of the 5-HT_{1F} receptors involved the localization of its mRNA by *in situ* hybridization histochemistry in the mouse *(82)*, rat *(83)*, and guinea pig *(14,75)* brains. 5-HT_{1F} receptor mRNA is found in layer V of cortex, claustrum, striatum, several thalamic nuclei, hippocampus, some nuclei of the amygdala, and in the dorsal raphe. There are no reports on the localization of 5-HT_{1F} receptor mRNA in human brain by *in situ* hybridization histochemistry.

The anatomical visualization of 5-ht_{1E} receptor mRNA has been done in monkey and human brain by *in situ* hybridization histochemistry *(75)* (*see also* Fig. 4) and in the guinea pig brain (Fig. 4). The strongest signals are seen in the caudate and putamen, islands of Calleja, external and internal cortical layers (layers II and V in the monkey brain), CA fields and dentate gyrus of the hippocampal formation, dorsal and ventral parts of the lateral septal nuclei, and some hypothalamic nuclei.

The lack of specific ligands for 5-ht_{1E} receptors has hindered the study of their distribution in the brain. By using [^{3}H]5-HT and blocking 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{2C} receptors, the distribution of 5-HT_1-like receptors (5-$ht_{1E/1F}$ receptors) has been determined in rat and guinea pig brains *(85–87)*.

There is no selective radioligand for the 5-HT_{1F} receptor. The regional distribution of 5-HT_{1F} receptors has been studied using [^{3}H]sumatriptan in the presence of a suitable concentration of 5-CT to block 5-HT_{1B} and 5-HT_{1D} receptors in the guinea pig brain *(14,88)* (*see also* Fig. 5) and in the human brain *(69,70,89)*. 5-HT_{1F} receptor-binding sites are found in the intermediate layers of the cortex, in some thalamic nuclei and striatum of the rat and guinea pig brain, and in olfactory tubercle in rat and claustrum in guinea pig. In the human brain, 5-HT_{1F} receptors, (i.e., [^{3}H]sumatriptan-binding sites insensitive to 5-CT) are present in several forebrain areas, such as layer V of the frontal cortex and the CA1 field of the hippocampus. On the other hand, those binding sites were absent from the globus pallidus. In addition, this receptor subtype is found in significant quantities in several brainstem nuclei such as the substantia nigra, nucleus of the tractus solitarius, and substantia gelatinosa of the upper cervical spinal cord.

When the sites of expression of 5-HT_{1F} receptor mRNA in guinea pig brain are compared to the [^{3}H]sumatriptan-binding sites insensitive to 5-CT, a clear correspondence between both distributions is observed, an indication that 5-HT_{1F} receptors are localized in the cell bodies and/or dendrites (*see* Fig. 5).

3. 5-HT_2 Receptor Family

The 5-HT_2 receptors are G protein–coupled receptors (GPCRs) comprising 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. These three subtypes are integrated into the same family because of their high degree of structural homology and their

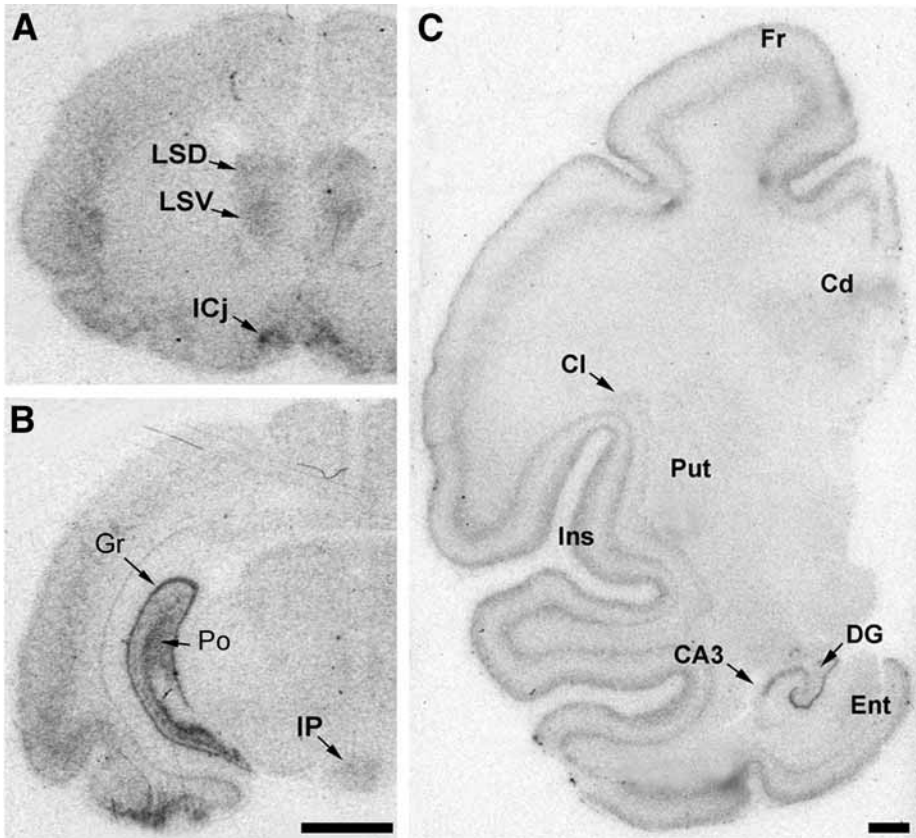


Fig. 4. 5-ht_{1E} receptors in the guinea pig and monkey brain. **(A**,**B)** Autoradiographic images showing the hybridization signal obtained with a ^{32}P-labeled oligonucleotide probe complementary to the mRNA encoding 5-ht_{1E} receptors in the guinea pig brain. **(C)** Visualization 5-ht_{1E} receptor mRNA in an autoradiographic image from a coronal section from the monkey (*Macaca fascicularis*) brain. Abbreviations: CA3: CA3 hippocampal field; Cd: caudate nucleus; Cl: claustrum; DG: dentate gyrus; Ent: entorhinal cortex; Fr: frontal cortex; Gr: granule cell layer of the dentate gyrus; ICj: islands of Calleja; Ins: insula; IP: interpeduncular nucleus; LSD: lateral septal nucleus, dorsal part; LSV: lateral septal nucleus, ventral part; Po: polymorph layer of the dentate gyrus; Put: putamen. Scale bar: 3 mm.

coupling by means of G proteins to the same effector system, namely phospholipase C (PLC) *(2,90)*. This similitude in their structure has made the pharmacological differentiation of these three receptor subtypes difficult, because many agonist or antagonist molecules present similar affinities for each one of them, although, lately, new compounds have appeared, improving the pharmacological characterization of each one of the receptors belonging to the 5-HT_2 family *(91,92)*.

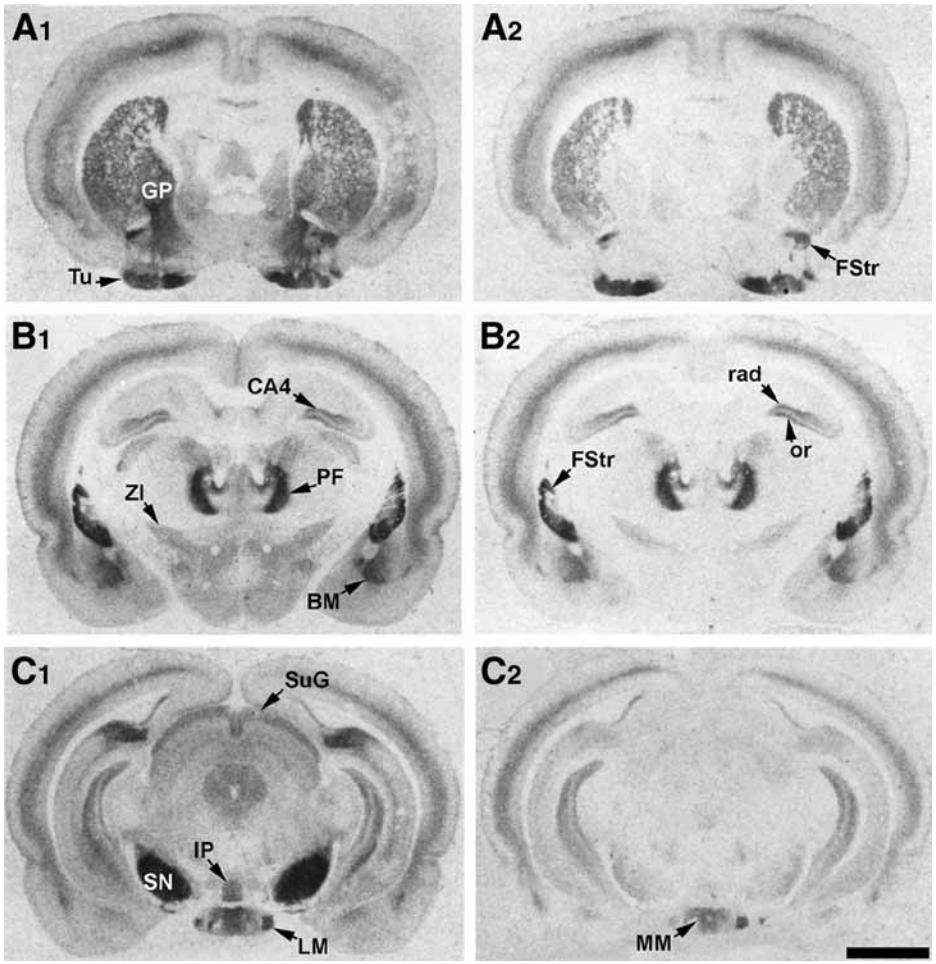


Fig. 5. 5-HT_{1F} receptors in the rat brain. The 5-HT agonist [^{3}H]sumatriptan displays high affinity for 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors. 5-HT_{1F} receptors can be selectively visualized using [^{3}H]sumatriptan in combination with appropriate drugs that block binding to 5-HT_{1B} and 5-HT_{1D}, as illustrated here. **(A1–C1)** Autoradiographic images of the total binding of [^{3}H]sumatriptan in the rat brain. In these sections, 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors are simultaneously labeled. **(A2–C2)** When [^{3}H]sumatriptan is incubated in the presence of 10 n*M* of 5-CT only 5-HT_{1F} receptors remain labeled. Abbreviations: BM: basomedial amygdaloid nucleus; CA4: CA4 hippocampal field; FStr: fundus striati; GP: globus pallidus; IP: interpeduncular nucleus; LM: lateral mammillary nucleus; MM: medial mammillary nucleus; or: stratum oriens of the hippocampus; PF: parafascicular nucleus of the thalamus; rad: stratum radiatum of the hippocampus; SN: substantia nigra; SuG: superficial gray layer of the superior colliculus; Tu: olfactory tubercle; ZI: zona incerta. Scale bar: 3 mm.

3.1. 5-HT$_{2A}$ Receptors

The first member of the family to be described was the 5-HT$_{2A}$ receptor by means of radioligand-binding techniques *(6)*. Since then, this receptor has become one of the most extensively studied in this family because of the efficacy of 5-HT$_{2A}$ antagonists in the treatment of schizophrenia.

Initial studies on 5-HT$_{2A}$ receptor localization used high-affinity, but not selective, radioligands, such as the antagonists [^{3}H]spiperone, [^{3}H]ketanserin, [^{125}I]-7-amino-8-iodo-ketanserin ([^{125}I]AMIK), and [^{3}H]RP62203, or the agonists [^{3}H]LSD, 4-bromo-2,5-dimethoryphenyl-isopropylamine ([^{3}H]DOB), or [^{125}I]DOI *(6,93–101)*. Many of these compounds have been shown later to present high affinity for other receptors, particularly for 5-HT$_{2C}$ receptors and other binding sites. The development of [^{3}H]MDL100,907 (also called M100907), a highly potent selective antagonist with subnanomolar affinity for 5-HT$_{2A}$ receptors *(102,103)*, allowed the selective detection of these receptors by quantitative receptor autoradiography *(104–108)*.

A comparative study of the distribution in the rat brain of 5-HT$_{2A}$ receptor as labeled with four different high-affinity ligands has been published by López-Giménez et al. *(105)*. This study shows that [^{3}H]MDL100,907 appears to be a truly selective 5-HT$_{2A}$ radioligand, which allows for the direct visualization and quantification of these sites without the need for the blockade of additional sites that are labeled by all the other radioligands used until now to label these receptors. Similar selectivity has been found when using this ligand to label 5-HT$_{2A}$ receptors in the primate and human brain. Unfortunately, this ligand is no longer commercially available.

The distribution of 5-HT$_{2A}$-binding sites in all of the mammalian species studied until now showed an enrichment of these sites in the neocortex, which is the brain region with the highest densities of 5-HT$_{2A}$ receptors (*see also* Fig. 6). Other regions with significant densities of 5-HT$_{2A}$ receptor are the olfactory tubercle, striatum, substantia nigra, hilus, dentate gyrus hippocampus, and some brainstem nuclei, particularly the facial nucleus and the motor trigeminal nucleus. Species differences have been found between rodents and primates. The rat claustrum contains very high densities of [^{3}H]ketanserin-labeled 5-HT$_{2A}$ receptors, whereas in the monkey, claustrum intermediate densities were detected with [^{3}H]MDL100,907, this difference not being attributable to the use of two different radioligands *(108)*. In the rat brain, high densities of 5-HT$_{2A}$ receptors are located in layer Va of the cortex *(109)*. In the human brain, these receptors are seen mainly in three layers (I, III, and V) *(104)* and in all six cortical layers in the monkey brain *(108)*. Caudate-putamen and nucleus accumbens in the rat present high [^{3}H]MDL100,907-binding sites *(105)*, whereas in the monkey, these nuclei present very low and homogeneous

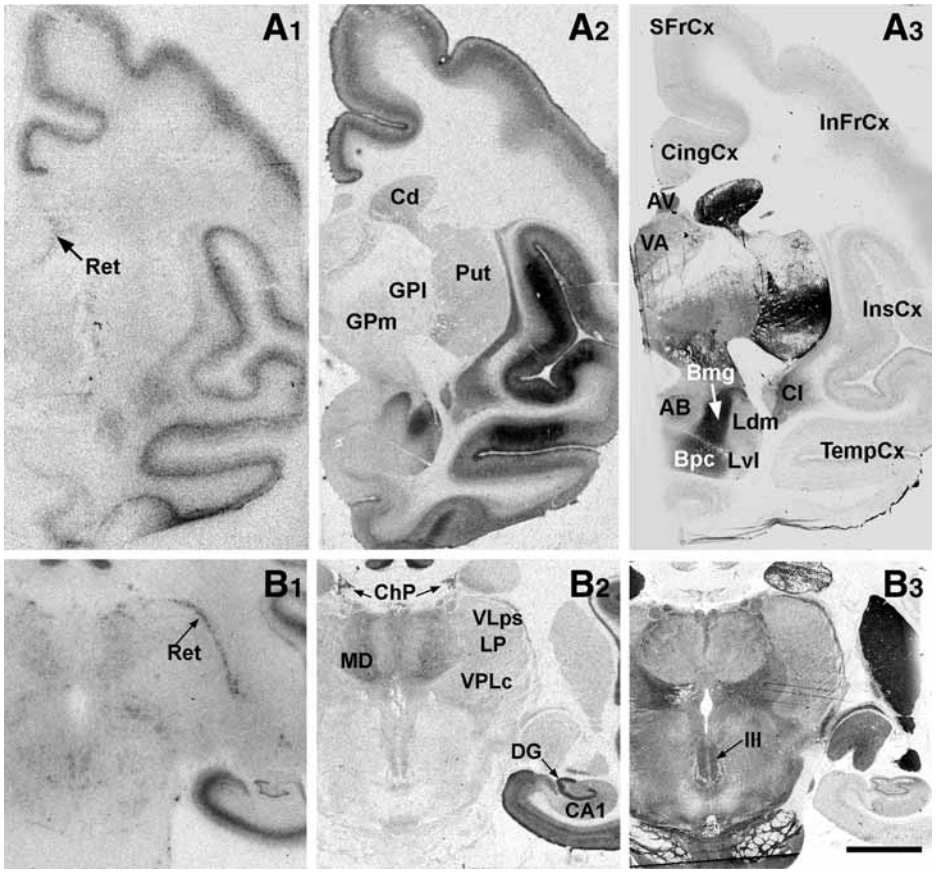


Fig. 6. 5-HT_{2A} receptors in the monkey brain. Regional distribution of 5-HT_{2A} receptor mRNA and binding sites in adjacent sections of the monkey *(Macaca* sp.*)* brain. (**A_1,B_1**) Hybridization signal obtained with a ^{32}P-labeled oligonucleotide probe complementary to the mRNA encoding 5-HT_{2A} receptors. (**A_2,B_2**) 5-HT_{2A} receptor-binding sites labeled by 0.14 n*M* [^{3}H]MDL100,907. Nonspecific binding was determined by the addition of 10 μ*M* spiperone and was very low homogeneous (not shown). (**A_3,B_3**) Acetylcholinesterase-stained sections to provide anatomical reference. Pictures are digital photographs from film autoradiograms or stained sections. Abbreviations: AB: accesory basal nucleus of the amygdala; AV: anteroventral thalamic nucleus; Bmg: basal nucleus of the amygdala, magnocellular part; Bpc: basal nucleus of the amygdala, parvicellular part; CA1: CA1 field of Ammon's horn; Cd: caudate nucleus; CingCx: cingulate cortex; Cl: claustrum; ChP: choroid plexus; DG: dentate gyrus; GPl: globus pallidus lateralis; GPm: globus pallidus medialis; InFrCx: inferior frontal cortex; InsCx: insular cortex; Ldm: lateral nucleus of the amygdala, dorsomedial part; LP: lateral posterior nucleus of the thalamus; Lvl: lateral nucleus of the amygdala, ventrolateral part; MD: dorsomedial nucleus of the thalamus; Put: putamen; Ret: reticular nucleus of the thalamus; SFrCx: superior frontal cortex; TempCx: temporal cortex; VA: ventral anterior

densities *(108)*. In the human, these binding sites show a characteristic patchy pattern with strong labeling corresponding to striosomes *(107)*, similar to that observed in the mouse caudate-putamen *(110)*.

The development of antibodies against 5-HT_{2A} receptor peptides allowed the study of the brain distribution of the receptor protein by immunohistochemistry. By using a polyclonal antibody against a receptor peptide *(111)*, 5-HT_{2A} receptor immunoreactivity in the rat brain has been found in the basal forebrain and olfactory system, neostriatum, dorsal hippocampus, layers II–VI of the neocortex, and some thalamic nuclei. Few 5-HT_{2A} receptor immunoreactive cells are present in the diencephalon, midbrain, and hindbrain. Most of the cells in the cortex display a pyramidal morphology *(112)*. By using two polyclonal antibodies, Hamada and co-workers *(113)* have also described a localization of the 5-HT_{2A}-positive neurons in the rat brain very similar to the one previously described. The authors found 5-HT_{2A} immunoreactivity also in glial cells. With a monoclonal antibody raised against the entire N-terminal region of the 5-HT_{2A} receptor, Cornea-Hébert and collaborators *(114)* found somatodendritic and axonal 5-HT_{2A} immunoreactivity, cytoplasmic rather than membrane bound, in most of the brain regions analyzed by the two previously discussed publications. In the primate cerebral cortex, 5-HT_{2A} receptor immunoreactivity is detected in layers II–III and V–VI *(115)*. In all cortical regions, pyramidal cells represented the majority of the immunoreactive cells. Some recent reports identified dopaminergic cells in the midbrain, showing 5-HT_{2A} immunoreactivity in the rat *(116)* and human *(117)*.

The 5-HT_{2A} receptor gene has been cloned from several mammalian species: the mouse, rat, hamster, pig, sheep, monkey, and human (for references, *see* ref. *1*). The presence and distribution mRNA for 5-HT_{2A} receptors have been determined in the rat brain *(118–122)*, the primate brain *(108)* (*see also* Fig. 6), and the human brain *(29,30)*. 5-HT_{2A} receptor mRNA is detected in the rat caudate-putamen, substantia nigra, and pontine nuclei, but it is absent from these regions in monkey and human brains. In contrast, other regions such as the habenular complex, parabigeminal nucleus, and ventral tegmental nucleus present higher 5-HT_{2A} receptor mRNA labeling in the monkey brain than in the rat brain. 5-HT_{2A} receptor mRNA is present in the prefrontal cortex of the rat brain *(119)*, being more often expressed in glutamatergic cells than in GABAergic cells *(123)*. In the human brain, 5-HT_{2A} receptor transcripts are visualized in cortical layers III and V *(29,30)*, whereas in the monkey brain, 5-HT_{2A} receptor mRNA has been detected in layers III, IV, and upper V *(108)*.

Fig. 6. *(continued)* thalamic nucleus; VLps: ventral lateral nucleus of the thalamus, pars postrema; VPLc: ventral posterolateral nucleus of the thalamus, caudal part; III: oculomotor nucleus. Scale bar: 5 mm.

Although at first sight it could appear that the distribution of 5-HT_{2A} receptors in the mammalian species studied so far is very similar, significant differences exist when receptor distribution is examined in detail and some of these differences could be of important functional consequences. As mentioned above, and just to cite one as an example, striosomal localization in the human caudate and putamen nuclei is not seen in the monkey brain nor in the rat; however, it is observed in the mouse. This fact should be taken into account when extrapolating functional results from one species to another.

3.2. 5-HT_{2B} Receptors

Rat, mouse, and human 5-HT_{2B} receptor genes have been cloned *(124–128)*. By Northern analysis, high levels of 5-HT_{2B} receptor mRNA are detected in the stomach, appearing to be the pharmacologically defined 5-HT receptor in the fundus. The presence of the mRNA coding for this receptor in the human brain has been described by reverse transcription–polymerase chain reaction (RT-PCR) at very low levels only *(125,126)* and by Northern analysis *(129)*. There is only one recent report on the localization in the rat brain of the mRNA coding for the 5-HT_{2B} receptor by *in situ* hybridization *(130)*. The cells expressing this receptor are visualized in the dorsal raphe nuclei and in some layers on the cortex and faint hybridization in the habenula and hippocampal formation. The presence of the 5-HT_{2B} receptor protein has been shown, by immunohistochemistry, in dorsal hypothalamic nucleus, multipolar neurons of the septum, multipolar and bipolar neurons of the medial amygdala, and Purkinje cells of the cerebellum in the rat brain *(131)* and in Purkinje cells, granular cells, and the interpositus cell layer of the cerebellum in the mouse brain *(132)*. The lack of selective ligands (and radioligands) specific for 5-HT_{2B} receptors has hampered the pharmacological characterization and the localization of their binding sites in the brain. There are some ligands, such as SB 200646, SB 204741, and BW 723C86, showing some selectivity for 5-HT_{2B} receptors that have to be further evaluated in different assays with tissues from different species in order to prove their selectivity *(133)*.

Much work is still necessary to understand the distribution of 5-HT_{2B} receptors in the brain as well as its functional role.

3.3. 5-HT_{2C} Receptors

The 5-HT_{2C} receptors were initially visualized and characterized using [^{3}H]mesulergine, a dopaminergic and 5-HT_2 receptor ligand *(134)*. The main feature of the initial autoradiograms with this ligand was an intense labeling of the choroid plexus. Because sites were labeled with [^{3}H]5-HT but not with [^{3}H]spiperone, they were classified as belonging to the 5-HT_1 family and, consequently, named 5-HT_{1C} receptors. It was only after the cloning of the 5-HT_{1C}

receptor gene that the structural similarity with the 5-HT_2 receptors was obvious and they were properly reclassified as 5-HT_{2C} receptors.

One of the differential characteristics of the distribution of 5-HT_{2C} receptors is the low density of binding sites in the gray matter of the brain as compared to the choroid plexus and with the abundance of mRNA coding for this receptor in different regions of the mammalian brain, as we will see below.

The first detailed description of 5-HT_{2C} receptors (at the time, 5-HT_{1C} receptors) *(8)* with [^{3}H]mesulergine showed the highest concentration in the choroid plexus and high densities in the anterior olfactory nucleus, olfactory tubercle, lateral amygdaloid nucleus, CA1 field of hippocampus, central medial thalamic nucleus, claustrum, cortex, and nucleus of the solitary tract. A similar distribution is found in the mouse brain *(118)*. In the monkey brain, [^{3}H]mesulergine-binding sites *(135)* (*see also* Fig. 7) are distributed similarly to those in the rat and mouse brain. However, there are differences when monkey [^{3}H]mesulergine-binding sites are compared with those in the human brain. High densities of binding sites are observed in the human globus pallidus and substantia nigra *(17)*, whereas they are absent from the monkey globus pallidus and low in the substantia nigra *(135)*. Similarly, in the monkey neocortex, very low levels of [^{3}H]mesulergine-specific binding are detected on layer V, whereas in human cortical areas, [^{3}H]mesulergine labeling is located predominantly in layer III.

Immunohistochemical localization of the 5-HT_{2C} receptor has been reported by several groups *(136,137)*. In the rat brain, these receptors are found in the olfactory system, CA1 field of the hippocampus, dorsal hippocampal area, several thalamic nuclei, olfactory tubercle, caudate-putamen, ventral pallidum, several areas of the cortex (including piriform and frontal), and dorsal raphe nucleus.

The cloning of partial or full-length sequences of 5-HT_{2C} receptor genes in the rat *(138)*, mouse *(139)*, and human *(140)* has allowed the determination of the regional distribution of the mRNA coding for these receptors in the rat *(119,120,141)*, mouse *(142)*, monkey *(135)* (*see also* Fig. 7), and human brain *(143)*. 5-HT_{2C} recepto

r mRNA localization in the brain of the mammalian species studied shows a very similar widespread and heterogeneous distribution. There are, however, some important differences to be mentioned. In rodent neocortical regions, 5-HT_{2C} receptor mRNA is present in prefrontal, cingulate, and retrosplenial cortices, whereas in the monkey brain, the mRNA is detected in all neocortical areas, with the exception of the calcarine sulcus within the occipital cortex. In rat, mouse, and human 5-HT_{2C} receptor mRNA is present in the CA3 field of the hippocampus, but it cannot be detected in the monkey CA3 field. Interestingly, the substantia nigra also presents differences: Whereas in the human brain, 5-HT_{2C} receptor mRNA is detected in its pars compacta, in the monkey brain the hybridization signal is confined in the lateral part of this nucleus.

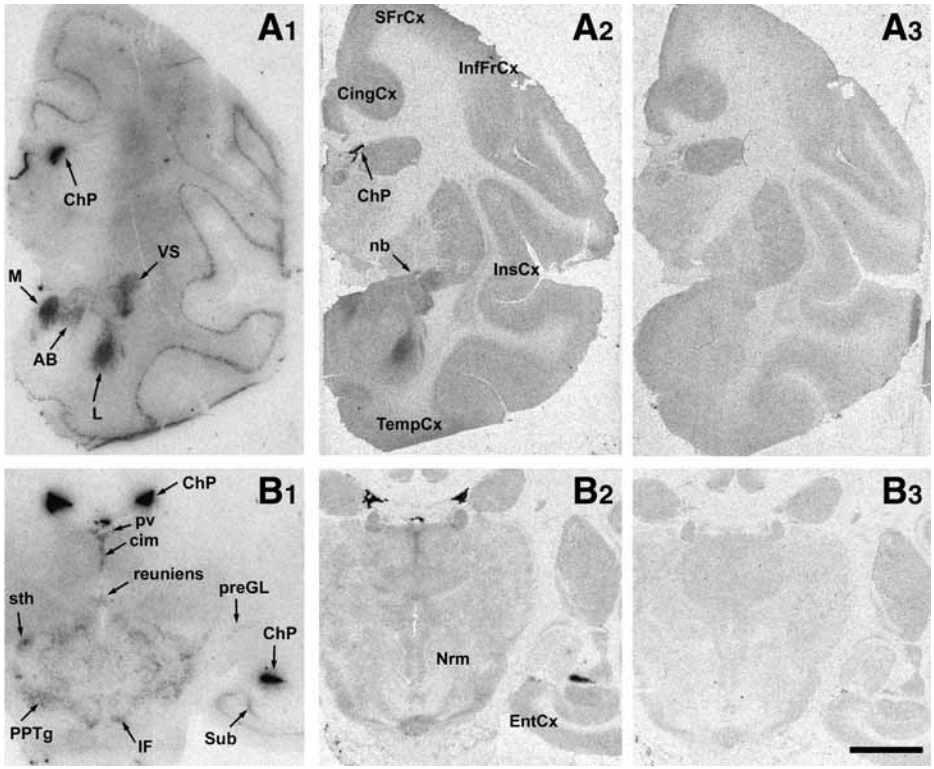


Fig. 7. 5-HT_{2C} receptors in the monkey brain. Regional distribution of 5-HT_{2C} receptor mRNA and [^{3}H]mesulergine-binding sites in adjacent sections of the monkey (*Macaca* sp.) brain. **(A1,B1)** Hybridization signal obtained with a ^{32}P-labeled oligonucleotide probe complementary to the mRNA encoding 5-HT_{2C} receptors. **(A2,B2)** Receptor-binding sites labeled by 5 n*M* [^{3}H]mesulergine. **(A3,B3)** Nonspecific binding remaining in the presence of 1 μ*M* mianserin. Pictures are digital photographs from film autoradiograms. Abbreviations: AB: accesory basal nucleus of the amygdala; cim: nucleus centralis intermedialis of the thalamus; CingCx: cingulate cortex; ChP: choroid plexus; EntCx: entorhinal cortex; IF: interfascicular nucleus; InfFrCx: inferior frontal cortex; InsCx: insular cortex; L: lateral nucleus of the amygdala; M: medial nucleus of the amygdala; nb: nucleus basalis of Meynert; Nrm: nucleus reticularis mesencephali; PPTg: pedunculopontine tegmental nucleus; preGL: lateral pre-geniculate nucleus; pv: paraventricular thalamic nucleus; SFrCx: superior frontal cortex; sth: subthalamic nucleus; Sub: subiculum; TempCx: temporal cortex; VS: ventral striatum. Scale bar: 5 mm.

There is, in general, a good correspondence between the patterns of distribution of [^{3}H]mesulergine-specific binding sites and of mRNA for 5-HT_{2C} receptors in all of the species examined. However, there is less agreement with regard to the intensity of both signals: With the exception of the choroid plexus,

the density of [^{3}H]mesulergine-labeled sites would be expected to be higher in view of the high amounts of 5-HT_{2C} receptor mRNA detected in most brain regions. The overlapping distributions of both autoradiographic signals suggest a predominantly somatodendritic localization of these receptors.

4. 5-HT_3 Receptors

The 5-HT_3 receptor is the only serotonin receptor that belongs to the ligand-gated ion channel superfamily of receptors in mammals *(144)*. It was first identified by its rapidly desensitizing and depolarizing pharmacological effects on enteric and peripheral sensory neurons and named the "M" receptor by Gaddum and Picarelli *(145)*. 5-HT_3 sites appear to be responsible for the depolarizing effects of 5-HT and to mediate peripheral neuronal transmitter release, emesis, and nociception. However, direct evidence of the presence of 5-HT_3 receptors in both peripheral and central tissues has been provided only during the last decade.

In the rat brain, 5-HT_3 receptor-binding sites have been identified using [^{3}H]GR65630 *(146–148)*, [^{3}H]zacopride *(149–151)*, [^{125}I]zacopride *(150,152)*, [^{3}H]ICS205–930 *(153)*, [^{3}H]BRL 43694 *(154)*, and [^{3}H]LY278584 *(155)*.

The distribution in the rat brain of 5-HT_3 receptor-binding sites obtained with all of these different radioligands is very similar. High densities of 5-HT_3 receptors are detected in the nuclei of the solitary tract, dorsal motor nucleus of the vagus nerve, spinal trigeminal nucleus, and area postrema. Moderate levels of binding are found in the glomerular layer of the olfactory bulb, the anterior olfactory nucleus, the cortical and basolateral nuclei of the amygdaloid complex, the granular cell layer of the CA1 subfield of the hippocampus, the dentate gyrus, substantia gelatinosa of the trigeminal nucleus, and spinal cord. Low levels of binding are found in the superficial laminae of the cerebral cortex.

In the human brain 5-HT_3 receptor-binding sites have been identified using [^{3}H]zacopride *(156)* and [^{3}H]ICS205–930 *(157)*. The highest densities of binding sites are observed in several discrete nuclei of the lower medulla, such as the area postrema, the nucleus of the solitary tract, the dorsal nucleus of the vagus nerve, and the spinal trigeminal nucleus and in the substantia gelatinosa at all levels of the spinal cord. In the forebrain, 5-HT_3 receptor-binding sites are visualized, but at a much lower concentration than in the medulla. In the hippocampal formation, 5-HT_3 receptor-binding sites are concentrated in the molecular and granular layers of the dentate gyrus and the pyramidal layer of the CA1, CA2, and CA3 subfields. They are present at a much lower concentration in the entorhinal cortex and the amygdala. In all other regions examined, such as the different components of the basal ganglia, neocortex, thalamus, cerebellum, and pons, no significant specific binding is detected. Neurons of the peripheral ganglia also contain 5-HT_3 receptor-binding sites *(148,158)*.

Two 5-HT_3 receptor subunits have been cloned: 5-HT_{3A} in the rat *(159)* and human *(160)* and 5-HT_{3B} in the rat *(161,162)* and human *(163)*. The 5-HT_{3A} subunit could assemble into functional homomeric receptors, whereas expression of the recombinant receptor subunit 5-HT_{3B} alone does not produce a functional 5-HT_3 receptor. However, in heteromeric receptor complexes, the 5-HT_{3B} subunit confers distinct functional characteristics *(164,165)*.

In the mouse brain, 5-HT_{3A} receptor mRNA *(166)* is found in the glomerular layer of the olfactory bulb, superficial layers of the medial cingulate cortex, entorhinal and piriform cortices, amygdaloid complex, medial and lateral preoptic regions of the hypothalamus, stratum lacunosum moleculare, radiatum and oriens of CA1 subfield, pyramidal cell layer and stratum radiatum of CA3, granule cell layer of the dentate gyrus, trochlear nerve nuclei, dorsal and laterodorsal tegmental nuclei, and nucleus of the spinal tract of the trigeminal nerve. Scattered cells containing 5-HT_{3A} subunit mRNA are observed in the rat brain throughout the cortical areas, with the highest densities in the cingulate, entorhinal, and piriform areas *(167)*, and in the prelimbic area of the prefrontal cortex *(168)*. Most of these cortical cells are GABAergic *(168,169)*. Neurons of the peripheral ganglia contain 5-HT_{3A} subunit receptor mRNA *(166,167,170)*. In contrast, 5-HT_{3B} receptor subunit transcripts are restricted to peripheral neurons *(167)* and are not detected in neurons of the central nervous system. In superior cervical and nodose ganglia, 90% of all 5HT_{3B} expressing neurons coexpressed the 5-HT_{3A} subunit *(167)*.

In the human brain, the knowledge of the distribution of the mRNAs coding for 5-HT_{3A} and 5-HT_{3B} receptor subunits is much more limited. By *in situ* RT-PCR, the co-localization of both subunit mRNAs in a population of neurons in monkey lateral amygdala, and entorhinal cortex and in pyramidal cells of the human cerebral cortex *(163)* has been described.

5. 5-HT_4 Receptors

The existence of 5-HT_4 receptors was postulated in 1988 based on functional and transductional criteria. In primary cultures of embryonic mouse colliculi, a serotonin receptor was identified that coupled positively with adenylate cyclase and had a pharmacological profile distinct from those expected for the other three families of serotonin receptors known at that moment (5-HT_1,5-HT_2, and 5-HT_3) *(171)*. In a short period of time, this novel receptor subtype was identified functionally in several other brain regions and peripheral tissues *(172,173)*. The development and radiolabeling of the selective 5-HT_4 receptor antagonist [^{3}H]-GR 113808 *(174)* opened the way to the analysis of the distribution of 5-HT_4 receptors in the brain. Autoradiographic or binding studies with this radioligand in several mammalian species showed that 5-HT_4 receptors were highly

enriched in the olfactory tubercle, islands of Calleja, and several components of the basal ganglia, in particular nucleus accumbens, caudate and putamen nuclei, globus pallidus, and substantia nigra (Fig. 8). Lower densities of 5-HT_4 receptor-binding sites were also present in other brain areas (e.g., hippocampal formation, amygdala, cortical areas, thalamus, hypothalamus, and several midbrain structures) *(175–179)*. Other radioligands developed later have also been used for the autoradiographic localization of 5-HT_4 receptors. These include the 5-HT_4-selective antagonists [^{125}I]SB 207710 *(13,14,180,181)* and [^{3}H]-R116712 *(182)* and the 5-HT_4-selective agonist [^{3}H]prucalopride *(182)*. The distribution observed with these radioligands in the different species is practically identical to that obtained with [^{3}H]GR 113808. Altogether, the autoradiographic studies have shown that the above-mentioned distribution of brain 5-HT_4 receptors is highly conserved across species. There are, however, a few differences. First, the interpeduncular nucleus is highly enriched in 5-HT_4 receptors in the rat and mouse, whereas the guinea pig *(176,181)* and human brain *(13)* exhibit low to very low binding in this region. Second, 5-HT_4 receptors in the rat and mouse substantia nigra are concentrated in its lateral part, whereas in the guinea pig, monkey and human, the whole of the pars reticulata is enriched in these receptors *(176,181–183)*.

Serotonin 5-HT_4 receptors were first cloned in the rat *(184)*. Two splice variants were described, differing from Leu358 in the length and sequence of the carboxy terminus and termed 5-HT_{4S} and 5-HT_{4L} for short and long, respectively. Subsequent cloning efforts in other species (reviewed in ref. *185*) showed the existence of additional C-terminal splice variants, and this prompted the adoption of the alphabetical nomenclature by which 5-HT_{4S} and 5-HT_{4L} became 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$, respectively. At present, and including all of the isoforms cloned in three mammalian species (human, rat, and mouse), nine different C-terminal splice variants and one internal splice variant have been cloned *(185,186)*. The distribution in brain of the mRNA encoding 5-HT_4 receptors has been studied by *in situ* hybridization. In the rat brain, probes that do not discriminate the different splice variants reveal high levels of 5-HT_4 receptor mRNA expression in the caudate-putamen, nucleus accumbens, olfactory tubercle, islands of Calleja, medial habenula, and hippocampus. Lower hybridization signals are observed in several other regions, including septal nuclei, some thalamic, hypothalamic, and amygdaloid nuclei, cortical areas, periaqueductal gray, zona incerta, and subthalamic nucleus. No hybridization signal has been detected in the globus pallidus or in the substantia nigra compacta, whereas some labeled cells are present in the substantia nigra reticulata *(181,187,188)*. A similar distribution of the mRNA has been observed in the guinea pig brain, the main difference being the lack of mRNA expression in the medial habenula *(181)*. Only one *in situ* hybridization study

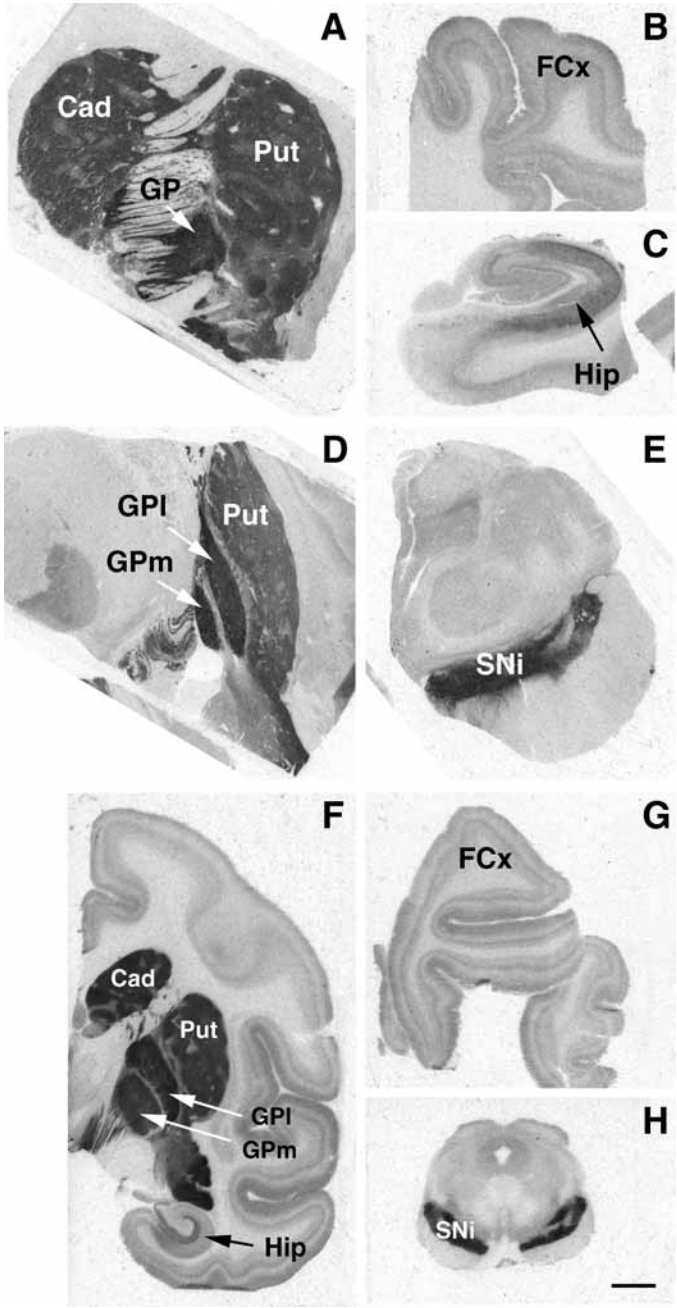


Fig. 8. 5-HT_4 receptors in the human and monkey brain. Autoradiographic visualization of 5-HT_4 receptors labelled with 0.02 n*M* [^{125}I]SB 207710 in several regions of the

in the human brain has been reported thus far *(182)*. It shows high levels of 5-HT_4 receptor mRNA expression in the caudate nucleus, putamen, nucleus accumbens, and hippocampal formation. Low levels are detected in the entorhinal cortex and no expression is detected in the globus pallidus and substantia nigra. Comparison of the distributions of 5-HT_4 receptor mRNA and 5-HT_4 receptor sites labeled by radioligands indicates that these receptors might have both a somatodendritic and an axonal localization. For example, the coincidence in the pattern of distribution of both mRNA and receptor sites in regions such as the nucleus accumbens and the caudate-putamen, indicates that 5-HT_4 receptors present in these areas are most likely located in the somatodendritic domain of neurons whose cell bodies are situated in these nuclei. In contrast, there are several nuclei where the presence of very high densities of 5-HT_4 receptor sites is accompanied by very restricted or undetectable levels of 5-HT_4 receptor mRNA. This is the case of the globus pallidus and substantia nigra in all of the species examined and in the interpeduncular nucleus in rat brain. In these instances, evidence favors a predominant location of 5-HT_4 receptors on the axonal terminals of neurons that project to these areas from distant brain nuclei. For the cases mentioned, good candidates are the neurons of the striato-pallidal, striato-nigral, and habenulo-interpeduncular projections, which contain high levels of 5-HT_4 receptor mRNA. In fact, lesion studies performed in the rat *(180,189)* and guinea pig brain *(181)* support the localization of 5-HT_4 receptors on the terminals of the striato-pallidal and striato-nigral pathways.

The distribution of the mRNAs encoding the different C-terminal splice variants of 5-HT_4 receptors has been studied by *in situ* hybridization or by RT-PCR. Although early RT-PCR studies appeared to indicate that 5-HT_{4S} mRNA was expressed only in the striatum and 5-HT_{4L} mRNA was more widely expressed in the rat brain *(184)*, subsequent *in situ* hybridization *(188)* or RT-PCR *(190)* studies showed that both variants presented a widespread and very similar regional distribution in the brain. A recent RT-PCR study of the distribution of the three C-terminal variants known at present in the rat has shown that r5-$HT_{4(b)}$ mRNA presents the widest distribution, being strongly detected in all

Fig. 8. *(continued)* human **(A–E)** and monkey **(F–H)** brain. Images are digital photographs from film autoradiograms, where dark regions indicate the presence of labeled receptors. Nonspecific binding was determined by the addition of 1 μ*M* GR 113808 and was very low and homogeneous (not shown). Abbreviations: Cad: caudate nucleus; FCx: frontal cortex; GP: globus pallidus; GPl: globus pallidus, pars lateralis; GPm: globus pallidus, pars medialis; Hip: hippocampus; Put: putamen; SNi: substantia nigra. Scale bar: 4 mm.

the regions examined (olfactory tubercle, striatum, hippocampus, parietal cortex, substantia nigra, and inferior colliculus). Both r5-$HT_{4(a)}$ and r5-$HT_{4(e)}$ mRNAs present a somewhat more restricted distribution, hardly being detected in the substantia nigra, and in the case of r5-$HT_{4(e)}$ mRNA, not detected in the parietal cortex *(181)*. Thus, variant r5-$HT_{4(e)}$ has a more restricted distribution in the rat brain when compared to the other two variants, in keeping with earlier observations in the mouse brain *(191)*. The distribution of splice variant mRNAs has also been studied by *in situ* hybridization in the rat brain *(181,188)*. In general, the regional patterns of the hybridization signal obtained with probes for r5-$HT_{4(b)}$ mRNA and with probes that recognize both r5-$HT_{4(a)}$ and r5-$HT_{4(e)}$ mRNAs (here referred to as r5-$HT_{4(a)+(e)}$) were similar to each other and to the pattern obtained with probes that recognize all three splice variants simultaneously. Some differences were observed in the relative intensities of hybridization signals between regions. Thus, in the hippocampus, the signal obtained for r5-$HT_{4(b)}$ mRNA was stronger in the dentate gyrus than in the cornu ammonis, whereas the intensity of the signal obtained for r5-$HT_{4(a)+4(e)}$ mRNAs was very similar in these two structures. In other regions (e.g., the periaqueductal gray, midbrain reticular formation, superior and inferior colliculi, medial septum, and diagonal band), the faint signals observed with the probes common to all splice variants seemed to be contributed to a greater extent by 5-$HT_{4(a)}$ and/or 5-$HT_{4(e)}$ mRNAs than by 5-$HT_{4(b)}$ mRNA. In the regions where more than one variant is present, it remains to be established whether the variants are expressed by the same or by different subpopulations of cells.

In the human brain, the study of the distribution of splice variant mRNAs has only been addressed by means of RT-PCR *(192,193)* or quantitative real-time RT-PCR *(194)*. Some conclusions can be drawn from these studies. All variants except h5-$HT_{4(d)}$ are detected in the brain, some of them (h5-$HT_{4(b)}$, h5-$HT_{4(a)}$, h5-$HT_{4(n)}$) being more widely distributed than others (h5-$HT_{4(c)}$, h5-$HT_{4(g)}$). In terms of abundance, variant h5-$HT_{4(b)}$ appears to be, by far, the most abundant in all of the brain regions examined *(194)*. None of the regions analyzed expresses one single variant, and, conversely, none of the variants is expressed in a single region, thus resulting in complex patterns of variant expression. Here again, much work is still to be done in order to obtain a more precise picture of the regional and cellular localization of the different 5-HT_4 receptor splice variants in the human brain.

6. 5-ht_5 Receptors

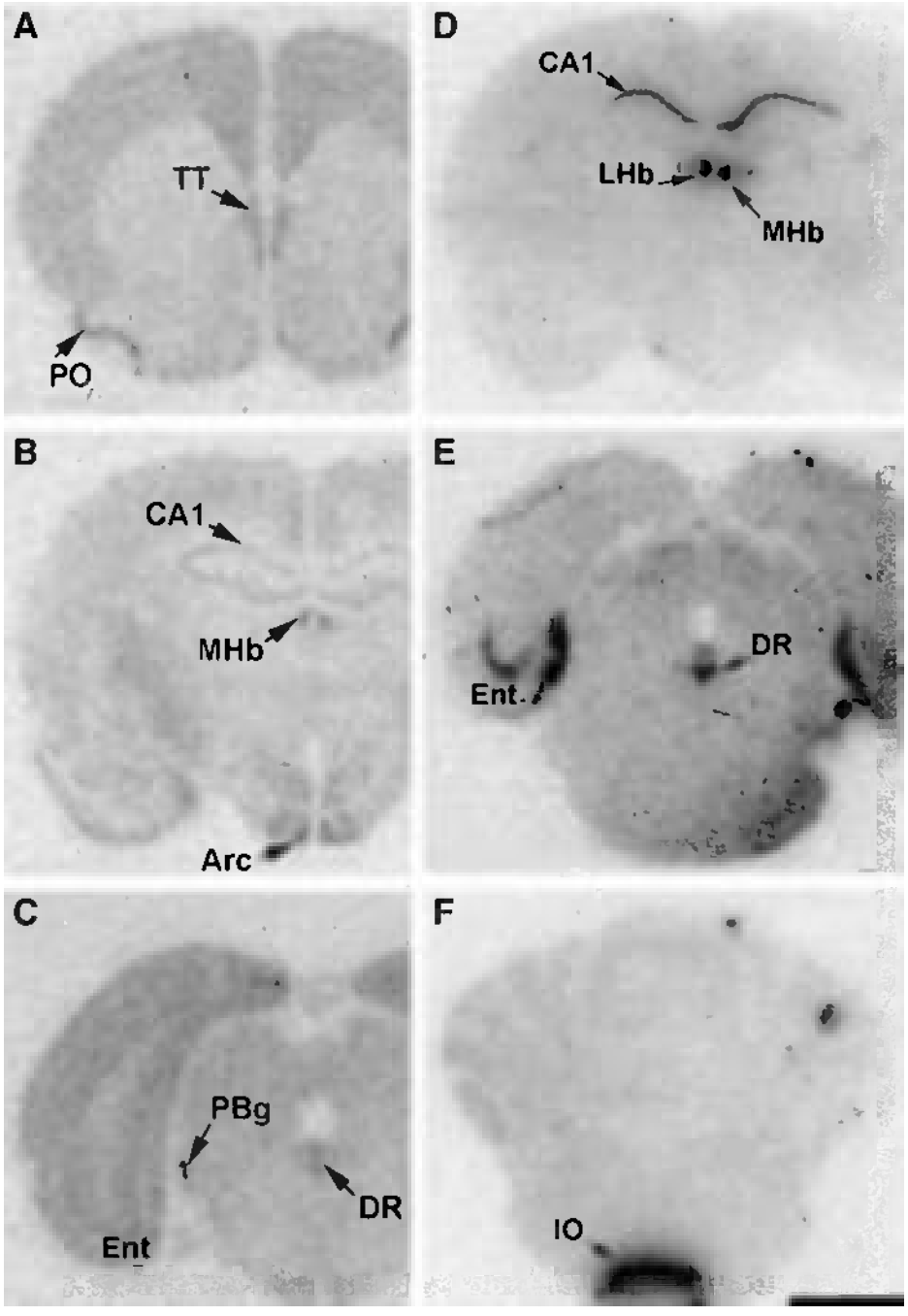
These receptors were cloned by nucleotide sequence homology with other previously identified 5-HT receptor subtypes, yet their function is not known. Until now, no evidence has been provided to confirm the function of the

endogenous receptor. For this reason, 5-ht$_5$ receptors are written in lowercase. Two members of this family have been described: 5-ht$_{5A}$ and 5-ht$_{5B}$. They have been cloned from different mammalian species *(195–200)*. The human 5-ht$_{5B}$ receptor gene does not encode a functional protein because of the presence of stop codons in its coding sequence *(200)*. The pharmacological profile of recombinant 5-ht$_{5A}$ and 5-ht$_{5B}$ is very limited and different from the rest of the 5-HT receptors. In a recent review by Nelson *(201)*, he has listed in great detail the affinities of the recombinant 5-ht$_{5A}$ and 5-ht$_{5B}$ receptors for several 5-HT ligands. It is worth mentioning the high affinity for [^{3}H]5-CT, a ligand for 5-HT$_{1A}$ and 5-HT$_{1B/1D}$ receptors, and for several ergot alkaloid derivatives, such as LSD. The lack of selective ligands has delayed the pharmacological characterization and the analysis of the localization of the binding sites in the brain. There have been attempts to visualize the 5-ht$_5$ receptor-binding sites in the mouse brain. Waeber and colleagues *(202)* and Graihle and co-workers *(203)* by using 5-ht$_{5B}$ receptor gene knockout mice have identified [^{3}H]LSD-binding sites corresponding to 5-ht$_5$ receptors in the olfactory bulb, and medial habenula with lower densities in the neocortex, hippocampus, and caudate-putamen. The same authors obtained similar results when the ligand used was [^{3}H]5-CT, with the only exception being the hippocampus where no differences between both mice types could be found with [^{3}H]5-CT *(203)*. This distribution is similar to that obtained in the mouse brain by *in situ* hybridization *(195)* for 5-ht$_{5A}$ mRNA. The expression of 5-ht$_{5A}$ and 5-ht$_{5B}$ mRNA in the rat brain *(196,197, 204)* (*see also* Fig. 9) has been described. Thus, 5-ht$_{5A}$ is found in the piriform cortex, habenula, some parts of the hippocampal formation, arcuate nucleus, dorsal raphe nucleus, and parabigeminal nucleus, all cortical regions, and prepositus nucleus. 5-ht$_{5B}$ mRNA is expressed in the CA1 field of the hippocampus, the inferior olivary nucleus, and raphe nuclei. In a more detailed analysis *(205)*, it is found that 5-ht$_{5B}$ receptor mRNA is abundantly expressed in the medial portion of the dorsal raphe nucleus. The cells expressing this receptor also express the 5-HT transporter mRNA, a marker for serotonergic cells, whereas in the lateral wings of the dorsal raphe nucleus, no 5-ht$_{5B}$ receptor mRNA was found. This suggests that 5-ht$_{5B}$ receptors could be acting as autoreceptors operating in the serotonergic projections to the cortex.

At present, there are no reports on the expression of this receptor in the human brain.

7. 5-HT$_6$ Receptors

The 5-HT$_6$ receptor was first cloned by sequence homology with another GPCR receptor, the histamine H2 receptor, from a rat brain cDNA library *(206)* and by PCR from a rat striatal cDNA library *(207)*. Later, the human *(208)* and the mouse *(209)* 5-HT$_6$ receptors were also cloned. This receptor


A
TT
PO
D
CA1
LHb
MHb
B
CA1
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Arc
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DR
Ent
C
PBg
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IO

shows a characteristic pharmacological profile, with a high affinity for both typical and atypical antipsychotics *(207,208,210)*. Several 5-HT_6 receptor antagonists, [^{3}H]Ro 63-0563 and [^{125}I]SB-258585, have been recently developed and characterized, showing selective labeling of native receptors *(211,212)*.

The mRNA coding for 5-HT_6 receptor has been localized in the rat brain by Northern blot, PCR, and *in situ* hybridization *(206,207,213,214)* (*see also* Fig. 10) and the protein by immunohistochemistry *(213)*. The first and more detailed description on the localization of mRNA coding for 5-HT_6 receptor was published by Ward and co-workers *(215)*, reporting that the main rat brain regions where this receptor is expressed is the pyramidal layer of the olfactory tubercle, islands of Calleja, nucleus accumbens, striatum, hippocampus, and piriform cortex. At moderate levels, it is expressed in other cortical areas, the olfactory bulb, some nuclei of the hypothalamus and amygdale, the habenula, and the cerebellum. No mRNA expression is found in the raphe nucleus. These results were confirmed later *(204)*.

The first attempts to determine the distribution of 5-HT_6 receptor-binding sites were done by taking advantage of the high affinity exhibited by clozapine for those receptors *(216)*. Glatt and co-workers characterized the binding of [^{3}H]clozapine to rat brain membranes and concluded that clozapine labels muscarinic cholinergic receptors and also binding sites with properties resembling 5-HT_6 receptors. The sites labeled by the compound [3H]Ro 63–0563 in rat and porcine striatal membranes have a pharmacological profile similar to that of the 5-HT_6 receptor, but because of its low affinity, it was impossible to use it for autoradiographic studies *(211)*. By receptor autoradiography and using a selective radioligand, Roberts and colleagues *(217)* have found that [^{125}I]SB-258585-binding sites in the rat brain are located at high densities in the striatum, nucleus accumbens, islands of Calleja, and olfactory tubercle, at moderate densities in the hippocampal formation, cerebral cortex, thalamus, hypothalamus, and substantia nigra, and at very low levels in the globus pallidus, cerebellum, and other mesencephalic regions. The 5-HT_6 receptor-binding sites of the nucleus accumbens and striatum are not located on dopaminergic neuron

Fig. 9. *(previous page)* 5-HT_{5A} and 5-HT_{5B} receptors in the rat brain. Regional distribution of 5-HT_{5A} receptor mRNA **(A–C)** and of 5-HT_{5B} receptor mRNA **(D–F)**. The autoradiographic images shown were obtained by *in situ* hybridization using ^{32}P-labeled oligonucleotide probes specific for each mRNA species. Abbreviations: Arc: arcuate nucleus; CA1: CA1 hippocampal field; DR: dorsal raphe nucleus; Ent; entorhinal cortex; IO: inferior olive; LHb: lateral habenular nucleus; MHb: medial habenular nucleus; PBg: parabigeminal nucleus; PO: primary olfactory cortex; TT: taenia tecta. Scale bar: 3 mm.

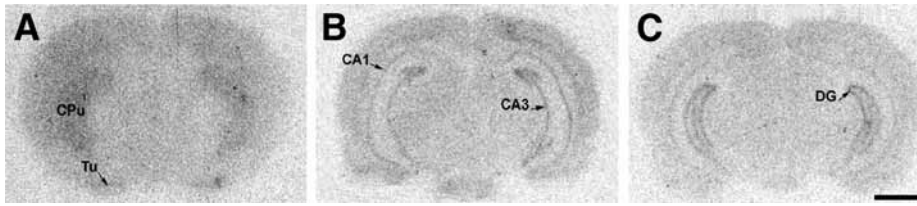

Fig. 10. 5-HT_6 receptors in the rat brain. **(A–C)** Autoradiographic detection of 5-HT_6 mRNA as labeled by *in situ* hybridization using a ^{32}P-labeled oligonucleotide probe. Autoradiographic dark silver grains denote the presence of hybridizing 5-HT_6 mRNA. Abbreviations: CA1: CA1 hippocampal field; CA3: CA3 hippocampal field; CPu: caudate putamen; DG: dentate gyrus; Tu: olfactory tubercle. Scale bar: 3 mm.

terminals of the nigrostriatal pathway because 6-OHDA lesions in the medial forebrain bundle failed to alter the densities of binding sites in those areas *(217)*. A direct comparison of the regional distribution of receptor-binding sites, using the selective ligand [^{125}I]SB-258585, and mRNA expression has been performed in the human and rat brain *(218)*. The highest densities of [^{125}I]SB-258585-binding sites in the human brain are found in the caudate, putamen, and ventral striatum; moderate densities are observed in the hippocampal formation, specially in the molecular layer of dentate gyrus and CA3, subiculum and parahippocampal gyrus, dorsolateral prefrontal, visual and temporal cortices but not in the cerebellum, similar to what could be observed in the rat brain. The regional distribution of the mRNA coding for 5-HT_6 receptors was almost identical to that of the radioligand-binding sites, except in the cerebellum, where the mRNA was present. These observations are in agreement with the postulated somatodendritic localization of these receptors.

8. 5-HT_7 Receptors

The 5-HT_7 receptor is the most recently identified member of the family of GPCR 5-HT receptors. It was first cloned by sequence homology to other 5-HT receptors in the mouse *(219)*. Consequently, it was cloned from the rat *(220–223)*, guinea pig *(224)*, pig *(225)*, and human *(226,227)*. Alternative splicing of the gene coding for 5-HT_7 receptors originates four isoforms (5-$HT_{7A–D}$) in the rat and human, which differ in their C-termini *(228)*.

One way to visualize 5-HT_7 receptor-binding sites by autoradiography in the brain is by using [^{3}H]-5-CT as the radioligand. [^{3}H]-5-CT labels several 5-HT receptors with high affinity, namely 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and 5-HT_7 sites *(88,229–231)*. Thus, it has been possible to visualize 5-HT_7 receptor sites with [^{3}H]-5-CT in the presence of selective blockers *(14,88,232,233)* in rat and

guinea pig brains (*see also* Fig. 11). 5-HT_7 receptor-binding sites are located in layers 1–3 of the cortex, septum, thalamus, hypothalamus (including the suprachiasmatic nucleus), centromedial amygdala, periaqueductal gray, and superior colliculus. Recently, two different approaches have been used to visualize 5-HT_7 receptor-binding sites in the human brain by receptor autoradiography. One is by using a 5-HT_7 receptor antagonist radioligand, [3H]SB-269970 *(234)*. A second approach has used [^{3}H]mesulergine as a ligand *(235)*. The regional distribution of these receptors in the human brain is very similar to that described in rat and guinea pig brains, but with some minor differences such as the higher densities in the inner cortical layers and in the substantia nigra in the human brain. In the same report *(235)*, the authors analyzed in parallel the localization of 5-HT_7 receptors in the rat and guinea pig and found similar results to those described earlier. Interestingly, in all three species, they found high 5-HT_7 receptor densities in the raphe nuclei. Taking advantage of the 5-HT_{1A} and 5-$HT_{1A/1B}$ receptors knockout mice, Bonaventure and co-workers *(236)* have reanalyzed the distribution of 5-HT_7 receptors using [^{3}H]-5-CT and [^{3}H]-8-OH-DPAT. The results obtained were similar to those described by Martin-Cora and Pazos *(235)*.

The mRNA coding for 5-HT_7 receptors, visualized by *in situ* hybridization histochemistry in the rat and guinea pig brain *(229,232)* (*see also* Fig. 11), is present at high levels in discrete nuclei of the thalamus and hippocampus. Moderate levels are found in the cortex, septum, hypothalamus, centromedial amygdala, and periaqueductal gray.

A clear correspondence between 5-HT_7-binding sites and mRNA-containing cells exists in some nuclei of the rat amygdala, CA2 layer, induseum griseum, and fasciola cinereum of the hippocampal formation, CA3 layer, dentate gyrus, paraventricular nucleus, centromedial nucleus, centrolateral nucleus, and reuniens nucleus of the guinea pig.

9. Concluding Remarks

The application of receptor autoradiography, *in situ* hybridization histochemistry, and immunohistochemistry to the study of the brain 5-HT receptors during the last two decades has generated useful information to help understand the pharmacology of ligand binding, by adding the anatomical component. In this way, the localization of 5-HT receptors at the level of the serotonergic neuron and in other brain cells has facilitated a better knowledge of the possible effects of drugs acting on these receptors, of 5-HT receptor alteration with disease, and so forth.

The number of open questions is enormous. We still have limited information on the subcellular localization of some 5-HT receptors and the relationship between membrane-bound receptors and the newly synthesized receptors. A clear

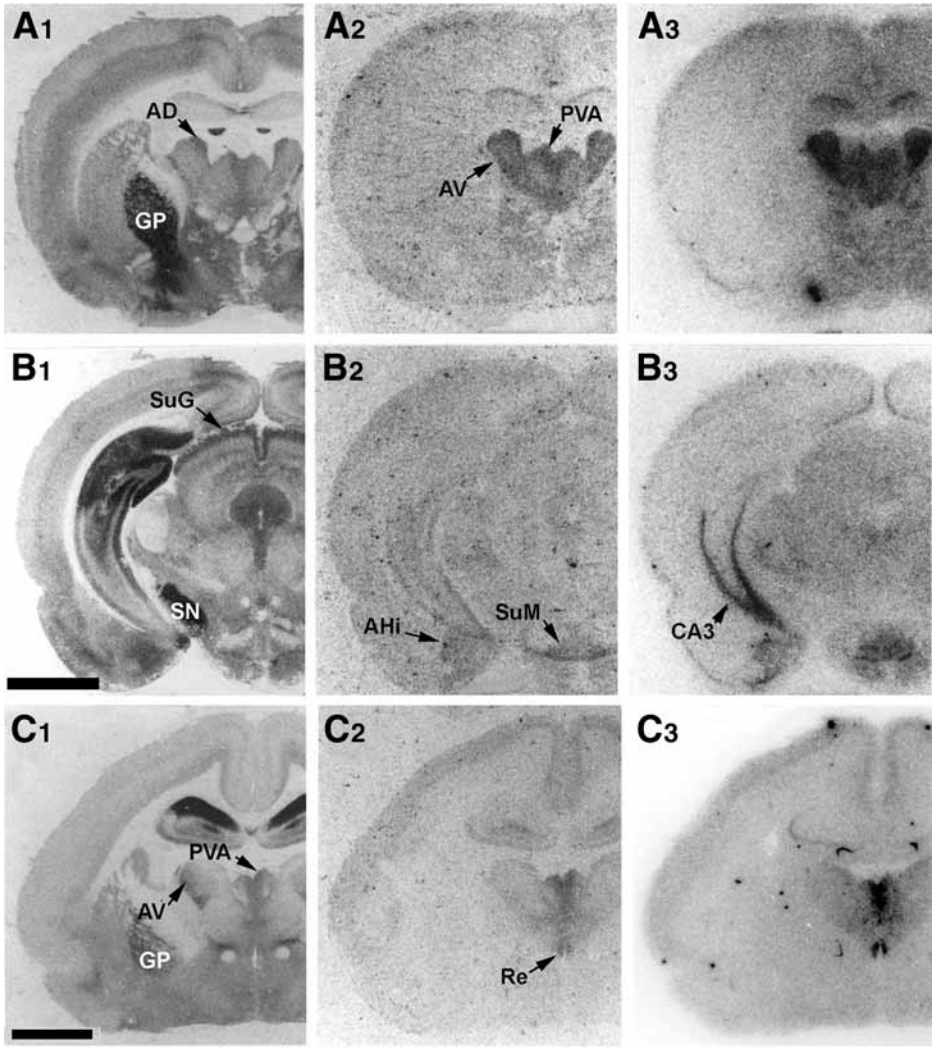

Fig. 11. 5-HT_7 receptors in the rat and guinea pig brain. Autoradiographic images were obtained from coronal sections from the rat brain **(A–B)** and the guinea pig brain **(C1–C3)**. **(A1–C1)** [^{3}H]5-CT used alone (total binding) labels various subtypes of 5-HT receptors, mainly 5-HT_{1A} (i.e., labeling in the hippocampus) and 5-$HT_{1B/1D}$ (i.e., GP, SN) sites, but it also binds to 5-HT_7 receptors. **(A2–C2)** 5-HT_7 receptors can be selectively visualized using [^{3}H]5-CT in the presence of high concentrations of WAY 100135 (0.01 m*M*) and GR 127935 (0.25 m*M*), which block the binding of [^{3}H]5-HT to 5-HT_{1A} and 5-$HT_{1B/1D}$, respectively. Note the presence of 5-HT_7 receptors in some nuclei of the anterior thalamus (AV, PVA, Re), posterior hypothalamus (SuM), amygdala (AHi), and parts of the ventral hippocampus. **(A3–C3)** The localization of 5-HT_7 receptor mRNA by *in situ* hybridization demonstrates that the distribution of the receptor

function is still not yet described for many 5-HT receptors. The dissection of the involvement of particular 5-HT receptors in important human brain diseases such as depression, schizophrenia, and so forth, where serotonergic mechanisms are clearly involved, still offers challenges to those interested in 5-HT receptors. As has been shown, with the application of imaging techniques, molecular neuroanatomical methods will certainly contribute to progress in this endeavor.

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Fig. 11. *(continued)* and its mRNA are coincident. This suggests that 5-HT$_7$ receptors are mainly expressed at the level of cell bodies and dendrites. Abbreviations: AD: anterodorsal thalamic nucleus; AHi: amygdalohippocampal area; AV: anteroventral thalamic nucleus; CA3: CA3 hippocampal field; GP: globus pallidus; PVA: paraventricular thalamic nucleus, anterior part; Re: reuniens thalamic nucleus; SN: substantia nigra; SuG: superficial grey layer of the superior colliculus; SuM: supramammillary nucleus. Scale bar: 3 mm.

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11

Modulation of the Neuronal Activity and Neurotransmitter Release by 5-HT_{1A} and 5-$HT_{1B/1D}$ Receptors

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Summary

The 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors are major determinants of the activity of serotonergic cells and of serotonin, 5-hydroxytrytamine (5-HT), release because of their role as somatodendritic and terminal autoreceptors, respectively. As such, their physiological role is to limit unwanted increases in serotonergic activity and 5-HT release and play an important role in the action of drugs used to treat psychiatric diseases, like anxiety or depression. Additionally, those receptors are located postsynaptically to serotonergic axons, in cortical, limbic, and hypothalamic areas (5-HT_{1A} receptors), and in the basal ganglia (5-HT_{1B} receptors). 5-HT, acting on those postsynaptic receptors, is involved in cognition, mood, impulse control, and motor functions by modulating the activity of different neuronal types and inhibiting the release of various neurotransmitters, such as glutamate, GABA, acetylcholine, and dopamine.

Key Words: 5-HT_{1A} receptors; 5-HT_{1B} receptors; 5-HT_{1D} receptors; extracellular recordings; hippocampus; intracellular recordings; microdialysis; potassium channels; prefrontal cortex; raphe nuclei.

1. Introduction

The serotonergic system is involved in a large number of physiological functions, resulting from its widespread innervation of the brain. The axons of serotonergic neurons of the midbrain raphe nuclei reach almost every brain structure. Action potentials traveling along these axons release 5-hydroxytryptamine

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(5-HT), which can act on 14 different receptors, coupled to different signal transduction mechanisms. With the exception of the 5-HT_3 receptor, a ligand-gated ion channel, the 5-HT receptors belong to the superfamily of G protein–coupled receptors (GPRCs) and their activation results mainly in modulatory actions in the neurons expressing these receptors. Given such a widespread innervation of the brain and the richness of signals evoked by 5-HT, it is not surprising that the 5-HT system is the target of many drugs used to treat mental illnesses. For instance, most antidepressant treatments block the 5-HT transporter and increase the extracellular (or synaptic) 5-HT concentration and, hence, they indirectly elevate the serotonergic tone at presynaptic and postsynaptic 5-HT receptors. Drugs of abuse such as cocaine, amphetamine, or MDMA (ecstasy) target monoaminergic transporters, including the 5-HT transporter. On the other hand hallucinogens like LSD, DOI, DOB, or DOM are agonists of the 5-HT_2 receptor family, whereas atypical antipsychotics act as preferential antagonists of these receptors. Moreover, 5-HT_3 receptor antagonists are commonly used to treat emesis induced by anticancer treatments. However, among the various 5-HT receptors, the 5-HT_1 family has probably received the largest attention because of the large density in various brain areas and the various roles subserved by some of their members. Thus, 5-HT_{1A} and 5-HT_{1B} receptors are autoreceptors in 5-HT neurons and, therefore, control the overall (5-HT_{1A}) or local (5-HT_{1B}) activity of the system. 5-HT_{1B} receptors are also terminal heteroreceptors and control the release of various transmitters, including glutamate, GABA, and acetylcholine. They are densely expressed in the basal ganglia, which suggests a role in the control of motor function. On the other hand, 5-HT_{1A} receptors are expressed in high density by different neuronal types (mainly pyramidal but also GABAergic) in the limbic system and in the prefrontal cortex, which suggests an important role in the control of mood and emotions as well as in cognition. An extensive review of the characteristics of the serotonergic system is beyond the scope of the present chapter. The reader is referred to several review articles dealing with the anatomy, physiology, neurochemistry, and neuropharmacology of 5-HT and its receptors *(1–3)*. In the following sections, we focus on the role of 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors in the control of neurotransmitter release and the direct and indirect electrophysiological actions produced on neurons postsynaptic to 5-HT. Unfortunately, there is still little information on other receptors of the 5-HT_1 family (5-HT_{1E} and 5-HT_{1F}) so as to be included in this review.

2. 5-HT_{1A} Receptors

2.1. Regional Distribution and Cellular Localization of 5-HT_{1A} Receptors

The highest density of 5-HT_{1A} receptors is found in limbic structures (the lateral septum, hippocampal formation, frontal and entorhinal cortices) and the

dorsal (DR) and median raphe (MnR) nucleus *(4–7)*. These receptors are expressed by serotonergic neurons in the DR and by 5-HT and some non-5-HT neurons in the MnR *(8)*. In the DR, 5-HT_{1A} receptors are distributed in the plasma membrane of perikarya and dendrites *(9,10)*. This is also the localization in some forebrain areas such as the septum or the cortex, whereas they appear to predominate on dendritic spines in the hippocampal formation *(9)*. The distribution of mRNA encoding the 5-HT_{1A} receptor matches that of the receptor protein, which also suggests a somatodendritic location *(4,6,7)*. However, in immunostaining studies using a different antibody, several groups have reported the localization of 5-HT_{1A} receptors in the axon hillock of pyramidal neurons in the frontal cortex of the rat, monkey, and human brain *(11–13)*.

2.2. *Coupling Mechanisms and Functional Correlates*

The 5-HT_{1A} receptor has been characterized biochemically and electrophysiologically as being coupled to the $G_{i/o}$ family of heterotrimeric G proteins. $G_{i/o}$ proteins coupled to 5-HT_{1A} receptors are composed of αi/αo-subunits that are inactivated by pertussis toxin (PTX), which catalyzes the ADP-ribosylation of these α-subunits. This coupling mechanism was demonstrated in vivo and in vitro in the dorsal raphe *(14,15)*. 5-HT_{1A} and $GABA_B$ receptors in hippocampal cells (as well as in 5-HT cells) share the same mechanism *(14–16)*. 5-HT_{1A} receptors can be linked to different G proteins in different areas of the brain. Unlike in the hippocampus, in the raphe nuclei the 5-HT_{1A} autoreceptor is not coupled to adenylate cyclase *(17)* but to a K^+ channel through a pertussis toxin–sensitive G protein (GiRK) *(15,18)* (*see also* Chapter 5).

Therefore, the activation of 5-HT_{1A} receptors in the 5-HT cells by the endogenous transmitter or 5-HT_{1A} receptor agonists increases K^+ conductance, which hyperpolarizes the neuronal membrane (*see* below), thus inhibiting 5-HT cell firing *(19–22)* (*see* below) and reducing 5-HT release in the cell body area *(23–27)* and projection structures *(24,25,28–30)* (Fig. 1).

Several studies have examined the existence of possible differences in the sensitivity of 5-HT_{1A} autoreceptors between DR and MnR 5-HT neurons. Using extracellular recordings, two studies have reported that 8-OH-DPAT, administered either intravenously (iv) *(19)* or by microiontophoresis *(31)*, is more potent in inhibiting serotonergic cell firing in the DR than in the MnR. However, a more recent study failed to find this difference and showed an equal potency of 8-OH-DPAT and paroxetine to suppress the firing of DR and MnR serotonergic neurons *(32)*. Likewise, the local administration of the highly selective 5-HT_{1A} receptor agonist BAY × 3702 results in similar reductions of 5-HT release in DR and MnR *(3,33)*. However, the systemic administration of various 5-HT_{1A} agonists reduced the impulse-dependent 5-HT release in various forebrain areas in an uneven manner *(33–35)* (i.e., more in areas innervated by the DR than in the hippocampal formation [particularly the dorsal hippocampus]), which

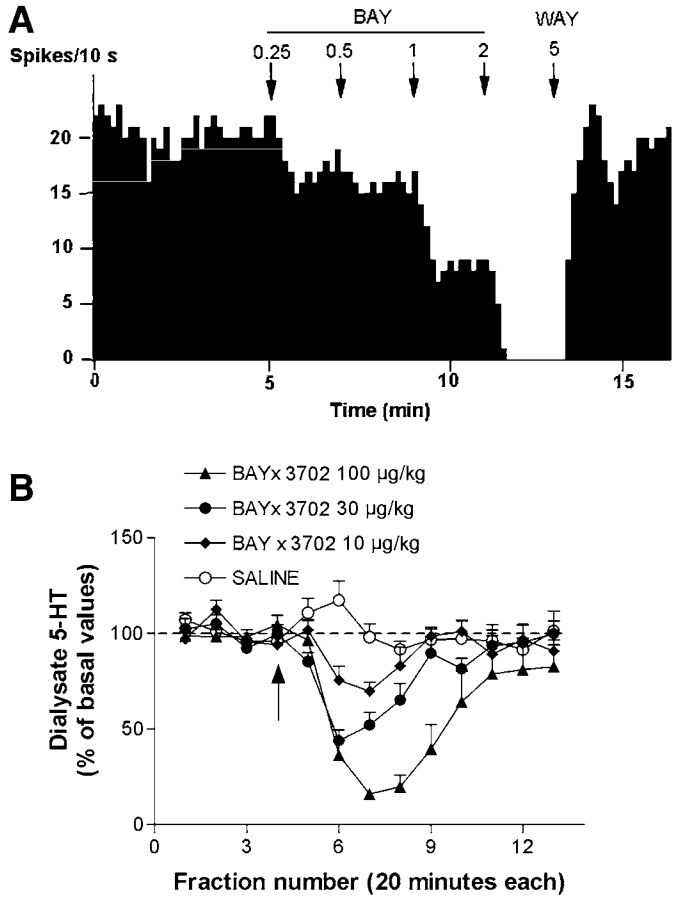

Fig. 1. The systemic administration of the 5-HT_{1A} receptor agonist BAY × 3702 inhibited the firing rate of dorsal raphe 5-HT neurons and decreased the 5-HT release in medial prefrontal cortex. **(A)** Integrated firing rate histogram showing the effect of sequential intravenous injections of BAY × 3702 on the firing rate of a 5-HT neuron in the DR. Arrows show the injections of BAY × 3702 at the indicated cumulative doses (in µg/kg) (iv). The calculated ED_{50} was 0.9 µg/kg iv ($n = 5$ rats). The injection of the selective 5-HT_{1A} receptor antagonist WAY-100635 (5 µg/kg iv, shown by an arrow) completely reversed the inhibition elicited by BAY × 3702. **(B)** Effects of the systemic administration of saline (open circles) or BAY × 3702 (10, 30, and 100 µg/kg sc; filled diamonds, circles, and triangles, respectively) on the 5-HT release in the medial prefrontal cortex of freely moving rats ($n = 7, 6, 5, 7$ respectively). Reproduced with permission from ref. *33*.

is mainly innervated by MnR axons. The exact reasons for these regional differences are still unclear, yet it is possible that the control of 5-HT release by postsynaptic 5-HT_{1A} receptors is different in each raphe nucleus and their projection areas.

Long-term administration of some 5-HT_{1A} receptor agonists *(36–39)* or selective serotonin reuptake inhibitors (SSRIs) *(40–43)* decreases the responsiveness of somatodendritic (but not hippocampus) 5-HT_{1A} receptors, resulting in a tonic activation of forebrain 5-HT_{1A} receptors *(44)*. Yet, hypothalamic 5-HT_{1A} receptors are also desensitized by repeated treatment with direct and indirect agonists *(45)*. These observations are consistent with the demonstration that acute administration of a 5-HT_{1A} receptor agonist elicits an internalization of this receptor in the DR 5-HT but not hippocampal (non-5-HT) neurons *(46)*. This process might also be viewed as an indirect mechanism in the control of 5-HT neuronal activity.

Although the activity of 5-HT cells is tightly regulated, the basal release of 5-HT in the raphe nuclei does not appear to be under tonic control by 5-HT_{1A} receptors. Several experimental conditions have confirmed this contention. First, the administration of the selective 5-HT_{1A} receptor antagonist WAY-100635 *(23,47–50)* or the inactivation of 5-HT_{1A} receptors by EEDQ *(23)* fail to alter basal 5-HT release in raphe nuclei. Second, in the raphe nuclei, the tissue concentration of 5-HT *(51)*, the in vitro [^{3}H]5-HT release in slices *(52,53)*, the in vivo 5-HT release *(54)*, and the basal firing of 5-HT neurons in the DR *(55)* are not altered in 5-HT_{1A} (–/–) receptor (knockout) mice compared with the corresponding wild-type controls. However, a study reported an increase in the firing rate of 5-HT cells after the iv administration of WAY-100635 *(56)*. Overall, these observations suggest that the physiological role of the 5-HT_{1A} autoreceptor is to function as a sensor responding with a reduction of 5-HT cell firing and release to the excess of 5-HT produced by excessive excitatory inputs to 5-HT cells *(57)*.

2.3. 5-HT_{1A} Receptor Electrophysiology

Most electrophysiological studies on 5-HT_{1A} receptors have been conducted in the DR, the hippocampal CA region and the prefrontal cortex, three brain areas with a high density of 5-HT_{1A} receptors. 5-HT_{1A} receptors belong to the subfamily of GPCRs that commonly hyperpolarize neurons by increasing potassium conductance and/or reducing calcium conductance *(18,58)*.

2.3.1. 5-HT_{1A} Receptor-Mediated Currents

The 5-HT_{1A} receptors are coupled to potassium and calcium channels. Intracellular current-clamp recordings in slices containing the DR established that the 5-HT-mediated inhibition involved an increase in potassium conductance, which exhibits inward rectification *(18,58)*. This induced a membrane hyperpolarization leading to a decrease in action potential frequency. Similar responses to 5-HT_{1A} receptor activation have been reported in other neuronal types, such as hippocampal pyramidal cells *(16,59)* or 5-HT neurons of the caudal raphe nuclei *(60)*.

Studies using voltage-clamp recordings in DR neurons revealed the existence of at least three types of inwardly rectifying K^+ current at potentials more negative than the resting potential (−60 mV): I_Q, I_{IR}, $I_{5\text{-HT}}$. 5-HT-induced conductance ($I_{5\text{-HT}}$) is different from I_{IR} or I_Q in its range of voltage dependence and its sensivity to PTX, which is identical to that induced by baclofen on $GABA_B$ receptors *(58)*. In the hippocampus, the action of 5-HT on 5-HT_{1A} receptors increased the potassium conductance, which also showed inward rectification *(61)* and was blocked by pretreatment with PTX *(16)*. The recording of this current has sometimes been used to determine whether the action of a drug is involved 5-HT_{1A} receptor activation *(62,63)*.

In addition to its action on an inward rectifying current, a second K^+ conductance activated by $5HT_{1A}$ receptors has been reported on prepositus hypoglossi neurons *(64)*, where both I_{IR} and I_{OR} (an outwardly rectifying slowly activated K current) contribute to generating the current that underlies inhibitory postsynaptic potentials (IPSPs).

The 5-HT, acting on 5-HT_{1A} receptors, also inhibits a calcium current through a PTX-sensitive, G protein coupling to voltage-dependent calcium channels. This 5-HT-induced inhibition of high-voltage-activated Ca^{2+} channels has been described in various cell types, such as DR 5-HT neurons (N-current) *(65,66)*, hypothalamic neurons (N- and Q-type currents) *(67)*, dorsal root ganglion cells (N-type currents) *(68,69)*, and hypoglossal motoneurons (N-P-type currents) *(70)*.

The inhibition of calcium currents through a PTX-sensitive G protein coupled to voltage-dependent calcium channels has also been studied in 5-HT neurons of the caudal raphe nuclei, where 5-HT, acting on 5-HT_{1A} receptors, inhibited N- and P/Q-type channels. Under current-clamp conditions, Ca^{2+} channel toxins and 5-HT each caused a decrease in the spike afterhyperpolarization (AHP), which is the result of Ca^{2+}-activated K^+ channels, and enhanced repetitive firing in response to injected current. The reduction of AHP would be able to modulate the firing pattern and would serve to magnify the neuronal response to suprathreshold inputs *(71)*. Hence, 5-HT acting through 5-HT_{1A} receptors would hyperpolarize the neuronal membrane by increasing K^+ conductance and would reduce AHP by reducing Ca^{2+} currents. Both actions would contribute to the increase of the firing response to strong suprathreshold inputs while inhibiting weaker inputs *(71)*. However, in hippocampal CA1 neurons, 5-HT suppresses the slow Ca^{2+}-activated K^+-conductance I_{AHP} independently of an action on Ca^{2+} influx, probably by acting on non 5-HT_{1A} receptors *(61,72)*. Thus, unlike the hyperpolarizing action of 5-HT through other currents, that on I_{AHP} was found to be insensitive fo PTX *(59)*. This was also supported by the inability of 8-OH-DPAT to mimic the action of 5-HT on I_{AHP} *(61)*.

2.3.2. Modulation of Neuronal Activity by 5-HT$_{1A}$ Receptors: In Vivo and In Vitro Studies with 5-HT$_{1A}$ Agonists

In vitro intracellular recordings of dorsal raphe 5-HT neurons showed that exogenously applied 5-HT and 5-HT$_{1A}$ agonists hyperpolarized the cells and decreased the membrane input resistance *(18,21)*. Studies in the neocortex showed that 5-HT application induced, often in the same neuron, both a hyperpolarization and a depolarization *(73–75)*. These effects were mediated, respectively, by an action of 5-HT on 5-HT$_{1A}$ and 5-HT$_2$ receptors *(75)* and likely can be accounted for by the high coexpression of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in cortical neurons *(55)*. Likewise (Fig. 2), bath application of 5-HT hyperpolarized and decreased the membrane input resistance of hippocampal CA1 cells, an effect blocked by the selective 5-HT$_{1A}$ receptor antagonist WAY-100635 *(76)*.

Similar to in vitro studies, early in vivo reports using extracellular recordings showed that the local or systemic application of 5-HT and selective 5-HT$_{1A}$ receptor agonists such as 8-OH-DPAT, ipsapirone, and buspirone induced a marked inhibition of 5-HT cell firing *(21,31,77,78)*. These effects were blocked by 5-HT$_{1A}$ receptor antagonists *(20,76,79–81)* (Fig. 3). Recent studies in 5-HT$_{1A}$ knockout mice showed that loss of 5-HT$_{1A}$ autoreceptors leads to a loss of the inhibitory actions of 5-HT$_{1A}$ receptor agonists and SSRIs on 5-HT cells *(53,55–57)* (Fig. 4).

In cortical neurons, 5-HT application excited and inhibited different cortical neurons *(82,83)*, although the predominant effect of 5-HT was an inhibition of spontaneous firing *(82,84–86)*. Similar actions have been described in other neuronal types such as midbrain periaqueductal gray neurons *(87)* and hippocampal pyramidal neurons *(88–90)*.

A preferential sensitivity of presynaptic vs postsynaptic 5-HT$_{1A}$ receptors to the action of 5-HT$_{1A}$ agonists has been reported by several studies *(36,77,91)*. In general, 5-HT$_{1A}$ ligands tend to behave preferentially as full agonists at 5-HT$_{1A}$ autoreceptors while displaying partial agonist or antagonist properties at postsynaptic (hippocampal) 5-HT$_{1A}$ receptors. Also, sensitivity differences have been reported between 5-HT$_{1A}$ receptors in CA1 and CA3 hippocampal regions *(92)*. The reasons for these differences are not likely the result of the existence of variants of the receptor but, instead, to differences in coupling mechanisms and/or a higher receptor reserve in the raphe, as demonstrated by neurochemical and electrophysiological studies *(93,94)*.

2.3.3. Modulation of the Neuronal Activity by 5-HT$_{1A}$ Receptors: Effects of Endogenous 5-HT

2.3.3.1. Presynaptic 5-HT$_{1A}$ Autoreceptors

A very important mechanism of control of 5-HT neurons is self-inhibition through 5-HT$_{1A}$ receptors. Initial studies found that there is a poststimulus

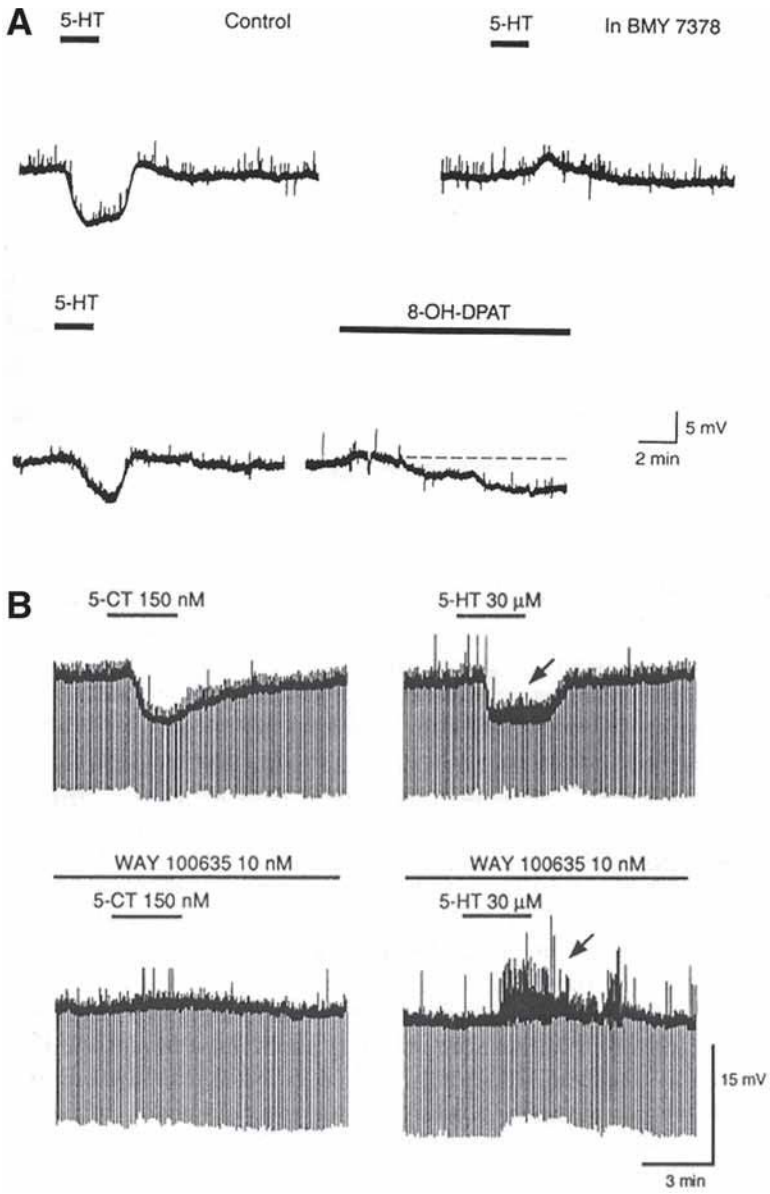

Fig. 2. Hyperpolarizing effects of 5-HT on the medial prefrontal cortex and hippocampal pyramidal neurons. **(A)** Bath application of 5-HT (30 µ*M*) elicited a hyperpolarizing response that was blocked by BMY 7378 (3 µ*M*). The lower trace shows that the selective 5-HT_{1A} agonist 8-OH-DPAT (30 µ*M*) induced a membrane hyperpolarization comparable to that elicited by 5-HT (30 µ*M*). Reproduced with permission from ref. *75*. **(B)** Bath application of 5-carboxyamidotryptamine (5-CT) and 5-HT produced a similar membrane hyperpolarization in CA1 hippocampal pyramidal cells. The lower panels show the inability of 5-HT and 5-CT to elicit the hyperpolarizing response in presence of the 5-HT_{1A} receptor antagonist WAY-100635. Reproduced with permission from ref. *76*.

period of inhibition in the activity of 5-HT neurons following antidromic activation mediated by 5-HT axon collaterals, suggesting the existence of a collateral inhibitory system *(95)*. This inhibitory system was reported in DR 5-HT neurons as a recurrent IPSP associated with an increase in membrane conductance *(96)*. The involvement of 5-HT in this IPSP (collateral inhibition) was suggested in experiments using focal electrical stimulation in rat dorsal raphe neurons *(97)*.

Subsequent studies using single-unit extracellular recordings have examined the effect of an increased activation of 5-HT_{1A} autoreceptors on 5-HT neuron activity. To this end, endogenous 5-HT concentrations have been increased both pharmacologically and physiologically. Hence, the local or systemic administration of compounds that block 5-HT reuptake (e.g., tricyclic antidepressants, SSRIs) or prevent its inactivation via monoamine oxidase (MAO) inhibitors suppress the activity of 5-HT neurons in the DR and MnR *(98–101)*. This effect is mediated by an increase in the extracellular concentration of 5-HT in these nuclei, as shown in microdialysis studies in the raphe area *(102–104)* and involves the activation of 5-HT_{1A} receptors, because it is reversed by 5-HT_{1A} receptor antagonists *(105)*. Other receptor subtypes do not appear to participate because the administration of the SSRI fluoxetine did not reduce 5-HT neuronal firing in 5-HT_{1A} knockout mice *(55)* (Fig. 4).

The activity of 5-HT neurons is controlled by neuronal afferents from several brain areas *(3)*, among which the prefrontal cortex plays an important role. Several recent anatomical and electrophysiological studies have helped to delineate the relationship between these two areas, which are involved in the pathophysiology and treatment of psychiatric diseases. Pyramidal neurons of the medial prefrontal cortex project to 5-HT and non-5-HT neurons in the DR *(106–112)*. The electrical stimulation of the infralimbic area in the medial prefrontal cortex results in a complex pattern of excitations and inhibitions in DR 5-HT neurons with a predominance of the latter responses *(57,108)*. Excitations are the result of the activation of direct prefrontal inputs onto DR 5-HT neurons and involve the activation of AMPA and NMDA receptors *(57)*, consistent with previous in vitro studies *(113)*. Inhibitory responses are partly mediated by prefrontal excitatory afferents onto DR GABAergic neurons, which, in turn, might synapse onto 5-HT neurons *(110,111)*. Consequently, inhibitory responses are blocked not only by the $GABA_A$ receptor antagonists *(57,110)* but also by the 5-HT_{1A} receptor antagonist WAY-100635, suggesting the additional involvement of 5-HT_{1A} receptors *(57)* (Fig. 3). Likewise, 5-HT synthesis inhibition with *p*CPA reduced the frequency of inhibitory responses elicited by prefrontal stimulation *(57)*, which is consistent with early studies suggesting the existence of a recurrent collateral inhibition on 5-HT neurons *(95)*.

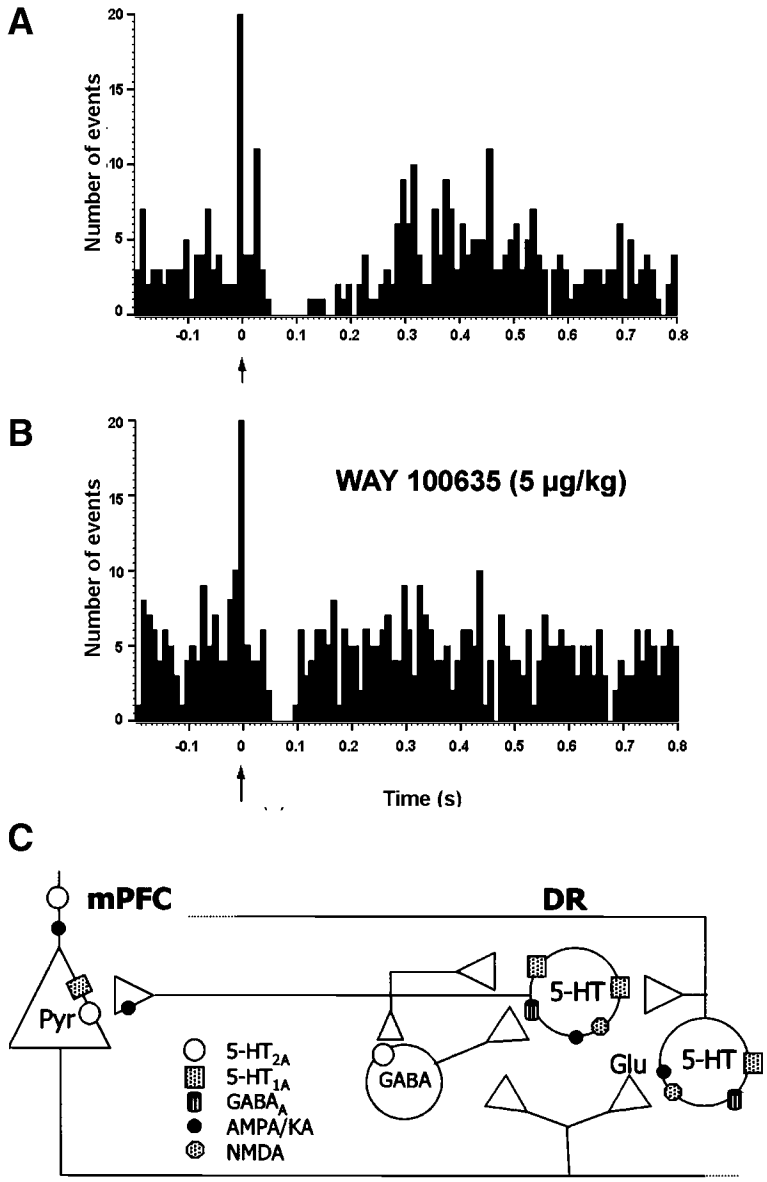

Fig. 3. Effect of the systemic administration of the 5-HT_{1A} receptor antagonist, WAY-100635 on the poststimulus inhibition of DR 5-HT neurons in response to the electrical stimulation of the medial prefrontal cortex. **(A)** Peristimulus time histogram showing the inhibitory response of a DR 5-HT neuron produced by prefrontal stimulation (arrow). **(B)** WAY-100635 (5 µg/kg iv) partially blocked the inhibition, suggesting that it was partly produced by 5-HT acting on 5-HT_{1A} autoreceptors. **(C)** Schematic representation of the putative relationships between medial prefrontal cortex projection neurons and DR 5-HT neurons. Descending excitatory afferents from the medial prefrontal cortex control the activity of 5-HT neurons directly, via NMDA and AMPA-KA receptors, and

2.3.3.2. Postsynaptic 5-HT$_{1A}$ Receptors

The effect of endogenous 5-HT on postsynaptic 5-HT$_{1A}$ receptors has been typically examined in two forebrain areas, the CA region of the hippocampal formation and the medial prefrontal cortex, which contain a high density of 5-HT$_{1A}$ receptors expressed mainly by pyramidal neurons *(5,6,114)*. The electrical stimulation of the medial forebrain bundle inhibits hippocampal pyramidal neurons (Fig. 5), an effect reversed by nonselective (BMY 7378, spiperone) and selective (WAY-100635) antagonists of 5-HT$_{1A}$ receptors *(89, 115,116)*. Based on the differential response of presynaptic and postsynaptic 5-HT$_{1A}$ receptors to spiperone and to cholera and pertussis toxin (which inactivate G_s and $G_{i/o}$ proteins, respectively), the existence of two different types of postsynaptic hippocampal 5-HT$_{1A}$ receptor (intrasynaptic and extrasynaptic) was suggested *(88)*.

Interestingly, chronic, but not acute, treatment with different types of antidepressant drug, including SSRIs, increased the tonic activation of 5-HT$_{1A}$ receptors by endogenous 5-HT in the hippocampal CA region, as assessed by the increase in pyramidal firing rate induced by the administration of the selective 5-HT$_{1A}$ receptor antagonist WAY-100635 *(44)* This supports the proposal that an increased serotonergic transmission through hippocampal 5-HT$_{1A}$ receptors might be involved in antidepressant drug action.

As previously observed with the microiontophoretic application of 5-HT (*see* above), the electrical stimulation of DR/MnR at a physiological rate (approx 1 spike/s) mainly evoked inhibitory responses in prefrontal cells in vivo, which were partly or totally blocked by the selective 5-HT$_{1A}$ antagonist WAY-100635 *(117,118)* (Fig. 5). In pyramidal neurons of the medial prefrontal cortex, identified by antidromic stimulation from the midbrain, the DR/MnR stimulation evoked inhibitory responses in two-thirds of the cases. The rest of the responses were orthodromic excitations, either pure (13%) or preceded by short-latency inhibitions (20%) *(118)*. Excitatory responses were blocked by the selective 5-HT$_{2A}$ receptor antagonist M100907 *(55,119)*. Intriguingly, the proportion of excitatory responses was markedly lower than that of inhibitory responses despite the approx 80% coexpression of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor mRNAs

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Fig. 3. *(continued)* indirectly, via activation of local inhibitory (5-HT$_{1A}$ and GABA$_A$) receptors. The stimulus-induced excitation of neurons receiving a direct input from the medial prefrontal cortex (either 5-HT or GABAergic) releases 5-HT or GABA, which inhibit other 5-HT neurons via 5-HT$_{1A}$ or GABA$_A$ receptors. The involvement of 5-HT$_{1A}$ receptors in the medial prefrontal cortex-induced inhibitions of 5-HT neurons is supported by the decrease in the proportion of inhibitions in rats depleted of 5-HT and by the reversal of the inhibitions induced by 5-HT$_{1A}$ receptor blockade with WAY-100635. Reproduced with permission from ref. *57*.

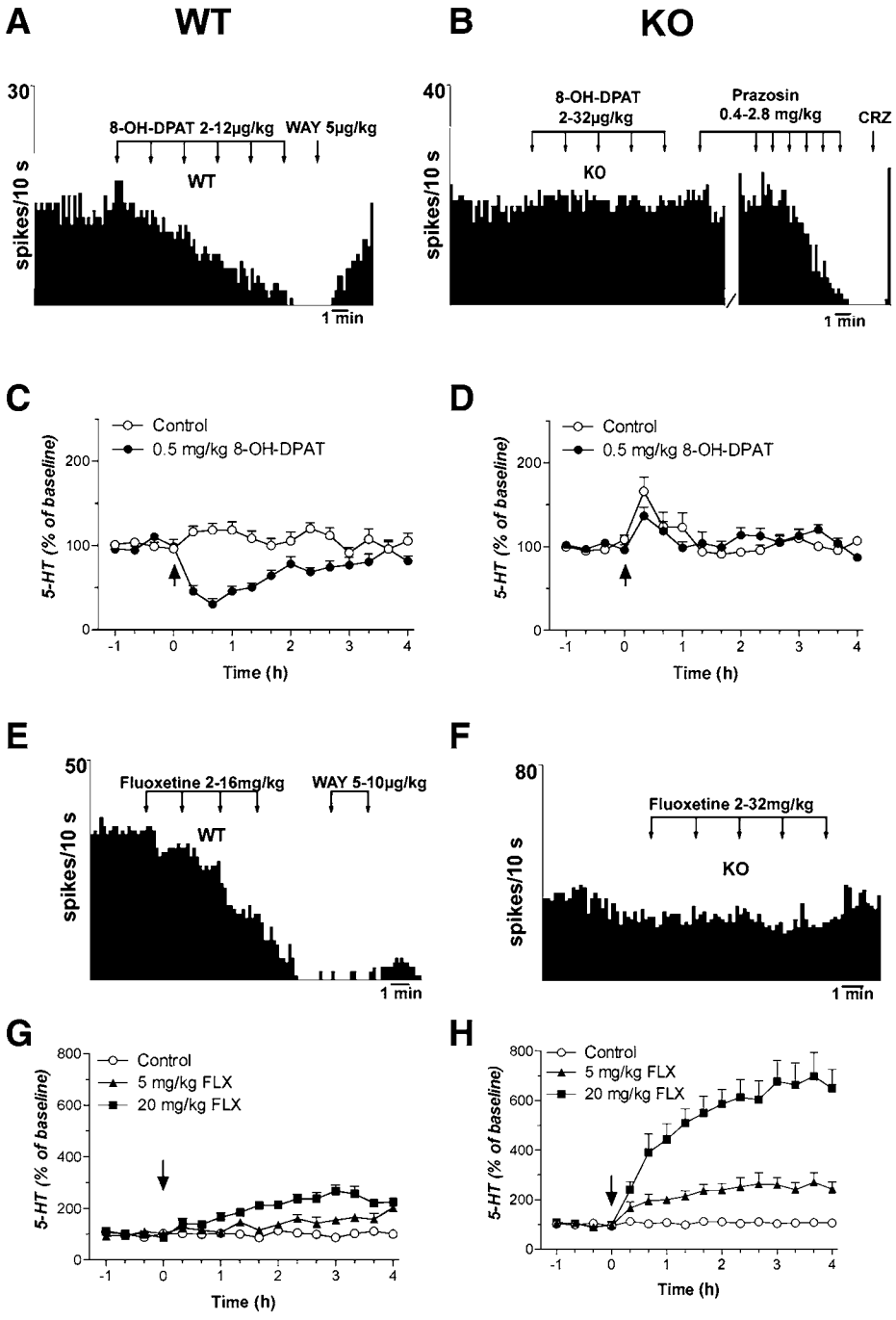
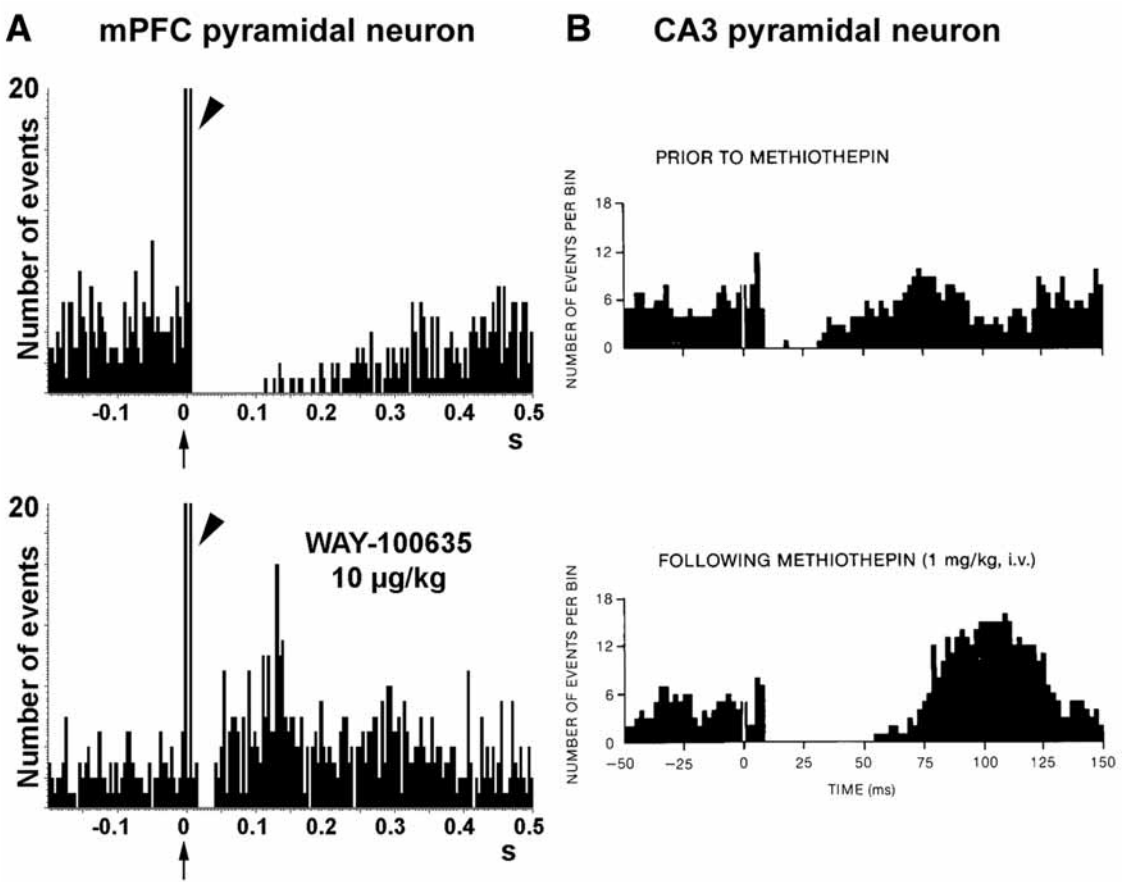


Fig. 4. 5-HT neuron activity and 5-HT release in wild-type (WT) and 5-HT_{1A} knockout (KO) mice in response to the systemic administration of the 5-HT_{1A} agonist 8-OH-DPAT and the SSRI fluoxetine. **(A,C)** 8-OH-DPAT inhibited the firing rate of

in prefrontal neurons *(55)*. Several observations could account for such a mismatch between receptor expression and functional responses. On the one hand, 5-HT_{2A} and 5-HT_{1A} receptors have been reported to be present in different compartments of pyramidal neurons. 5-HT_{2A} receptors have been consistently found in the cell body and apical dendrite *(120–122)* and the application of 5-HT near the apical dendrite elicited 5-HT_{2A} receptor-mediated excitatory postsynaptic currents (EPSCs) *(123)* (*see* Chapter 12). On the other hand, 5-HT_{1A} receptors have been reported to be localized in the axon hillock of cortical pyramidal neurons *(11–13,124)*. The latter localization matches with that of $GABA_A$ receptors activated by endogenous GABA released by chandelier interneurons, which exert a profound inhibitory action on pyramidal cells *(11)*. Such a respective localization of 5-HT_{1A} and 5-HT_{2A} receptors (although that of 5-HT_{1A} receptors is controversial; *see* refs. *5,9,* and *10)* might explain the predominant inhibitory action of 5-HT on cortical neurons.

In addition, inhibitory responses might be produced by GABA. In support of this possibility, inhibitions evoked by DR/MnR stimulation were partly blocked by WAY-100635 and by the $GABA_A$ antagonist picrotoxinin *(118)*. Moreover, the latency of inhibitory responses in peristimulus time histograms was shorter (9 ms on average) than the time required for action potentials to travel along 5-HT axons (orthodromic potentials; approx 25 ms) or pyramidal axons (antidromic potentials; approx 15 ms) *(118)* (Fig. 6). These observations are consistent with the existence of a GABAergic projection from the midbrain raphe to the medial prefrontal cortex (mPFC), suggested by anatomical studies *(125)*. This pathway would be analogous to the ascending GABAergic pathway between the ventral tegmental area and the mPFC *(126,127)*.

Fig. 4. *(continued)* DR 5-HT neurons and decreased the 5-HT release in the medial prefrontal cortex of WT mice but not in 5-HT_{1A} KO mice **(B**,**D)**. The iv administration of the 5-HT_{1A} antagonist WAY-100635 fully reversed the inhibition of 5-HT neuronal activity induced by 8-OH-DPAT **(A)**. **(B)** The sensitivity of 5-HT neurons in 5-HT_{1A} KO mice to other inputs is shown by the suppression of the firing rate following the administration of the α_1-adrenoceptor antagonist prazosin. The subsequent iv administration of the α_1-adrenoceptor agonist cirazoline (CRZ) could reverse the prazosin-induced suppression of cell firing. **(E**,**G)** Fluoxetine inhibited the firing rate of 5-HT neurons **(E)** while increasing 5-HT release in the DR **(G)** in WT mice. **(F**,**H)** In 5-HT_{1A} KO mice, fluoxetine did not modify 5-HT neuronal firing rate and increased extracellular 5-HT more than in WT mice. This observation is consistent with previous evidence in rats indicating that the 5-HT release in the DR is controlled by 5-HT_{1A} receptors. The suppressing effect of fluoxetine in WT mice **(E)** was antagonized by subsequent iv administration of the 5-HT_{1A} receptor antagonist WAY-100635. Reproduced with permission from ref. *55*.


A
mPFC pyramidal neuron
B
CA3 pyramidal neuron
20
Number of events
-0.1
0
0.1
0.2
0.3
0.4
0.5
s
WAY-100635
10 µg/kg
PRIOR TO METHIOTHEPIN
NUMBER OF EVENTS PER BIN
18
12
6
0
FOLLOWING METHIOTHEPIN (1 mg/kg, i.v.)
−50
−25
0
25
50
75
100
125
150
TIME (ms)

In summary, both possibilities (differential localization of 5-HT_{1A} and 5-HT_{2A} receptors and existence of a raphe–prefrontal pathway) are not mutually exclusive and both might account for the predominantly inhibitory responses elicited by raphe stimulation on pyramidal neurons of the medial prefrontal cortex.

3. 5-$HT_{1B/1D}$ Receptors

For several years, the study of the role of 5-HT_{1B} and 5-HT_{1D} receptors in serotonergic transmission has been hampered by the lack of selective compounds able to discriminate them pharmacologically. Yet, this situation has changed recently because a few selective 5-HT_{1B} receptor agonists such as CP-93,129 *(128–130)* and CP-94,253 *(131)* and antagonists such as SB-224289 *(132,133)* and NAS-181 *(134,135)* have been developed. In addition, L-775,606 *(136)* and BRL 15572 *(137)* have been claimed to be a 5-HT receptor agonist and antagonist, respectively, with a high affinity and a good selectivity for the 5-HT_{1D} subtype.

3.1. Regional Distribution, Cellular Localization and Coupling Mechanisms

Radioligand-binding and autoradiographic studies have detected the presence of 5-HT_{1B} receptors in many areas of the brain *(138,139)*. The 5-HT_{1B} receptor was initially claimed to exist only in rodents, but subsequent cloning and sequencing studies demonstrated that it is, in fact, the homologous species of the human 5-$HT1_{D\beta}$ receptor *(140,141)*. Contrary to 5-HT_{1A} receptors, the presence of 5-HT_{1B} and 5-HT_{1D} receptors in the raphe nuclei has not been unequivocally demonstrated in histochemical studies. Initial autoradiographic studies described a noticeable amount of putative 5-HT_{1B} receptors in the raphe nuclei of the rat *(138,142)*. However, the results of these studies were tentative because of the lack of selective ligands and the non-5-HT_{1A} binding detected

Fig. 5. *(previous page)* Peristimulus time histograms showing the inhibitory response of a medial prefrontal cortex (mPFC) pyramidal neuron **(A)** and a CA3 hippocampal pyramidal neuron **(B)** to the electrical stimulation of ascending 5-HT pathways. **(A)** mPFC pyramidal neuron inhibited by DR stimulation (arrow). The lower panel shows the blockade of the inhibition by the iv administration of the 5-HT_{1A} receptor antagonist WAY-100635. **(B)** CA3 pyramidal neuron inhibited by the electrical stimulation of the ventromedial tegmentum (stimulus delivered at time = 0). The lower panel shows the increase in the duration of the suppression of firing activity after the intravenous administration of the terminal receptor antagonist methiothepin. The increase in the duration of the inhibitory response was taken as an evidence that 5-HT inhibits its own release in the hippocampus through the activation of 5-HT_{1B} autoreceptors. Reproduced with permission from refs. *55* and *89*.

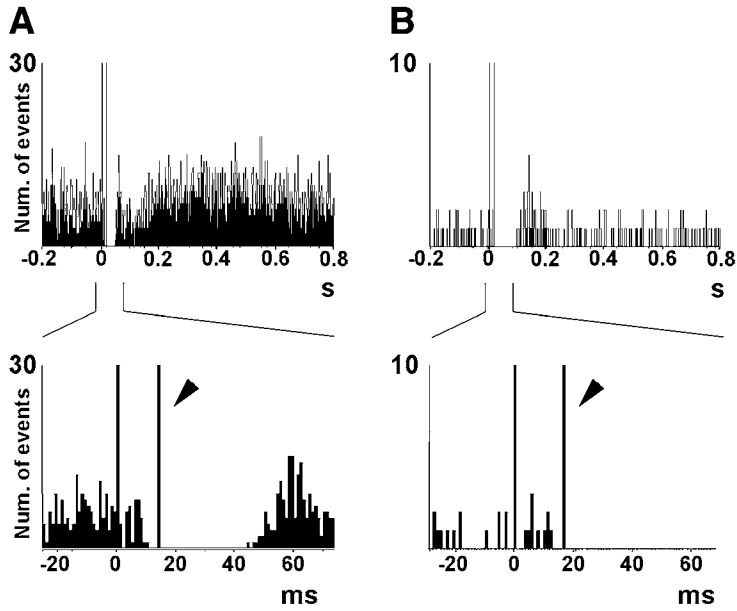


Fig. 6. (A,B) Peristimulus time histograms of mPFC pyramidal neurons showing inhibitory responses elicited by the electrical stimulation of the DR. The lower panels show an enlargement of the time period around the stimulus. **(A)** The stimulation of the DR at 1 mA evoked a short-latency, short-duration inhibition in this pyramidal neuron (32 ms; 8% of prestimulus firing rate). The latency of the inhibition was 11 ms, whereas the antidromic spike (arrowhead) had a latency of 15 ms. Peristimulus time histogram made of 718 triggers (approx 13 min recording). **(B)** Short-latency inhibition evoked in medial prefrontal cortex by DR stimulation at 1 mA (duration, 78 ms; 3% of prestimulus firing rate). The latency of the inhibition was 12 ms and that of the antidromic spike was 15 ms. Peristumulus time histogram made of 617 triggers (approx 11 min). Note the onset of the inhibitions (absence of orthodromic spikes) in both examples, which precludes that the these inhibitory responses might be triggered by antidromic invasion of pyramidal collaterals and further activation of local GABA interneurons. Bin size is 1 ms. Reproduced with permission from ref. *118*.

5-$HT_{1B/1D}$, and also 5-HT_{1F}, 5-HT_7, and possibly 5-HT_{1E} receptors. *In situ* hybridization studies have reported an abundant expression of 5-HT_{1B} and 5-HT_{1D} receptors in the raphe nuclei *(143–146)*, thus indicating that the receptor proteins are synthesized in 5-HT cells. More recent immunocytochemical studies have failed to visualize 5-HT_{1B} receptor protein in the DR of the rat *(147–149)*. Presynaptic 5-HT_{1B} autoreceptors, however, represent a small proportion of the entire population of 5-HT_{1B} receptors in the brain because the lesion of 5-HT neurons does not generally result in a reduction of their

density *(150,151)*. Therefore, it is possible that a small concentration of 5-HT_{1B} receptors is present in the raphe nuclei that cannot be detected by current immunocytochemical techniques. The question remains as to the possible cellular localization of 5-HT_{1B} and 5-HT_{1D} receptors in the raphe area. The 5-HT_{1B} receptor has been described chiefly in axonal varicosities and terminals but not in the soma or dendrites of 5-HT neurons *(147,149)*. Furthermore, the 5-HT_{1B} receptor protein has been shown to migrate along the axon of hippocampal neurons in primary cultures *(152)*. On the other hand, it has been suggested that 5-HT_{1D} receptors could be directed to dendrites instead of axon terminals *(153,154)*. More research is needed, however, to verify this possibility. As mentioned earlier, varicosities and/or terminals have been observed in the DR *(155)*. Therefore, it is conceivable that the 5-HT_{1B} receptors are localized in those structures regardless of their origin within recurrent axons or 5-HT fibers coming from the same or other raphe nuclei.

The 5-HT_{1B} receptors are predominantly localized at the presynaptic level, they are coupled negatively to adenylate cyclase, and their activation by selective agonists induces a decrease in the forskolin-stimulated adenylate cyclase *(156)*; for review, *see* ref. *157*. Consequently, their activation reduces neurotransmitter release (*see* below).

3.2. Assessment of 5-$HT_{1B/1D}$ Receptor Function

There is anatomical and pharmacological evidence that 5-HT_{1B} receptors have an axonal localization in different cerebral pathways and inhibit neurotransmitter release *(10,149,158,159)*. Therefore, most electrophysiological evidence of the role of 5-HT_{1B} receptors in neuronal function is based on the assessment of inhibitory actions on evoked synaptic potentials or currents in target neurons, whereas neurochemical studies have examined direct effects on neurotransmitter release.

3.2.1. 5-$HT_{1B/1D}$ Receptors: Control of the Activity of 5-HT Neurons and Midbrain 5-HT Release

The role of 5-HT_{1B} receptors in the control of 5-HT cell firing is controversial. Initial studies showed that intravenous or local administration of 5-HT_{1B} receptor agonists displayed only weak or irregular actions on the spontaneous 5-HT neuronal firing in the dorsal raphe nucleus *(21,160)* as well as on electrically evoked 5-HT release *(161)*. Using in vivo extracellular recordings in different species, it has been reported that 5-$HT_{1B/D}$ receptor agonists enhanced 5-HT cell firing in the dorsal *(162,163)* and median raphe nucleus *(19,164)*. In studies using guinea pig midbrain slices containing the DR, the inhibitory effect of exogenous 5-HT on neuronal firing was abolished by WAY-100635, but not blocked by GR 127935, a 5-$HT_{1B/1D}$ receptor antagonists *(81)*.

Whole-cell recordings of synaptic potentials from DR neurons in wild-type and 5-HT_{1B} receptor knockout mice showed that presynaptic 5-HT_{1B} receptors mediate an inhibition of 5-HT_{1A} receptor-dependent IPSPs evoked by electrical stimulation, an effect absent in knockout mice *(165)*. Moreover, it has been reported that the firing of 5-HT neurons in the DR is under an excitatory influence mediated by 5-HT_{1B} receptors. However, because the spontaneous firing of DR 5-HT neurons in these knockout mice is identical to that in wild-type controls, it appears that 5-HT cell firing is not tonically controlled by 5-HT_{1B} receptors *(162)*. This is in agreement with the absence of effect of 5-HT_{1B} antagonists on 5-HT cell firing in mice and guinea pig *(160,162)*.

Notwithstanding the above inconclusive histochemical studies, evidence exists that 5-HT_{1B} and 5-HT_{1D} receptors modulating local 5-HT release are present in the raphe nuclei. Thus, local application of 5-HT_{1B} receptor agonists decrease the release of 5-HT in DR and MnR and this effect is prevented by 5-HT_{1B} receptor antagonists *(48,163,164,166,167)*. Yet, 5-HT_{1B} receptor antagonists alone do not affect basal 5-HT release *(50,164)*. This is additional evidence suggesting that endogenous 5-HT does not exert a tonic action upon 5-HT_{1B} autoreceptors in DR and MnR. It thus seems that 5-HT_{1B} autoreceptors in the raphe nuclei are only activated when an excess of 5-HT is present in the extracellular space, similarly to 5-HT_{1A} autoreceptors (*see* above). Two studies examined the existence of differences in the 5-HT_{1B} receptor-mediated regulation of 5-HT release between DR and MnR *(164,167)*. Hence, the reduction of 5-HT release evoked by CP-93,129 is more pronounced in the MnR compared to the DR *(164)*, which might suggest a different efficacy and/or density of 5-HT_{1B} receptors in each nucleus. Alternatively, assuming that 5-HT_{1B} receptors are located in axon terminals or varicosities, it might also be that the proportion of terminal-like 5-HT release in the MnR is higher than that in the DR. Other studies have claimed that 5-HT release in the DR is also under the control of 5-HT_{1D} receptors *(161,163,166,168,169)*. However, caution must be taken because of the lack of studies with selective 5-HT_{1D} receptor agents. In this regard, the allegedly selective 5-HT_{1D} receptor antagonist BRL 15572 has been shown to increase the electrically stimulated in vitro 5-HT release in the DR and MnR *(50,167)*. These findings seem to suggest that under experimental conditions of elevated extracellular 5-HT, the transmitter could activate tonically the 5-HT_{1D} receptor, although it has to be taken into consideration that BRL 15572 can act through other mechanisms rather than via 5-HT_{1D} receptors *(167)*. Taken together, all of the above-described findings seem to indicate that 5-HT_{1B} receptors might regulate 5-HT release preferentially in the MnR, whereas the function of 5-HT_{1D} receptors could be more important in the DR.

In addition to the 5-HT_{1B} receptors confined within the boundaries of raphe nuclei, recent electrophysiological studies have suggested the existence of a

long feedback loop responsible for the 5-HT_{1B} receptor-mediated changes in DR serotonergic cell firing *(162)*. We extended this finding to the MnR and showed that the 5-HT release in this nucleus increases following the systemic administration of the 5-HT_{1B} receptor agonist CP-94,253, in parallel with changes in the serotonergic firing rate *(164)* (Fig. 7). However, in dual-probe microdialysis experiments we were unable to show changes of 5-HT release in the MnR following the perfusion of a high concentration (300 μ*M*) of the 5-HT_{1B} receptor agonist CP-93,129 in areas of the brain enriched in 5-HT_{1B} receptors. These findings did not fully support the view of a long feedback loop to the MnR involving 5-HT_{1B} receptors in nonserotonergic elements. However, we could not rule out the possibility that the amount of the compound that diffuses into these areas is not sufficient to activate local 5-HT_{1B} receptors regulating MnR 5-HT release or that 5-HT_{1B} receptors outside of these areas might control the release of 5-HT in this nucleus. Alternatively, we suggested that 5-HT_{1B} heteroreceptors might be located on inhibitory elements within the MnR *(164)*.

3.2.2. 5-$HT_{1B/1D}$ Autoreceptors: Control of 5-HT Release and Postsynaptic 5-HT Actions in the Forebrain

In addition to the above-mentioned effects in the cell body area, a large number of studies have shown that 5-HT_{1B} receptors located on 5-HT axons exert a negative feedback control on 5-HT release of serotonin *(166,170–173)*. The electrophysiological consequences of serotonergic terminal autoreceptor activation have been examined by measuring changes in the excitability of 5-HT terminal fields to the electrical stimulation in the striatum. Following systemic or local administration of an autoreceptor agonist (5-MeODMT), a decrease in the excitability of 5-HT terminal fields in the striatum was reported *(174)*. Consistent with this, other in vivo electrophysiological studies have shown that the intravenous administration of methiothepin, a terminal 5-HT receptor antagonist, enhances the inhibitory effect of afferent 5-HT pathway stimulation on the firing activity of hippocampal pyramidal neurons *(89,175)*. This effect seemed to be mediated presynaptically (i.e., by removal of the 5-HT_{1B} receptor-mediated local negative feedback, without modifying the responsiveness of the hippocampal pyramidal neurons to microiontophoretically applied 5-HT) *(175)* (Fig. 5). Likewise, when the effect of microiontophoretic application of putative serotonin 5-HT_{1B} receptor agonists was evaluated on the spontaneous firing rate of CA1 pyramidal cells, it was found that they produced only weak and inconsistent inhibition of spontaneous firing *(77)*.

3.2.3. 5-HT_{1B} Heteroreceptors: Control of Neurotransmitter Release and Postsynaptic Actions in the Forebrain

The 5-HT_{1B} receptor subtype has also been described as a heteroreceptor located on nonserotonergic terminals of the brain where it controls the release

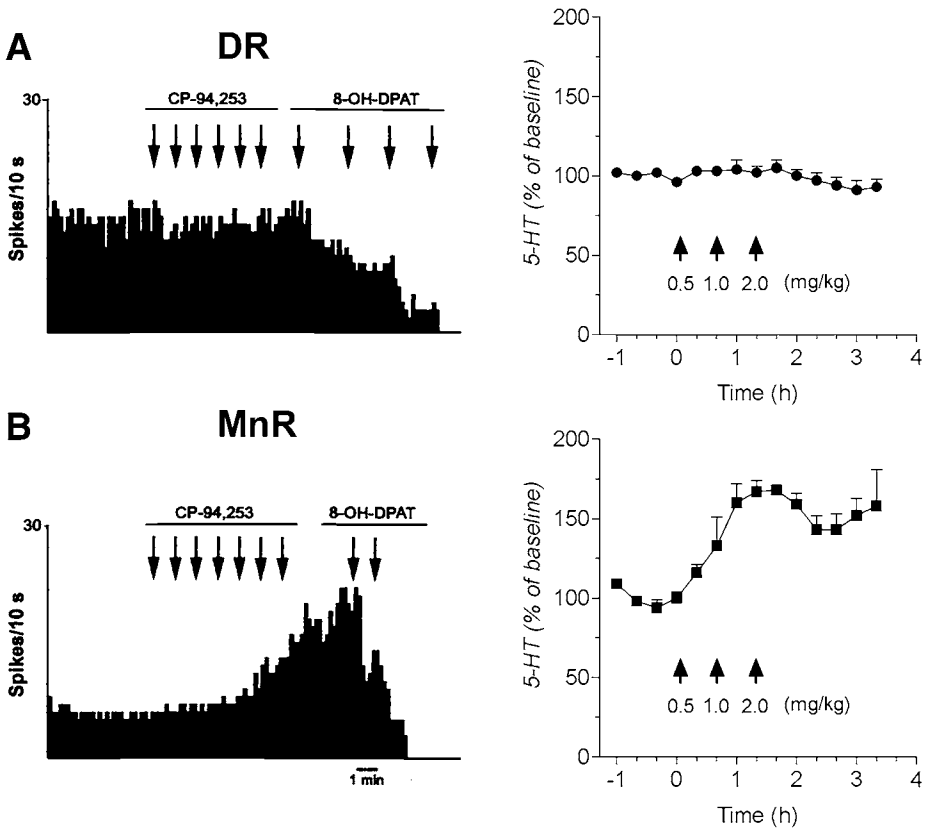


Fig. 7. Effect of the iv administration of the 5-HT_{1B} agonist CP-94,253 on the spontaneous firing rate of 5-HT neurons and 5-HT release in the DR and MnR nucleus. **(A)** The systemic administration of cumulative doses of CP-94,253 (0.05–1.6 mg/kg iv) did not change the firing activity of DR 5-HT neurons or the 5-HT release (right-hand panel). **(B)** In contrast, CP-94,253 increased the 5-HT firing activity and 5-HT release in MnR at the same doses. The administration of CP-94,253 in single-unit recording experiments (left-hand panels) is shown by arrows, which correspond to the following doses: 0.05, 0.05, 0.1,0.2, 0.4, and 0.8 mg/kg iv. An additional dose of 1.6 mg/kg CP-94,253 was injected when recording 5-HT neurons in the MnR. Both 5-HT neurons were inhibited by 8-OH-DPAT (up to 4 µg/kg iv). Data of 5-HT release in DR and MnR are from four and five rats, respectively. Redrawn from data in ref. *164*.

of other neurotransmitters. As for its terminal autoreceptor location, functional responses associated with its activation are usually the inhibition of evoked synaptic potentials or currents on target neurons.

3.2.3.1. Control of Acetylcholine Release

Presynaptic release-modulating 5-HT_{1B} receptors are found in cholinergic nerve endings in the hippocampus, where the K^+-evoked release of acetylcholine was inhibited by 5-HT and the 5-HT_{1B} receptor agonist RU 24969, but not by the 5-HT_{1A} receptor agonist 8-OH-DPAT *(176,177)*. The same group also characterized a terminal 5-HT autoreceptor in this brain area *(178)*. For a review of serotonergic modulation of cholinergic function, *see* ref. *179*.

3.2.3.2. Control of Glutamate Release

The control of glutamate release by 5-HT_{1B} receptors has been described in different brain areas. In caudal raphe neurons, presynaptic 5-HT_{1B} receptors inhibit glutamatergic synaptic inputs, as assessed by recording EPSCs *(180)*. Also, in slices of the cingulate cortex, 5-HT, acting on 5-HT_{1B} receptors, reduced the amplitude of NMDA and non-NMDA components of synaptic potentials recorded intracelluarly in layer V pyramidal neurons *(74)* (Fig. 8). This phenomenon was also observed in the locus coeruleus *(181)* and in the subiculum from CA1 pyramidal afferents *(182)*. Likewise, activation of 5-HT_{1B} receptors in the dorsal subiculum suppresses subicular transmission at low frequencies *(183)*. More recent work in the hippocampus using whole-cell voltage-clamp recordings in CA1 pyramidal neurons revealed that 5-HT reduces synaptic transmission by acting at 5-HT_{1B} receptors on local collaterals of CA1 pyramidal *(184)*. The same group also reported, using patch-clamp recordings on disinhibited rat CA1 minislices, that 5-HT_{1B} activation might represent a predominant component of the physiological response to endogenous 5-HT in the CA1 area *(185)*.

Finally, whole-cell recordings of membrane potential from medium spiny neurons in nucleus accumbens of wild-type and 5-HT_{1B} receptor knockout mice show that 5-HT_{1B} receptor agonists inhibited glutamate-induced EPSC in wild-type, but not knockout, mice *(165)*.

3.2.3.3. Control of Dopamine Release

In vitro studies showed that 5-HT_{1B} receptor agonists inhibited the K^+-evoked release of [^{3}H]dopamine from striatal synaptosomes *(186)*, an effect absent in synaptosomes obtained from 5-HT_{1B} receptor knockout mice *(187)*. In agreement, early in vivo microdialysis studies suggested the involvement of 5-HT_{1B} receptors in the control of striatal dopamine release *(188)*. However, more recent data from 5-HT_{1B} knockout mice do not support this contention *(189)*. To the best of our knowledge, data from evoked synaptic potential or dopamine-induced currents in target neurons have not been reported.

3.2.3.4. Control of GABA Release

The 5-HT_{1B} receptor control of GABA release has been suggested by electrophysiological studies in the substantia nigra, where 5-HT_{1B} receptor agonists

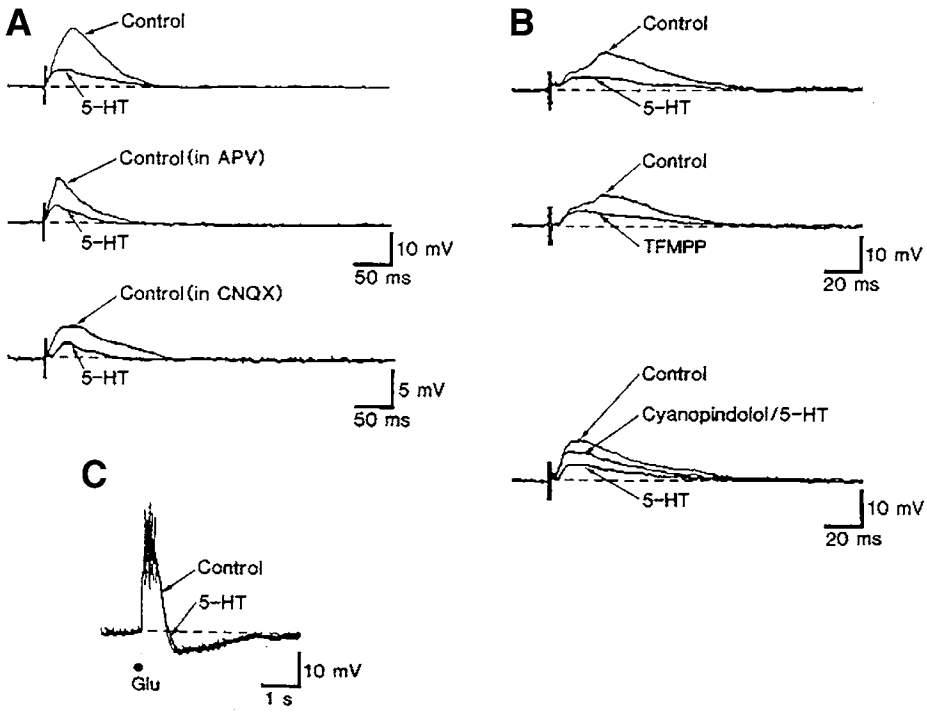


Fig. 8. The stimulation of 5-HT_{1B} receptors inhibit excitatory synaptic transmission in the cingulate cortex. **(A)** The stimulation of subcortical white matter elicited excitatory postsynaptic synaptic potentials (EPSP) in pyramidal neurons of the rat cingulated cortex that were dependent on NMDA and non-NMDA currents. The action of 5-HT partially depressed EPSPs. **(B)** The reduction of EPSPs by 5-HT was mimicked by the nonselective 5-HT_{1B} agonist TFMPP and the depressing action of 5-HT was antagonized by the 5-HT_{1B} antagonist cyanopindolol. **(C)** The above effects appear to be mediated by a presynaptic action of 5-HT because the depolarizing effect of glutamate was unaffected by 5-HT application. (Reproduced with permission from ref *74*.)

block $GABA_B$ synaptic potentials in rat dopamine neurons *(190)*. This observation has been confirmed using 5-HT_{1B} receptor knockout mice *(165)*. Likewise, using whole-cell patch-clamp recordings, it was reported that 5-HT also acts on 5-HT_{1B} receptors to depress the evoked GABA-mediated synaptic input to substantia nigra pars reticulata neurons *(191)*. In addition, the release of GABA in slices of the globus pallidus is inhibited by a 5-HT_{1B} agonist *(192)*. Also, the action of cocaine on the evoked $GABA_B$ synaptic potentials recorded in dopamine neurons of the ventral tegmental area of guinea pig is mediated by the 5-HT_{1D} receptor *(193)*.

4. Therapeutic Implications

The 5-HT_{1A} autoreceptors play an important role in the therapeutic delay observed during the treatment with antidepressant drugs. These markedly increase the extracellular 5-HT concentration in the DR and MnR *(102,194)*, which activates 5-HT_{1A} receptors and reduces the firing rate of serotonergic neurons *(40)* and axonal release in various forebrain areas *(102,195)*. The blockade of 5-HT_{1A} receptors with selective (e.g., WAY-100635) or nonselective (pindolol) agents has been shown to prevent the 5-HT_{1A} autoreceptor-mediated negative feedback and to potentiate the effects of SSRIs in experimental and clinical studies (*see* refs. *196* and *197* for review). More recent studies using 5-HT_{1A} knockout mice are fully consistent with this previous evidence. The firing activity of 5-HT cells is not suppressed by 5-HT_{1A} agonists or SSRIs in these mice and SSRIs increase extracellular 5-HT to a larger extent than in their wild-type counterparts *(53–55,198)*.

Likewise, postsynaptic 5-HT_{1A} receptors in the hippocampus have been suggested as potential targets for anxiolytic and antidepresant drugs *(199)*. However, the failure of several clinical trials using highly selective agents like ipsapirone or flesinoxan raises doubts about the use of direct 5-HT_{1A} agonists for the treatment of affective disorders.

Some atypical antipsychotic drugs, such as ziprasidone or aripiprazole, display affinity for 5-HT_{1A} receptors in the low nanomolar range. Other atypical drugs, lacking significant in vitro affinity for 5-HT_{1A} receptors, increase dopamine release in the mPFC by a 5-HT_{1A} receptor-dependent mechanism *(200)* and clozapine occupies 5-HT_{1A} receptors in vivo at therapeutic doses *(201)*. These observations might possibly be related to the efficacy of atypical antipsychotic drugs in the treatment of affective/negative and cognitive symptoms in schizophrenic patients and suggest that 5-HT_{1A} receptors might be a new potential target in antipsychotic drug development *(202)*.

On the other hand, 5-HT_{1A} receptor antagonists have been shown to attenuate or reverse the cognitive deficits induced by the cholinergic blocker scopolamine, which might suggest a new therapeutic activity for this drug class *(203,204)*.

Consistent with its role as autoreceptor and as previously observed for 5-HT_{1A} receptors, 5-HT_{1B} receptors also limit the increase in extracellular 5-HT produced by antidepressant treatments. Hence, several reports have shown that the addition of 5-HT_{1B} receptor antagonists potentiates the increase in extracellular 5-HT induced by SSRI administration *(205,206)*. Thus, blockade of 5-HT_{1B} autoreceptors might also be a novel therapeutic strategy to augment the clinical effects and possibly to accelerate the onset of action of antidepressant drugs.

On the other hand, the presence of abundant 5-HT_{1B} receptors in the basal ganglia and their role in modulating the activity of output GABAergic neurons in the substantia nigra pars reticulata *(191)* suggests a possible role of 5-HT_{1B}

ligands in the treatment of motor disorders, although this possibility has not been examined as yet.

Serotonin plays an important role in the pathogenesis of migraine, and 5-HT_{1B} receptors have been considered the preferred targets of antimigraine agents. Sumatriptan, a 5-HT_{1B} receptor agonist, inhibits the release of calcitonin-gene-related peptide (CGRP), which acts in the superior sagittal sinus following stimulation of the trigeminal ganglion *(207)*. In an in vivo cat model of trigeminovascular stimulation, it has been described that stimulation of the trigeminovascular system excites neurons in the nucleus tractus solitarius (NTS), which is involved in regulating vomiting. This excitatory trigeminovascular input is inhibited by iontophretic and intravenous administration of 5-$HT_{1B/1D}$ receptor agonists eletriptan and naratriptan, suggesting that the alleviation of the associated symptoms of nausea and vomiting by antimigraine compounds might be via an action at 5-$HT_{1B/1D}$ receptor in the NTS *(208)*.

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12

Electrophysiology of 5-HT_{2A} Receptors and Relevance for Hallucinogen and Atypical Antipsychotic Drug Actions

Evelyn K. Lambe and George K. Aghajanian

Summary

The feature that distinguishes psychedelic agents from some other classes of psychotomimetic drug is their capacity reliably to induce states of altered perception, thought, and feeling without producing marked disorientation or delirium. There is an accumulation of evidence that the 5-hydroxytryptamine$_{2A}$ (5-HT_{2A}) subtype of serotonin (5-HT) receptor mediates the actions of psychedelic hallucinogens. The majority of 5-HT_{2A} receptors are found in the cerebral cortex. Here, we explore the electrophysiology of 5-HT_{2A} receptors in the prefrontal cortex and their relevance for normal brain function and for hallucinogen and atypical antipsychotic drug actions.

Key Words: LSD; serotonin; 5-hydorxytryptamine (5-HT); glutamate; prefrontal cortex; brain slice; rat.

1. Introduction

Psychedelic ("mind manifesting") hallucinogens have profound effects on cognition, affect, and perception (*see* ref. *1*). The feature that distinguishes psychedelic agents from some other classes of psychotomimetic drug is their capacity reliably to induce states of altered perception, thought, and feeling without producing marked disorientation or delirium. Typically, the clinical effects of psychedelic hallucinogens (e.g., lysergic acid diethylamide [LSD] and mescaline) can be described in the following three categories: (1) perceptual symptoms—altered

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shapes and colors, difficulty in focusing on objects, sharpened sense of hearing, and synesthesias or the crossing of senses; (2) psychic symptoms—alterations in mood, distorted sense of time, difficulty in expressing thoughts, depersonalization, dreamlike feelings, and visual hallucinations; (3) somatic symptoms—dizziness, parathesias, and blurred vision (*1*). Remarkably, despite the great diversity of these symptoms, recent evidence shows that most, if not all, are mediated primarily through the action of hallucinogens at a *single receptor*: one subtype of serotonin 5-hydroxytrytamine [5-HT] receptor *(1)*.

2. Hallucinogens, 5-HT_{2A} Receptors, and Antipsychotic Drugs

There is now a convergence of basic and clinical evidence that the 5-HT_{2A} subtype of the 5-HT receptor mediates the actions of psychedelic hallucinogens. Originally, this idea was based on the demonstration of a high correlation between hallucinogenic potency in humans and the affinity of both phenethylamine (e.g., mescaline and DOI) and indoleamine (e.g., LSD and psilocybin) hallucinogens for 5-HT_2 receptors *(2)*. Indeed, among all known 5-HT receptor subtypes, only an affinity for 5-HT_2 receptors is shared by both the indoleamine and phenethylamine classes of hallucinogen. There is now abundant evidence from biochemical *(3)*, electrophysiological *(4)*, and animal behavior studies *(5)* that the effects of hallucinogens are mediated through a partial agonist action at 5-HT_2 receptors. In accord with preclinical studies, the psychotomimetic effect of the indoleamine hallucinogen psilocybin in human subjects have now been shown to be blocked completely by the preferential 5-HT_{2A} antagonist ketanserin and by a low dose of the high-affinity 5-HT_{2A} antagonist (and atypical antipsychotic) risperidone, but not by the typical antipsychotic haloperidol *(6)*.

Independently, interest in 5-HT_{2A} receptors arose from the finding that some antipsychotic drugs with atypical features such as clozapine interacted more potently with 5-HT_2 than with D_2 receptors. When antipsychotic drugs were classified as "typical" or "atypical," a high affinity for 5-HT_2 relative to D_2 receptors was proposed to be the defining characteristic of the atypical category *(7,8)*. An early clinical study suggested that a drug, setoperone, which has high affinity for 5-HT_2 relative to D_2 receptors in addition to having relatively low extrapyramidal side effects, might have enhanced efficacy in the treatment of negative symptoms (e.g., "emotional withdrawal" and "blunted affect") *(9)*. Interestingly, part of the rationale given in the latter study for testing 5-HT_2 antagonist drugs for possible antipsychotic properties was their ability to block various effects of LSD in animal model systems *(10)*. However, it has been suggested that a high occupancy of 5-HT_{2A} receptors alone is insufficient to achieve full antipsychotic efficacy *(11,12)*.

3. Localization of 5-HT$_{2A}$ Receptors in the Cerebral Cortex

Because 5-HT$_{2A}$ receptors have been implicated both in the action of psychedelic hallucinogens and atypical antipsychotics, there has been much interest in the location and function of these receptors in the central nervous system. Although autoradiographic studies show 5-HT$_{2A}$ receptor binding in a number of subcortical regions of the brain, the bulk of these receptors are found in the cerebral cortex *(13,14)*. Similarly, by *in situ* hybridization, the greatest expression of 5-HT$_{2A}$ receptor mRNA also has been found in the cerebral cortex *(15–17)*. Except for layer VI, the great majority of pyramidal cells in the prefrontal cortex express 5-HT$_{2A}$ receptor mRNA, consistent with the fact that hallucinogens disrupt many higher cortical functions such as perception, cognition, and affect. Immunocytochemical studies have demonstrated a particularly high density of 5-HT$_{2A}$ receptors within the apical dendrites of cortical pyramidal cells *(18–22)*, as shown in Fig. 1. Recent ultrastructural studies (using an alternative antibody) have shown 5-HT$_{2A}$ immunoreactivity postsynaptically in pyramidal cell dendritic shafts and spines in the midlayers of rat prelimbic cortex, but rarely presynaptically in glutamate axons *(23)*. This postsynaptic localization is consistent with the dendritic targeting and axonal exclusion of 5-HT$_{2A}$ receptors, as recently demonstrated with the use of green fluorescence protein (GFP)–tagged 5-HT$_{2A}$ receptors in primary cultures of cortical pyramidal cells *(24)*.

4. Studies on 5-HT$_{2A}$-Induced Spontaneous Excitatory Postsynaptic Currents

The application of 5-HT to brain slice of the prefrontal cortex elicits a dramatic increase in spontaneous excitatory postsynaptic currents (spEPSCs) in layer V pyramidal neurons, as shown in Fig. 2. These spEPSCs elicited by 5-HT can be suppressed by the selective 5-HT$_{2A}$ antagonist MDL100907 *(25)* and do not occur in a 5-HT$_{2A}$ knockout mouse *(26)*. Mice lacking 5-HT$_{2A}$ receptors are insensitive to many of the behavioral and physiological effects of hallucinogens *(26)*. Several lines of indirect evidence indicate that 5-HT-induced spEPSCs in prefrontal layer V pyramidal cells results from glutamate released from thalamocortical terminals, which, in this region, originate from the midline/intralaminar nuclei *(27)*. The latter pathway has long been regarded as the last link in the ascending arousal pathway, integrating sensory information with other functions of the prefrontal cortex *(28)*. We have found that 5-HT-induced spEPSCs in layer V pyramidal cells of the prefrontal cortex can be mimicked by hypocretin/orexin acting at hypocretin receptor-2 *(29)*, nicotine at $\alpha_4\beta_2$ nicotinic acetylcholine receptors *(30)*, and norepinephrine at α_1-receptors *(31)*. Among many parallels to 5-HT (e.g., TTX sensitivity), both hypocretin and nicotine-induced spEPSCs are suppressed by the μ-opioid agonist DAMGO *(29,30)*. This

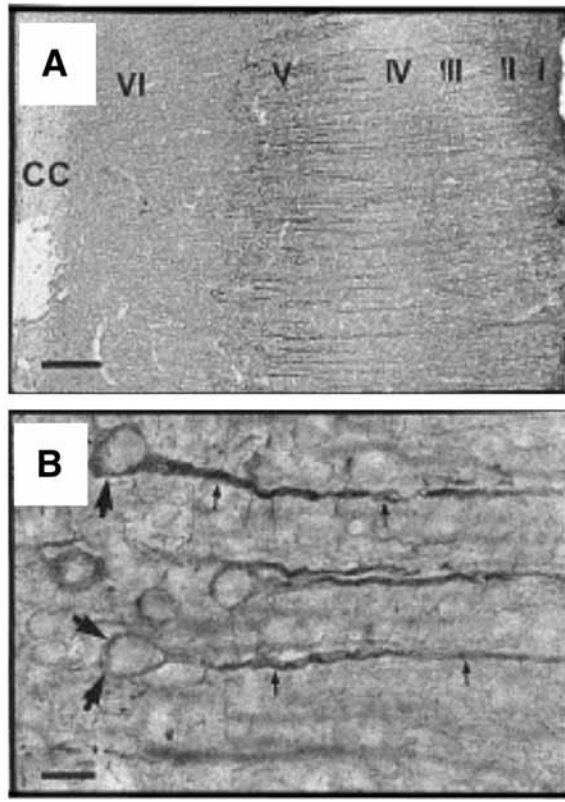

Fig. 1. Serotonin 5-HT_{2A} receptor immunolabeling in rat frontal cortex. **(A)** Labeling of 5-HT_{2A} receptors in the frontal cortex shown at low magnification. Note dense labeling of layer V pyramidal neurons and their apical dendrites. Scale bar: 100 µm. **(B)** Layer V pyramidal neurons shown at higher magnification. Scale bar: 20 µm. Adapted from ref. *22*.

suppression is significant because thalamocortical terminals have µ-opioid receptors, whereas most corticocortical terminals do not.

We have found that agonists at some other G_i/G_o-coupled receptors such as mGlu2/3 *(32)* and adenosine A_1 *(33)* also have a preferential action in suppressing thalamocortical vs corticocortical transmission, although not with the same degree of selectivity as µ agonists. Therefore, it is puzzling that ultrastructural immunohistochemistry has shown 5-HT_{2A} receptors to be located almost exclusively postsynaptically *(23)*. This conclusion fits with most *in situ* mRNA and immunohistochemical studies showing very little expression of 5-HT_{2A} receptors in midline thalamus (however, *see* ref. *34*). A model depicting the proposed location of inhibitory and excitatory receptors modulating

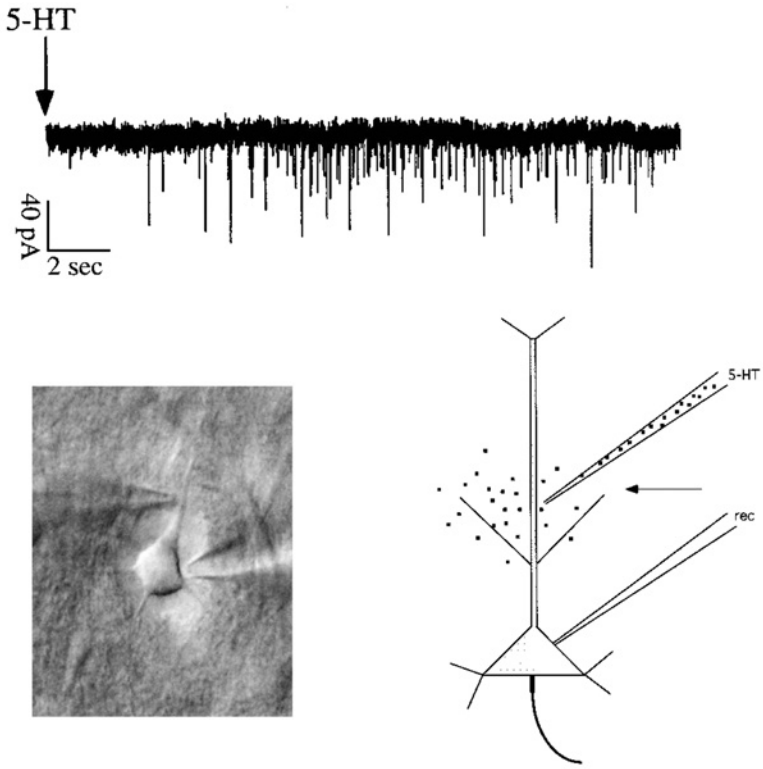

Fig. 2. Electrophysiological trace from a prefrontal layer V showing the rapid onset of spEPSCs in response to puff application (1 s) of 5-HT. Note the increase in spEPSCs begins within approx 3–4 s. The neuron is voltage-clamped close to its resting potential and is not directly depolarized by the application of 5-HT.

midline/intralaminar thalamocortical terminals in the prefrontal cortex is given in Fig. 3. As the model suggests, the simplest explanation for 5-HT$_{2A}$-induced spEPSCs would be a presynaptic population of receptors on thalamocortical terminals. If postsynaptic 5-HT$_{2A}$ receptors are responsible for mediating the effect on spEPSCs, it is necessary to explain how this could trigger the presynaptic release of glutamate. It is possible that a retrograde messenger is generated through 5-HT's action upon postsynaptic 5-HT$_{2A}$ receptors, which then has a selective effect upon thalamocortical nerve terminals. This selectivity might depend on an interaction with presynaptic Kv1.2-containing, subhreshold voltage-gated potassium channels *(35)*.

Another possible postsynaptic mechanism could involve a rapid switching of "silent synapses" to a functional state (e.g., by a translocation of AMPA receptors) *(36,37)*. The resulting increase in spEPSC frequency would then be the

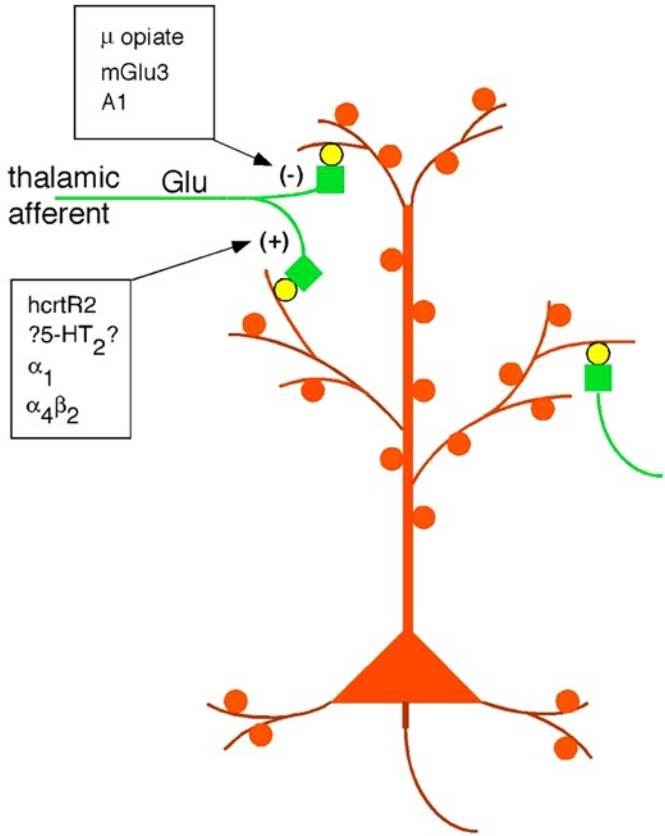

Fig. 3. Model depicting various excitatory and inhibitor influences upon glutamate release from midline/intralaminar terminals on layer V pyramidal cells in the medial prefrontal cortex. Inhibitory G_i/G_o-coupled receptors (μ-opioid receptor, metabotropic glutamate receptor 3 [mGlu3], and adenosine receptor 1 [A1]) are shown at the top. In the middle, a group of excitatory modulators, G_q-coupled excitatory receptors (hypocretin receptor 2 [hcrtR2], α_1-noradrenergic receptor, and $\alpha_4\beta_2$ nicotinic acetylcholine receptors) are shown. Thalamocortical boutons are colored light gray. The layer V pyramidal cell, including most dendritic spines, is colored dark gray. White colored spines denote putative postsynaptic calcium transients at thalamocortical synapses.

result of an increased detection of pre-existing low-amplitude spEPSCs. However, this possibility does not fit easily with several experimental observations. First, the elicitation of spEPSC by puff application of 5-HT shows that the effect is extremely rapid, occurring within a few seconds and recovering within 1 min. We know of no precedent for such an abrupt large-scale translocation of AMPA receptors in and out of postsynaptic sites. Second, a complete loss of the 5-HT response after TTX would not be expected if the increase in spEPSCs

were simply the result of the unmasking of pre-existing silent synapses. If additional receptors were inserted, 5-HT should increase the frequency of miniature EPSCs (in the presence of TTX), but this was not found to be the case *(25)*.

Alternatively, 5-HT-induced spEPSCs could result from an excitation of intrinsic glutamatergic neurons within the prefrontal cortex. However, in agreement with our own observations, after the first 3–4 wk of postnatal development, 5-HT tends to have a net inhibitory rather than excitatory effect upon pyramidal cell firing *(38,39)*. Nevertheless, in mature animals, there could be an undetected subpopulation of glutamatergic neurons that is excited by 5-HT, which is responsible for generating the spEPSC. Furthermore, it is conceivable that such cells, unlike most pyramidal cells, could express μ-opioid receptors, accounting for the suppression by DAMGO of 5-HT-induced spEPSCs. Rodrigo Andrade and colleagues (personal communication) have recently obtained preliminary evidence for the latter possibility.

5. Studies on Hallucinogen-Enhanced Late, Electrically Evoked EPSCs

Hallucinogens such as LSD or DOI do not induce a large increase in spEPSCs because they are only partial agonists at 5-HT$_{2A}$ receptors. Instead, the hallucinogens are highly effective in facilitating an unusual, prolonged late component of intracortical electrically evoked EPSCs (evEPSCs), most likely at corticocortical synapses *(33,40,41)* (Fig. 4). These late evEPSCs might be an electrophysiological marker of novelty, as they occur in the slice only when stimulation occurs after a long quiescent period, but rapidly habituate with repetitive stimulation. Application of a psychedelic hallucinogen shortens the refractory period, allowing late evEPSCs to occur with each stimulus (Fig. 5). Interestingly, the hallucinogen-enhanced late evEPSCs do not appear until relatively late in development, emerging in rats during adolescence (4–5 postnatal weeks). In some cases, the late evEPSC can last more than 1 s. Independent evidence for hallucinogen-enhanced increases in glutamate release comes from microdialysis studies in vivo, where systemic, and most significantly, local (intracortical) administration of DOI has been shown to increases extracellular glutamate levels *(42)*. Similar results have been reported with LSD *(43)*. Paradoxically, the main effect of 5-HT is to suppress the DOI-induced late component. We originally proposed that 5-HT suppresses the late evEPSC because of its opposing actions at non-5-HT$_2$ receptors *(44)*. 5-HT's action at non-5-HT$_{2A}$ receptors would help explain why 5-HT, despite being a highly efficacious 5-HT$_{2A}$ agonist, is not hallucinogenic even when brain levels are extremely high.

However, the identity of non-5-HT$_{2A}$ receptors mediating this effect is not well understood. Previous studies, using relatively nonselective drugs, have suggested

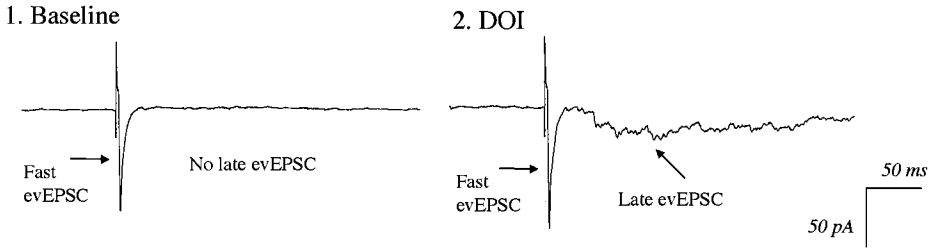

Fig. 4. Electrophysiological traces from a prefrontal layer V showing the response to nearby electrical stimulation of corticocortical afferents. Stimulus artifact appears as a vertical line. **(1)** The fast evoked excitatory postsynaptic current (evEPSC) follows immediately, as depicted by the arrow. Under normal conditions, stimulation at 0.1 Hz evokes only a fast evEPSC. **(2)** However, after the application of a psychedelic hallucinogen (3 μ*M* DOI, 15 min), stimulation at this frequency almost always evokes both a fast evEPSC and a late evEPSC, as depicted by the arrows. The neuron is voltage-clamped close to its resting potential and was not directly depolarized by DOI. It is not known what type of glutamate release accounts for the late evEPSC. Traces are averages of 10 sweeps taken during each of the conditions.

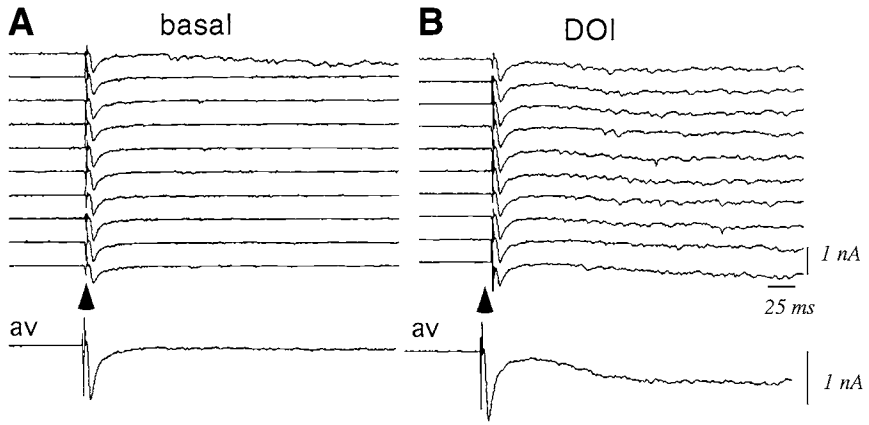

Fig. 5. Electrophysiological traces from a prefrontal layer V showing the response to repeated electrical stimulation. **(A)** Under basal conditions, a late evEPSC follows the fast evEPSC only after the first in a series of 10 stimuli at 0.1 Hz. **(B)** Following DOI (3 μ*M*; applied for 10 min), there is a dramatic increase in the proportion of sweeps with a persistent late component of EPSCs. Adapted from ref. *40*.

that suppression of the fast evEPSCs by 5-HT in cortical pyramidal cells is mediated by 5-HT_{1B} receptors *(45)*. In recent work, using a selective 5-HT_{1B} antagonist (SB224,289), we have confirmed that 5-HT_{1B} receptors mediate the 5-HT suppression of the fast evEPSCs in layer V pyramidal cells *(41)*. Unexpectedly,

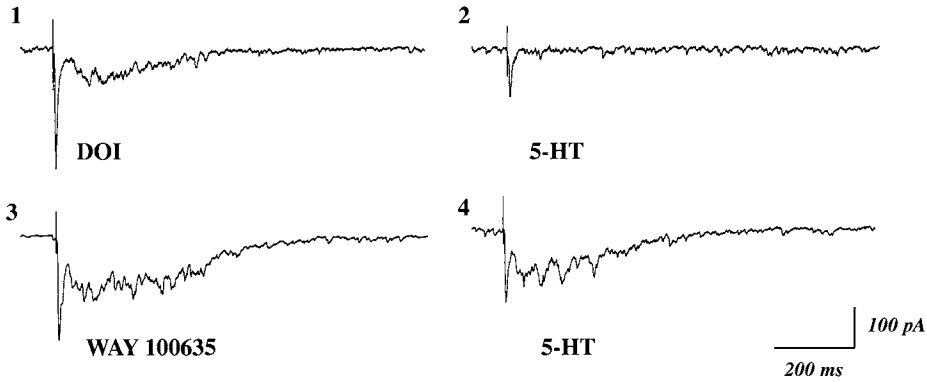

Fig. 6. Serotonin 5-HT$_{1A}$ stimulation suppresses DOI-enhanced late evEPSCs. **(1)** Late evEPSCs after application of DOI; **(2)** 5-HT suppressed both fast and late DOI-enhanced EPSC by 5-HT (3 μ*M*); **(3)** evEPSCs during application WAY 100,635 (100 n*M*); **(4)** following WAY 100,635, 5-HT suppressed the fast evEPSC but not the late evEPSC. Traces are averages of 10 sweeps for each condition (0.1 Hz frequency). Note: Slice had been pretreated with 3 μ*M* DOI for approx 15 min to allow the late EPSC to develop.

SB224,289 did not impair the ability of 5-HT to suppress the late evEPSC, implicating the involvement of an inhibitory 5-HT receptor other than 5-HT$_{1B}$. Preliminary investigations have now shown that 5-HT suppression of the late evEPSC can be blocked preferentially by the 5-HT$_{1A}$ antagonist WAY 100,635 (Fig. 6) *(41)*. These results suggest that the fast and late evEPSCs are not produced through the same mechanisms. Of interest in this regard is that a number of atypical antipsychotic drugs, including clozapine and two newly introduced drugs, ziprasidone and aripiperazole, have partial 5-HT$_{1A}$ agonist activity *(46,47)*.

Traditionally, late evEPSCs have been thought simply to represent polysynaptic activity. The suppression by 5-HT$_{1A}$ would be congruent with this hypothesis because 5-HT$_{1A}$ receptors hyperpolarize cortical pyramidal neurons *(38,39)* and, in particular, might inhibit spiking at the axon hillock *(48)*. However, in a number of respects, the properties of DOI-enhanced late components are distinct from conventional polysynaptic EPSCs *(49)*. This difference is seen, for example, in the long refractory period of hallucinogen-enhanced late evEPSCs as compared to typical polysynaptic activity. Another difference between the two is that the late evEPSC can be blocked by the broad-spectrum competitive NMDA antagonist AP-5 *(33)*. Nevertheless, AMPA/kainate antagonists also block the late evEPSC, which is expected, given that the postsynaptic current (at resting potential) is carried mainly by non-NMDA channels. [Note that the blockade of hallucinogen-enhanced late evEPSCs by NMDA antagonists distinguishes this phenomenon from 5-HT-induced spEPSCs, which are resistant to NMDA antagonists; *see* ref. *33*.]

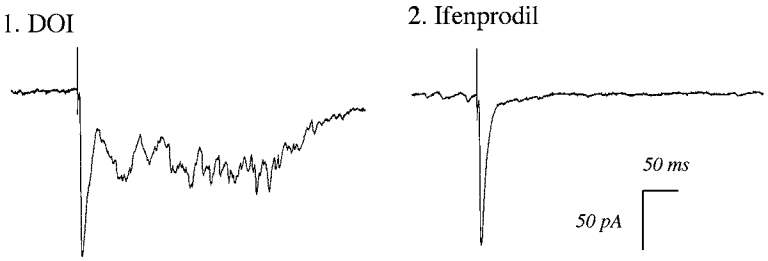

Fig. 7. The NR2B antagonist ifenprodil completely blocks DOI-enhanced late evEPSCs. **(1)** DOI-enhanced late evEPSCs following its application in the perfusate (3 μ*M*; 15 min). **(2)** Selective suppression of late evEPSC during application of ifenprodil (3 μ*M*). Traces are averages of 10 sweeps taken during each of the conditions.

In further studies, we have found that a selective NR2B antagonist ifenprodil (3–10 μ*M*) completely blocks the hallucinogen-enhanced late evEPSC without reducing the amplitude of the fast evEPSC (Fig. 7). Involvement of the NR2B receptor has broad implications because it has high glutamate affinity and slow deactivation kinetics and is located at extrajunctional sites both presynaptically and postsynaptically in the cerebral cortex as well as other regions *(50,51)*. NR2B receptors have been implicated in mediating intersynaptic crosstalk *(52)* or "superslow afterbursts" in the hippocampus *(53)*. In the neocortex, the location of NR2B receptors appears to be pathway-specific in that NR2B antagonists block selectively intracortical vs callosal NMDA evEPSCs *(54)*. NR2B receptors can function as presynaptic autoreceptors, facilitating asynchronous glutamate release from excitatory inputs onto layer V pyramidal cells in the entorhinal cortex *(55)*. Presynaptic NR2B autoreceptors have been implicated both in delayed (or asynchronous) release from the same terminals *(55)* and crosstalk between terminals from two different but overlapping pathways *(52)*. The NR2D-containing NMDA receptors might have a similar role, given their extremely slow deactivation kinetics and reduced sensitivity to a Mg^{2+} block *(56)*. Thus, binary or ternary complexes containing NR1, NR2B, and/or NR2D subunits, which are present in the cortex and thalamus *(57)*, are prime candidates for mediating the late and prolonged evEPSCs induced by hallucinogenic drugs. Interestingly, postmortem studies show selective alterations in certain brain glutamate receptor subtypes in schizophrenia *(58–60)*.

Of special significance is the fact that, unlike broad-spectrum NMDA antagonists, selective NR2B NMDA antagonists do *not* produce PCP-like psychotomimetic effects in normal volunteers or patients *(61)*, nor do they induce "PCP-like" gross behaviors, disrupt prepulse inhibition *(62)*, or disrupt LTP in rodents. The wider significance of determining NMDA subtypes involved in mediating the hallucinogen-enhanced late evEPSCs would be that it provides

a model for the development of therapeutic agents with enhanced potency and selectivity.

6. Significance

These two aspects of 5-HT$_{2A}$-mediated glutamatergic neurotransmission in the prefrontal cortex are quite different. The hallucinogen-induced late evEPSCs are not produced by 5-HT, the endogenous agonist at 5-HT$_{2A}$ receptors, because of counterbalancing actions of 5-HT$_{1A}$ receptors and from negative feedback resulting from glutamate released in spEPSCs. Without these counterbalancing forces, psychedelic hallucinogens lead to an unusual form of hyperglutamatergic transmission that is dependent on NMDA NR2B receptors. Selective NR2B antagonists have been found to markedly suppress the hyperglutamatergic effects of hallucinogens without altering normal, fast glutamatergic transmission. As hyperglutamatergic states might be involved in prodromal stages of schizophrenia *(63)*, our work suggests that NR2B antagonists might have potential as novel therapeutic or prophylactic agents in this condition. By contrast, the spEPSCs evoked by 5-HT give insight into the normal physiological role of 5-HT$_{2A}$ receptors in the medial prefrontal cortex. This effect of 5-HT is mimicked by hypocretin/orexin and nicotine, as well as by other activators of the midline/intralaminar arousal pathway. Thus, in that pathway, 5-HT$_{2A}$ receptors appear to act in concert with other transmitter systems that promote arousal and/or attention.

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13

The Emergence of 5-HT_{2B} Receptors as Targets to Avoid in Designing and Refining Pharmaceuticals

Vincent Setola and Bryan L. Roth

Summary

The long-term use of certain drugs, such as fenfluramine, dihydroergotamine, and pergolide, has been associated with an increased risk for valvular heart disease (VHD) and pulmonary hypertension (PH). Recent investigations have implicated the 5-hydroxytryptamine$_{2B}$ (5-HT_{2B}) receptor in the pathogenesis of VHD and PH. Specifically, the activation of 5-HT_{2B} receptors by VHD- and PH-associated drugs leads to proliferative lesions in the valves of the heart and the pulmonary arterial wall, respectively. For heart valve interstitial cells in vitro, mitotic responses to fenfluramine and its metabolite norfenfluramine have been demonstrated and shown to be mediated almost exclusively by 5-HT_{2B} receptor activation. Furthermore, the activity at recombinant 5-HT_{2B} receptors appears sufficient to predict the ability of a drug to induce mitosis in heart valve interstitial cells in vitro and, likely, VHD-inducing potential in humans.

In mice, hypoxia-induced smooth muscle cell proliferation that gives rise to pulmonary hypertension has been shown genetically and pharmacologically to require 5-HT_{2B} receptors. Additionally, hypoxia-induced pulmonary artery remodeling in mice is significantly exacerbated by prolonged treatment with the PH-associated drug fenfluramine, likely the result of high plasma levels of the metabolite norfenfluramine, a more potent and efficacious 5-HT_{2B} receptor agonist than the parent compound. Thus, drug-induced PH and VHD both seem to result from activation of mitogenic 5-HT_{2B} receptors by the parent compound and/or an active metabolite. As such, current and novel pharmaceuticals and their

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metabolites that are 5-HT_{2B} receptor agonists should not be used in humans because of their likelihood to induce fenfluraminelike VHD and PH.

Key Words: 3,4-Methylenedioxyamphetamine (MDA); 3,4-methylenedioxymethamphetamine (MDMA); 5-HT2B; ergotamine; fenfluramine; methyserdige; norfenfluramine; pergolide; pulmonary hypertension (PH); valvular heart disease (VHD).

1. Introduction

G Protein–coupled receptors (GPCRs) have emerged as major molecular targets for a myriad of effective, lucrative pharmacotherapies. Of the top 10 selling drugs in 2003 (IMS Health), 3 (Zyprexa® [olanzapine] [number 3], Plavix® [clopidogrel] [number 8], and Advair® [salmeterol/fluticasone] [number 9]) are known to exert their therapeutic actions mainly through direct modulation GPCR activity. Another (Zoloft® [sertraline] [number 10]) exerts its effects in part by indirect activation of GPCRs. Thus, GPCRs are a very frequent target of successful pharmaceuticals, and understandably so: GPCRs regulate numerous, diverse processes, such as vascular tone, blood pressure, and cardiac function (e.g., adrenergic GPCRs), inflammatory responses (e.g., histamine GPCRs), gastrointestinal secrections and motility (e.g., cholecystokinin GPCRs), sight (e.g., rhodopsin GPCRs), nociception (e.g., opioid GPCRs), and mood, cognition, and reality perception (e.g., 5-hydroxytryptamine [5-HT] receptors). The therapeutic—and economic—importance of 5-HT receptors among those mentioned is underscored by the fact that 2 of the 10 top-selling drugs of 2003, Zyprexa (olanzapine) and Zoloft (sertraline), target, directly or indirectly, one or more 5-HT receptors.

Although 5-HT receptors are most recognized for their role in treating psychiatric disorders, they are the targets of many nonpsychiatric medications as well. For example, antimigraine drugs such as Migranal® (dihydroergotamine), Sansert® (methysergide), and Imitrex® (sumatriptan) target 5-HT_1 receptors *(1)*. Drugs with activity at 5-HT_3 receptors (e.g., Zofran [ondansetron]) exhibit antiemetic/antinausea properties. The 5-HT_4 receptor agonist Propulsid® (cisapride) is an effective treatment for gastroesophageal reflux disease. In addition to their putative role in antipsychotic efficacy *(2)*, 5-HT_6 receptor antagonists have shown promise in preclinical studies as memory- and cognition-enhancing agents *(3–6)*. Thus, 5-HT receptors represent a microcosm of the GPCR superfamily in the breadth of illnesses/disorders that are effectively treated by targeting them. As such, 5-HT receptors are a frequent target for drug design efforts.

Despite the therapeutic utility of targeting 5-HT receptors, drug activity at some 5-HT receptors can lead to undesirable side effects. In recent years, one

receptor has emerged as an "antitarget" for the design of novel pharmacotherapies: the 5-HT$_{2B}$ receptor. In the sections that follow, we will review the case of fenfluramine, an effective appetite suppressant that was widely used in America the during the late 1980s and 1990s until its link to valvular heart disease (VHD) and pulmonary hypertension (PH) was established. Through a combination of in vitro pharmacology, cell biology, and genetic approaches, researchers were able to zero in on 5-HT$_{2B}$ receptors as the molecular culprit responsible for the adverse cardiopulmonary effects of fenfluramine. These studies, as well as their consequences on current drug screening and drug designing efforts, will be the focus of this chapter. We will also describe studies that have elucidated the signaling pathways that appear to underlie the pathophysiological changes resulting from 5-HT$_{2B}$ receptor activation. Finally, we will discuss the implications of the fenfluramine case on current and future drug design and screening efforts.

2. The Rise of Fenfluramine

In 1973, the US Food and Drug Administration (FDA) first approved the amphetamine derivative fenfluramine for use as an anorexigen, or appetite suppressant. Twenty-three years later, the (+) rotamer, dexfenfluramine, was approved for use as an anorexigen. However, prior to its introduction into the US marketplace, fenfluramine had a long history of use as an adjunct to calorie restriction and other methods of weight reduction *(7,8)*. A general trend was noted with the use of fenfluramine: In the first 3–6 mo, patients taking fenfluramine lost more weight (between 0.3 kg/mo and 4.6 kg/mo) than those taking the placebo *(9–23)*. Also, despite an apparent reduction in efficacy after 6 mo, patients who stopped taking fenfluramine regained more weight than patients taking placebo *(12,16,24)*. This latter observation implies that fenfluramine continues to have a weight-reducing effect beyond 6 mo of use.

In addition to its contributory effects on weight loss, fenfluramine exhibited, in many clinical trials, efficacy in improving metabolic and cardiovascular risk factors associated with obesity, such as blood levels of glucose, insulin, norepinephrine, glycosylated hemoglobin (HbA1c), free fatty acids, cholesterol, triglycerides, high-density lipoproteins, and both systolic and diastolic blood pressure *(15–17,19)*. Although some of the secondary effects were likely the result of weight loss induced by the drug, some studies reported fenfluramine-induced reductions in blood pressure, as well as decreases in plasma levels of norepinephrine, free fatty acids, and insulin, occurring within 1 wk of the treatment *(25,26)*. This early onset suggested that fenfluramine might be able to modify these parameters independent of their effects on weight loss.

Along with their salutary effects on the above-enumerated biochemical parameters, fenfluramine also appeared to have beneficial effects on food-associated

attitudes and behaviors. For instance, a few studies reported a fenfluramine-induced relief of the psychological symptoms of bulimia nervosa *(27,28)*, improvement in the food-related behaviors associated with Prader-Willi syndrome *(29)*, and reduction in anxiety about eating behaviors in obese individuals *(30)*. These limited studies suggested that in addition to its ability to modulate food intake and metabolic and cardiovascular processes, fenfluramine could also normalize disordered, food-centered affects.

Another amphetamine derivative, phentermine, has been in use as an appetite suppressant since its approval by the FDA in 1959. However, there are fewer clinical studies of phentermine's effectiveness in weight loss programs. Munro and colleagues *(31)* studied the effectiveness of phentermine during a 36-wk trial and reported a 13% reduction in initial body weight, compared to a 5.2% reduction for the placebo. The weight loss induced by phentermine over and above caloric restriction is comparable to that attained with fenfluramine.

Despite the equiefficacious appetite-suppressing effects of fenfluramine and phentermine, the two drugs had very different side effects that hindered patient compliance. Fenfluramine causes somnolence, diarrhea, and dry mouth, whereas phentermine induces insomnia and irritability *(32–34)*. In 1984, researchers at Rochester University first tested the hypothesis that short-term use of fenfluramine and phentermine in combination would result in a "canceling out" of side effects without compromising weight loss efficacy. By combining the two appetite suppressants, Weintraub et al. showed that half as much of each drug was as effective as a full dose of either and better tolerated in terms of side effects *(14)*. In 1992, Weintraub et al. studied the efficacy and tolerability of long-term fenfluramine and phentermine combination therapy *(35)*. In the first phase, a double-blind, 34-wk study, patients with a mean ± SEM ideal body weight of 154 ± 12% received either placebo or fenfluramine (60 mg) plus phentermine (15 mg) once a day as an adjunct to behavior modification, calorie restriction, and exercise. After 34 wk, the patients receiving the drug combination lost, on average, 14.2 ± 0.9 kg; the placebo group lost, on average, 4.6 ± 0.8 kg. This represents an almost threefold additional, statistically significant weight loss compared to diet and placebo. In addition, patients taking the drug combination reported the side effects to be similar to those of placebo (17.4 ± 0.3 vs 13.5 ± 0.2, visual analog scales). Many patients complained of dry mouth. Thus, the combination of fenfluramine and phentermine was three times more effective vis-à-vis weight loss and tolerated only slightly worse than placebo, demonstrating that combining the two poorly tolerated drugs reduces the side effects of both without compromising overall effectiveness.

In the second phase of the study, all participants from the first phase received the fenfluramine–phentermine combination, either daily or intermittently, for a

period of 70 wk *(36)*. During the entire, 2-yr study (phases 1 and 2), patients lost an average of 10.8 ± 0.8 kg, demonstrating the sustained, long-term effectiveness of the fenfluramine–phentermine combination. With respect to side effects, dry mouth continued to be the major complaint (41%); however, only 20 of the original 121 participants dropped out because of adverse effects, demonstrating that most patients tolerated well the fenfluramine–phentermine combination over the course of 2 yr. Later phases of the study followed patients for more than 3 yr and many maintained a weight loss greater than or equal to 10% of their original body weight *(33)*.

These findings precipitated the widespread use of a fenfluramine–phentermine combination therapy that came to be known as "Fen-Phen." The combined use of the two anorexigens had never been approved by the FDA, nor had the long-term safety of the therapy ever been established. Nevertheless, weight loss clinics specializing in Fen-Phen therapy were established throughout the country. In 1996, the FDA narrowly approved the more potent, less adverse-effect-prone (+)stereoisomer of fenfluramine, dexfenfluramine, for less-than-1-yr use in the treatment of obesity. *The New York Times* reported that in 1996, 18 million prescriptions had been written for fenfluramine alone or in combination with phentermine and that about 6 million Americans took the drug *(37)*.

3. The Fall of Fenfluramine

Fenfluramine's time at the top of the diet drug market ended in 1997 with a report in the *New England Journal of Medicine* from researchers at the Mayo Clinic. Connolly and colleagues *(38)* presented 24 cases of newly documented heart valve abnormalities in patients taking Fen-Phen for a duration of 1–28 mo. Specifically, Connolly et al. discovered, upon echocardiography, unusual valvular morphology and regurgitation affecting the valves on both sides of the heart, although in all patients at least one left-sided valve was affected. In those cases requiring surgical intervention, the abnormal valves displayed glistening white leaflets and chordae and diffuse thickening. Connolly et al. also noted that the histopathology was identical to the VHD associated with both carcinoid syndrome and ergotamine use. Microscopic examination of the thick, white lesions revealed proliferative myofibroblasts in an abundant extracellular matrix containing CD3-positive T-cells *(38,39)*.

In light of these findings, the FDA obtained echocardiographic data from several US health care facilities. An uncontrolled survey of 284 patients taking fenfluramine in some form for a median of 14 mo *(40)* revealed VHD among 34% of the patients, a much larger prevalence than in the general population *(41)*. Based on these data, the FDA recommended the voluntary withdrawal of

fenfluramine and dexfenfluramine from the US marketplace. In September 1997, Wyeth (a subsidiary of American Home Products), the manufacturer of fenfluramine, complied with the FDA's request. Since that time, retrospective studies have addressed the incidence of VHD among fenfluramine users, with widely disparate findings *(42)*. In five case-controlled studies of fenfluramine users finding significantly greater aortic and/or mitral valve regurgitation in the treated group compared to controls, the lowest incidence of VHD was 11 cases per 9765 patients (0.1%) treated for an average of 1 mo; the highest incidence was 25.2% among patients treated for an average duration of 20 mo *(43–47)*. Thus, several controlled studies reproduced the findings of the FDA and suggested that long-term fenfluramine use was associated with a significantly increased risk, although potentially less than that initially suggested by the FDA survey, for developing VHD.

Fenfluramine use has also been linked with PH. Brenot and colleagues found that 20% of 73 PH patients had a history of fenfluramine use *(48)*. This report precipitated a controlled study of 95 PH patients and 355 controls, which revealed a 23-fold increased risk for developing PH after 3 mo of fenfluramine use *(49)*. Connolly et al. also found eight cases of newly documented PH—a higher frequency (33%) than observed in the general population *(38)*. Like the lesions associated with VHD, pulmonary arteries from PH patients typically manifest plexiform arteriopathy (i.e., hyperplasia in both the intima [endothelial layer] and the media [the layer containing smooth muscle and elastic tissue]) *(50–52)*. Thus, VHD and PH are similar from a histopathological standpoint, in that proliferative plaques that compromise tissue integrity and function occur in both conditions.

4. Evidence Linking 5-HT$_{2B}$ Receptors to Valvular Heart Disease and Pulmonary Hypertension

Since 1997, researchers have sought to understand why fenfluramine and other drugs cause the fibrotic heart valve and pulmonary artery lesions described earlier. Because these lesions are also very frequent in patients with serotonin-secreting tumors (carcinoid syndrome) and in migraine headache sufferers taking serotonergic ergot drugs *(53–57)*, and given the serotonin-releasing properties of fenfluramine *(58–62)*, the involvement of serotonin, its receptors, and/or the 5-HT transporter in fenfluramine-induced VHD seemed likely. Indeed, many suspected that fenfluramine-induced increases in plasma 5-HT levels were responsible for the cardiopulmonary effects of the drug *(63)*. However, other drugs, such as lithium (a treatment for bipolar disorder) and monoamine oxidase inhibitors (antidepressant medications), increase plasma 5-HT levels about twofold and are not associated with VHD *(64–66)*.

Furthermore, long-term treatment with fenfluramine has been shown to reduce plasma 5-HT levels *(67–70)*. These findings negated the role of increased plasma 5-HT in fenfluramine-associated VHD. Thus, investigators began searching for other mechanisms underlying fenfluramine's cardiopulmonary side effects.

In 2000, our group profiled the in vitro pharmacology of the known VHD-associated drugs fenfluramine, ergotamine, and methysergide and of the in vivo metabolites norfenfluramine (a fenfluramine metabolite) and methylergonovine (a methysergide metabolite), as well as that of several serotonergic drugs not associated with VHD *(71)*. The Rothman et al. study revealed that all tested VHD-associated compounds (norfenfluramine, ergotamine, and methylergonovine) were potent agonists at recombinant serotonin 5-HT$_{2B}$ receptors; in contrast, none of the negative controls displayed affinity for or efficacy at these receptors *(71)* (Fig. 1). Around the same time, a group at DuPont-Merck independently reported that norfenfluramine induced a mitogenic marker (i.e., phosphorylation of mitogen-associated protein kinase) in tissue culture cells via activation of recombinant 5-HT$_{2B}$ receptors, the high-level expression of which they measured in both human and porcine heart valves *(72)*. The implication of these studies was that VHD-associated drugs and/or their metabolites activate 5-HT$_{2B}$ receptors on heart valve interstitial cells, leading to the formation of proliferative foci and subsequent changes (e.g., increased extracellular matrix deposition and leukocyte infiltration) that compromise tissue function. This hypothesis seemed reasonable, given the well-established crosstalk between 5-HT$_{2B}$ receptors and mitotic proteins and its role in cell proliferation in the developing heart (*see* below). However, direct proof that heart valve interstitial cells exhibit sustained, mitogenic responses to VHD-associated drugs and that those responses are sensitive to pharmacological blockade of 5-HT$_{2B}$ receptors was lacking.

In order to directly establish the activation of 5-HT$_{2B}$ receptors as the mechanism by which fenfluramine induces valvular interstitial cell proliferation, we measured drug-induced DNA synthesis in primary cultures of heart valve interstitial cells in the absence and presence of the 5-HT$_{2B}$ receptor-selective antagonist SB-206553 *(73)*. We found that fenfluramine and norfenfluramine both induced a significant increase in DNA synthesis compared to vehicle as measured in [^{3}H]thymidine deoxyribose incorporation assays; fenfluramine- and norfenfluramine-stimulated DNA synthesis were abrogated by cotreatment with SB-206553, demonstrating that the mitogenic response to the drugs required activation of 5-HT$_{2B}$ receptors. As an additional, separate measure of drug-induced mitogenesis, we assayed mitogen-activated protein kinase (MAPK) phosphorylation following a 10-min drug treatment *(73)*. The fenfluramine metabolite norfenfluramine induced a statistically significant increase in MAPK phosphorylation compared to the vehicle. These observations demonstrated that

Ki (nM)
EP-3
EP-1
ADENOSIN-A2
ADENOSIN-A1
ALPHA1B
ALPHA1A
BETA2
BETA1
ALPHA2C
ALPHA2B
ALPHA2A
AMPA
PCP
NMDA
H2
H1
OXYTOCIN
V3
V2
V1
BZP
GABA-B
GABA-A
DAT
NET
SERT
M5
M4
M3
M2
M1
KAPPA
DELTA
MU
D5
D4
D3
D2
D1
5-HT7
5-HT6
5-HT5
5-HT2C
5-HT2B
5-HT2A
5-HT1E
5-HT1D
5-HT1B
5-HT1A
5-HT2B
0.1
1
10
100
1000
10000
(+/-) Fenfluramine HCl
(+) Fenfluramine HCl
(-) Fenfluramine HCl
Methysergide Maleate
Ergotamine Tartrate
Phentermine
Fluoxetine
Norfluoxetine
(+/-) Norfenfluramine
(+) Norfenfluramine
(-) Norfenfluramine
Rothman et al. Circulation. 2000;102:2836; KIDB.

fenfluramine and norfenfluramine induce mitogenic responses in primary cultures of heart valve interstitial cells. These in vitro responses are consistent with the proliferative interstitial cell plaques observed in the valves of patients with fenfluramine-induced VHD *(38,39)*. Further, and more importantly, our results demonstrate that fenfluramine and norfenfluramine elicit mitogenic responses in heart valve interstitial cells via the activation of 5-HT$_{2B}$ receptors.

Because fenfluramine is a less potent and less efficacious agonist at recombinant 5-HT$_{2B}$ receptors, as measured by second-messenger (inositol phosphate) accumulation in cells, than is norfenfluramine *(73)*, our findings suggest that even a modest activation of 5-HT$_{2B}$ receptors is sufficient to initiate a mitotic response. Put another way, 5-HT$_{2B}$ receptor agonist efficacy, as measured by second-messenger accumulation assays, does not necessarily correlate with a drug's potential to induce heart valve interstitial cell mitosis and, as such, heart valve fibrosis and VHD. Our data do suggest, however, that drugs with *any* agonist activity at recombinant 5-HT$_{2B}$ receptors will induce heart valve interstitial cell proliferation. In this regard, two other drugs, 3,4-methylenedioxymethamphetamine (MDMA) and one of its in vivo metabolites (3,4-methylenedioxyamphetamine, MDA), identified in large-scale compound screens as agonists at recombinant 5-HT$_{2B}$ receptors, had a similar effect on DNA synthesis in primary cultures of heart valve interstitial cells as did fenfluramine and norfenfluramine. Like the response to fenfluramine and norfenfluramine, the response to MDMA and MDA was sensitive to 5-HT$_{2B}$ receptor blockade, indicating a dependence on 5-HT$_{2B}$ receptor activation. Thus, agonist activity at recombinant 5-HT$_{2B}$ receptors appears to be a reliable method to detect, in a high-throughput fashion, fenfluraminelike heart valve interstitial cell mitogens and potential VHD-associated drugs.

A role for 5-HT$_{2B}$ receptors in drug-induced PH was demonstrated in mice using both a genetic and pharmacological approach. Launay et al. *(74)* showed that mice maintained in hypoxic chambers for several weeks [an established method for inducing pulmonary hypertension *(75,76)*] developed PH as a result of pulmonary artery smooth muscle cell hyperplasia. Interestingly, mutant mice lacking 5-HT$_{2B}$ receptors did not develop PH *(74)*. In addition, hypoxia-induced

Fig. 1. *(previous page)* Three-dimensional representation of VHD-associated and non-VHD-associated drug affinity for a battery of GPCRs, ligand-gated ion channels, and biogenic amine transporters. Affinity constants (K_i) were measured by competition of various concentrations of test drug for [^{3}H]radioligand-binding sites. The VHD-associated drugs, but not the negative controls, displayed a high affinity for 5-HT$_{2B}$ receptors and α-adrenergic receptors (arrows). However, drugs with activity at α-adrenergic receptors are not associated with VHD. Thus, based on their in vitro pharmacology, VHD-associated drugs are characterized by affinity for 5-HT$_{2B}$ receptors. Reprinted with permission from ref. *66.* (Illustration appears in color in insert that follows p. 240.)

PH was exacerbated by a 5-wk treatment with therapeutic doses dexfenfluramine (and likely the result of the metabolite norfenfluramine) and was abrogated by the administration of a 5-HT_{2B} receptor-selective antagonist (RS-127445) *(74)*. Collectively, these data show that 5-HT_{2B} receptor function is required for the pulmonary artery smooth muscle cell hyperplasia that gives rise to PH. The findings of Launay et al. *(74)* suggested strongly that the increased risk for developing PH associated with fenfluramine *(38,48,49)* is largely the result of the activity of the metabolite norfenfluramine at 5-HT_{2B} receptors.

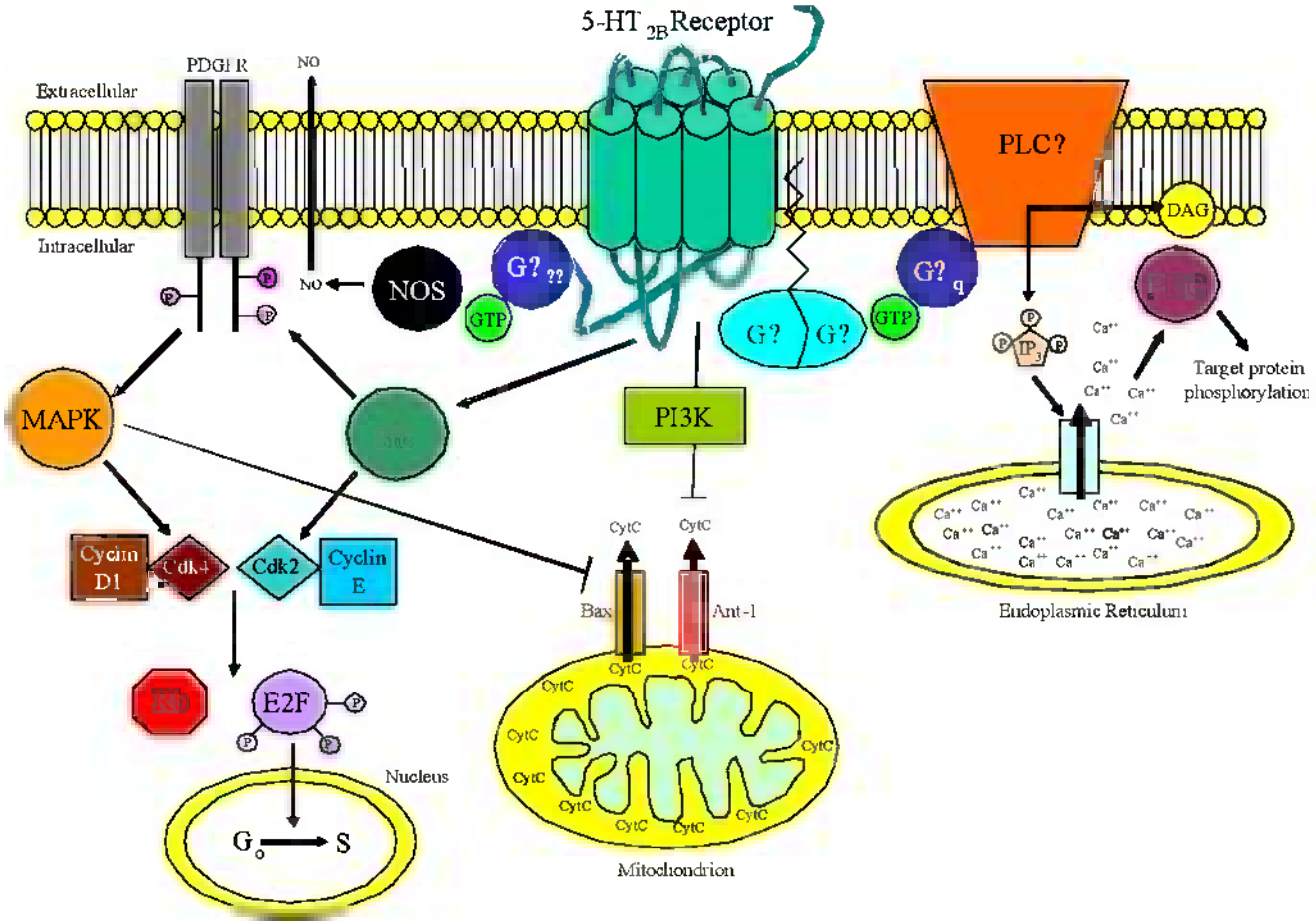
5. Pathways Linking 5-HT2B Receptor Activation to Mitosis

What is clear from the above discussion is that the mitogenic signals emanating from 5-HT_{2B} receptor activation contribute to at least two pathophysiological states. Thus, a better understanding of the molecular mechanisms underlying crosstalk between the 5-HT_{2B} receptor and downstream mitotic effectors is likely to reveal insights into—and potential therapeutic molecular targets for—the treatment of VHD and PH. Toward this end, several studies over the past decade have elucidated the complex mechanisms by which 5-HT_{2B} receptor activation leads to mitosis. Activation of endogenous *(73,77,78)* and exogenous *(72,79–81)* 5-HT_{2B} receptors has been shown to stimulate MAPKs Erk 1/2 (p42/44). In mouse fibroblasts stably expressing 5-HT_{2B} receptors, but not in nontransfected cells, Launay and colleagues showed that MAPK activation is downstream of $p21^{Ras}$ activation, which was sensitive to antibodies against $G\alpha_q$ and $G\beta\gamma$ *(81)*. Thus, in mouse fibroblasts at least, 5-HT_{2B} receptor-stimulated MAPK activation is dependent on G protein and $p21^{Ras}$. Furthermore, the 5-HT_2 receptor inverse agonist ritanserin, which completely blocks the 5-HT-stimulated activation of $p21^{Ras}$, also blocked both the constitutive and agonist-stimulated mitogenic response in mouse fibroblasts expressing the 5-HT_{2B} receptor *(81)*. This result indicates that 5-HT_{2B} receptors generate an agonist-independent, tonic mitogenic signal in mouse fibroblasts. Nebigil et al. *(80)* further detailed the mitogenic signaling of 5-HT_{2B} receptors in mouse fibroblasts. They demonstrated that activation of the 5-HT_{2B} receptor leads to activation of the oncogenic, cytoplasmic tyrosine kinase $p60^{Src}$, which, in turn, induces both cyclin E and cyclin D1 via separate pathways. Induction of cyclin D1 was shown, using pharmacological and genetic inhibitors, to occur through $p60^{Src}$-mediated activation of the platelet-derived growth factor receptor (PDGFR) tyrosine kinase, which, in turn, activates MAPK, a necessary step for the induction of cyclin D1 *(80)*. The induction of cyclin E, in contrast, although sensitive to inhibitors of $p60^{Src}$, was insensitive to inhibitors of PDGFR and MAPK *(80)*. The activated cyclins led to hyperphosphorylation of retinoblastoma protein (pRb) and disinhibition of the transcription factor E2F, which activated the transcription of genes involved in DNA replication *(80)*. In the

developing mouse myocardium, as in mouse fibroblasts, the mitogenic signaling of the 5-HT$_{2B}$ receptor appears to involve transactivation of a growth factor receptor tyrosine kinase, namely the epidermal growth factor receptor ErbB-2, because the expression of this enzyme is reduced in mutant mice lacking the receptor *(82)*. Thus, in terms of the downstream players linking 5-HT$_{2B}$ receptors to mitosis in various cell types, the proto-oncogene products p21^{Ras} and p60^{Src} appear to play pivotal roles. Growth factor receptors, such as PDGFR and ErbB-2, also contribute to 5-HT$_{2B}$ receptor-induced mitosis.

The 5-HT$_{2B}$ receptor has also been shown to display antiapoptotic activity, which is likely to contribute to the overall mitogenic response to receptor agonists. For instance, in mouse myocardial fibroblasts from wild-type mice, but not in those obtained from mutant mice lacking 5-HT$_{2B}$ receptors, treatment with 5-HT greatly reduced the apoptosis induced by serum deprivation *(79)*. The anti-apoptotic activity of 5-HT$_{2B}$ receptors was shown to be dependent on both the MAPK and PI3 kinase (PI3K) pathways and was the result of 5-HT-induced downregulation of the mitochondrial permeability regulators Bax and ANT-1, thereby decreasing cytochrome-*c* release *(79)*. The importance of the 5-HT$_{2B}$ receptor in cell proliferation and survival during myocardial development is further underscored by the embryonic and neonatal lethality of targeted disruption of 5-HT$_{2B}$ receptor expression. In mice bearing such a mutation, histological examination of the heart revealed increased apoptosis and decreased cell number in the ventricular trabeculae, as well as abnormal sarcomeric organization in the subepicardial layer *(79,82)*. In developing mouse embryos, treatment with the 5-HT$_2$ receptor inverse agonist ritanserin, at a time when 5-HT$_{2B}$ receptors are the main 5-HT$_2$ receptors expressed, induced apoptosis in the cephalic region, the heart, and the neural tube *(83)*, suggesting that 5-HT$_{2B}$ receptor function is important in the development of other tissues as well.

To summarize, although drug activity at some 5-HT receptors is therapeutic, activation of 5-HT$_{2B}$ receptors by drugs can also lead to potentially fatal cardiopulmonary side effects. The serious consequences of drug-induced 5-HT$_{2B}$ receptor activation are the result of the mitogenic and, perhaps, the anti-apoptotic signals generated by the 5-HT$_{2B}$ receptor. Although the "road" to mitogenesis appears to be cell type-specific, the classes of molecules involved appears to be somewhat conserved: Proto-oncogene products, growth factor receptors, cytosolic protein kinases, and mitochondrial permeability factors all seem to play a role (Fig. 2). These molecules might represent cell type-specific targets to modulate 5-HT$_{2B}$ receptor-mediated pathophysiology. For instance, although PDGFR is involved in the mitogenic response to 5-HT$_{2B}$ receptor activation in mouse fibroblasts, it does not appear to play a role in the developing mouse myocardium; rather, ErbB-2 seems to be a critical growth factor receptor. Thus, in cases where the therapeutic efficacy of drugs that activate 5-HT$_{2B}$ receptors outweighs the


5-HT 2B Receptor
PDGFR
NO
Extracellular
Intracellular
NOS
NO
GTP
G? ??
G? q
G? G?
GTP
PLC?
DAG
PKC
IP3
Target protein phosphorylation
MAPK
Src
PI3K
Cyclin D1
Cdk4
Cdk2
Cyclin E
Rb
E2F
Nucleus
G0 S
Bax
Ant-1
CytC
Mitochondrion
Endoplasmic Reticulum
Ca++

VHD- and PH-associated risks (e.g., the antiparkinsonian pergolide), drugs that inhibit, in a cell type-specific manner, the downstream mitogenic effectors of 5-HT$_{2B}$ receptor signaling might decrease the cardiopulmonary risks associated with such drugs.

6. Implications for Drug Design and Drug Screening

The fenfluramine case study contains several valuable lessons for those in the business of designing and evaluating the safety of novel drugs. The main lesson is clearly that agonist activity at 5-HT$_{2B}$ receptors should be avoided at all costs because of the clear link between activation of the receptor and the development of fibroproliferative cardiopulmonary disease. And a costly lesson, indeed, it has been for American Home Products, which has set aside $16.6 billion for settlements with fenfluramine users and legal costs (Associated Press Wire, 04/28/04). The company's reported net income in 2003 was approx $2 billion (United Business Media, 01/22/04). Thus, to say nothing of the human consequences, bringing a drug to market that activates 5-HT$_{2B}$ receptors can have dramatic financial consequences, as well.

A second valuable lesson from the fenfluramine story is that screening drugs for activity at 5-HT$_{2B}$ receptors is a relatively easy, reliable method to identify

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Fig. 2. *(previous page)* Mitogenic signal transduction from 5-HT$_{2B}$ receptors. The various pathways regulating cell proliferaction/survival shown to be affected by 5-HT$_{2B}$ receptors are schematized. The pathways can be divided into three categories: second-messenger generation, mitogenesis, and antiapoptosis. The second-messenger-generating pathway involves 5-HT$_{2B}$ receptor-mediated activation of phospholipase Cβ, which leads to hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate into inositol-1,4, 5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$, which is hydrophilic, activates ligand-gated Ca^{2+} channels on the membrane of the endoplasmic reticulum, causing the release of intracellular Ca^{2+} stores. DAG, which is hydrophobic, recruits to the plasma membrane and, along with the increased intracellular Ca^{++}, acitvates protein kinase C (PKC). Interactions between the 5-HT$_{2B}$ receptor with nitric oxide synthase stimulate the latter to generate the second-messenger nitric oxide. The mitogenic pathway has two branches, both of which start with 5-HT$_{2B}$ receptor-mediated activation of Src. The first branch involves Src-mediated transactivation of the PDGFR, which leads to activation of MAPK and, subsequently, cyclin D1/cdk4. The second branch involves Src-dependent activation of cyclin E/cdk2 via a MAPK-independent mechanism. Both branches lead to phosphorylation of retinoblastoma protein, which then dissociates from the transcription factor E2F. E2F then translocates to the nucleus, where it stiumlates transcription of genes involved in DNA synthesis. The antiapoptotic pathway involves the downregulation of the mitochondrial permeablility factors Bax and ANT-1 via 5-HT$_{2B}$ receptor-mediated activation of MAPK and PI3K, respectively. Downregulation of Bax and ANT-1 lead to decreased cytoplasmic levels of cytochrome-*c*, thereby reducing cytochrome-*c*-mediated activation of caspase-9 activity. (Illustration appears in color in insert that follows p. 240.)

drugs with the potential to induce VHD and PH. Ideally, assaying mitogenic responses in primary cultures of human heart valve interstitial cells would be the most informative index of a drug's likelihood to cause fenfluraminelike VHD. However, the difficulty in acquiring human hearts with healthy valves and establishing primary cultures preclude large-scale screening efforts using heart valve interstitial cells. Also, evaluating a drug's effect on hypoxia-induced pulmonary hypertension in rodents is also time-consuming and difficult for identifying a few compounds among many that increase the risk for PH. Thus, cell lines expressing 5-HT_{2B} receptors represent a reliable, low-resolution filter through which to isolate compounds with VHD- and PH-inducing potential in a high-throughput fashion.

Even prior to performing screens, it is possible to predict, based on the current evidence, classes of compounds likely to activate 5-HT_{2B} receptors and, as such, be high-risk compounds in terms of cardiopulmonary side effects. Drugs known or likely to cause VHD and PH can be grouped into two classes: ergolines (dihydroergotamine, methysergide, and pergolide) and phenylisopropylamines (fenfluramine, norfenflurmine, MDMA, and MDA). Thus, lead compounds in the ergot and phenylisopropylamine structural classes should be avoided unless great pains are taken to "design out" binding to and/or activation of 5-HT_{2B} receptors. In this regard, using molecular modeling and site-directed mutagenesis, we have recently shown that stabilizing, hydrophobic interactions between a valine in transmembrane helix II (V103) of the 5-HT_{2B} receptor and the α-methyl group of norfenfluramine are critical determinants of the compound's selective potency and efficacy at 5-HT_{2B} receptors compared to 5-HT_{2C} and 5-HT_{2A} receptors *(84)*. Thus, in order to be safe in terms of VHD and PH, phenylisopropylamines should be designed in such a way as to decrease hydrophobic interaction with V103. It is likely that ergot binding to and activation of 5-HT_{2B} receptors involve interactions that are uniquely important for 5-HT_{2B} receptor activity. However, as a general rule, ergot and phenylisopropylamine lead compounds are high risk in terms of VHD- and PH-inducing potential.

In conclusion, the current evidence linking activation of 5-HT_{2B} receptors to VHD and PH is very strong. We have suggested that all currently marketed and investigational pharmaceuticals be screened for agonist activity at recombinant 5-HT_{2B} receptors *(71)*, and we have shown that such an approach is likely to identify potential VHD- and PH-inducing drugs *(73)*. Although it is unclear "how much" activation of 5-HT_{2B} receptors by drugs is "enough" to set off the mitogenic responses that give rise to fibrotic lesions in vivo, available data suggest that even a modest agonist character is sufficient to do so, at least in heart valves. Further, not only the parent compounds but in vivo metabolites should also be screened for activity at 5-HT_{2B} receptors, as the metabolites

might contribute to the pathogenesis of VHD and PH. However, for now, because it is better to be safe than to be sorry, the best policy is to avoid the use in humans of any drug that is, or after metabolism yields, a 5-HT$_{2B}$ receptor agonist.

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14

The 5-HT_3 Receptor

Andrew J. Thompson, Li Zhang, and Sarah C. R. Lummis

Summary

The 5-HT_3 receptor is a member of the Cys-loop neurotransmitter-gated ion channel family. It has five symmetrically placed subunits surrounding a central ion-conducting pore. Each subunit consists of an extracellular N-terminal ligand-binding domain, and C-terminal domain containing four transmembrane α helices (M1–M4); M2 lines the channel and controls ion selectivity and gating. A long intracellular loop between M3 and M4 is responsible for channel conductance and intracellular modulation. In this chapter we look at each of these regions, exploring the structure of the ligand binding site and its pharmacophore model, the importance of M2 and the complex modulatory mechanisms within the intracellular region that underlie the regulation, assembly, targeting, and trafficking of the 5-HT_3 receptor.

Key Words: Serotonin$_3$ receptor; 5-hydroxytryptamine$_3$ receptor; Cys-loop receptor; ligand-gated ion channel; ionotropic receptor; neurotransmitter binding site.

1. Introduction

The 5-hydroxytryptamine$_3$ (5-HT_3) receptor contains an integral, agonist-gated ion channel and in this way differs from all other known serotonin receptors whose actions are mediated via G proteins *(1)*. 5-HT_3 receptors were one of the original two classes of serotonin-activated receptors defined by Gaddum and Picarrelli *(2)*. Seven distinct classes have now been defined, but, to date, the 5-HT_3 receptor is the only vertebrate 5-HT-gated ion channel known; indeed, it is more closely related to the nicotinic acetylcholine (nACh) receptor than to any of these other classes of 5-HT receptor.

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The 5-HT_3 receptors are found in both the peripheral nervous system and central nervous system (CNS), where they mediate fast synaptic transmission at synapses *(3)*. In the CNS, they are located predominantly at interneurones, where they modulate the release of a range of neurotransmitters *(4–9)*. There is some evidence that 5-HT_3 receptors play roles in brain reward mechanisms and in neurological phenomena such as anxiety, psychosis, nociception, and cognitive function *(10,11)*, and in the first few years following the discovery of these receptors, there was also much interest in the therapeutic potential of 5-HT_3 receptor antagonists for antipsychotic, antinociceptive, and other psychiatric disorders *(12–15)*. This potential has not yet been realized, but there is still active research in this area *(16)*, and their current major therapeutic target is against emesis in cancer chemotherapy and irritable bowel syndrome *(17,18)*.

The cloning of cDNAs encoding 5-HT_3 receptor subunits over the last decade has taken the study of 5-HT_3 receptor pharmacology, physiology, and pathophysiology to the molecular level, although there is still much to be discovered. The availability of the acetylcholine-binding protein crystal structure has substantially enhanced our understanding of the ligand-binding domain *(19)*, and functional regions involved in receptor gating have been mapped (e.g., by electrophysiological analysis of mutated receptors expressed in heterologous cell systems) *(20)*. This chapter focuses primarily on the structural and functional insights of 5-HT_3 receptors revealed by molecular biological techniques, with particular attention being drawn to developments since 1990.

2. Receptor Subtypes

The first cDNA clone encoding the 5-HT_3 receptor A-subunit was isolated by screening a mouse neuroblastoma (NCB20) expression library for functional 5-HT-gated currents in *Xenopus* oocytes *(1)*. Subsequently, the full-length cDNAs for the orthologous 5-HT_{3A} subunit have been cloned from human *(21)*, rat *(22)*, guinea pig *(23)*, and ferret *(24)* by polymerase chain reaction (PCR) screening of several libraries. Sequence analysis of the 5-HT_{3A} subunit places it in the Cys-loop ligand-gated ion channel family because it has significant sequence and predicted structural similarity to other members of the family, which includes nACh, $GABA_A$, and glycine receptors. Topologically, these receptors consist of a large extracellular N-terminal and C-terminal domain, four transmembrane regions, M1–M4, of which M2 lines the pore, and a large intracellular loop between M3 and M4 (Fig. 1). Since 1999, four more genes encoding 5-HT_3 receptor subunits have been identified: 5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D}, and 5-HT_{3E} subunits, although the latter two have not yet been expressed *(25,26)*. The 5-HT_{3B} and 5-HT_{3C} subunits have 45% and 39% sequence identities with their 5-HT_{3A} homologs, respectively, whereas the 5-HT_{3C} and 5-HT_{3D} predicted

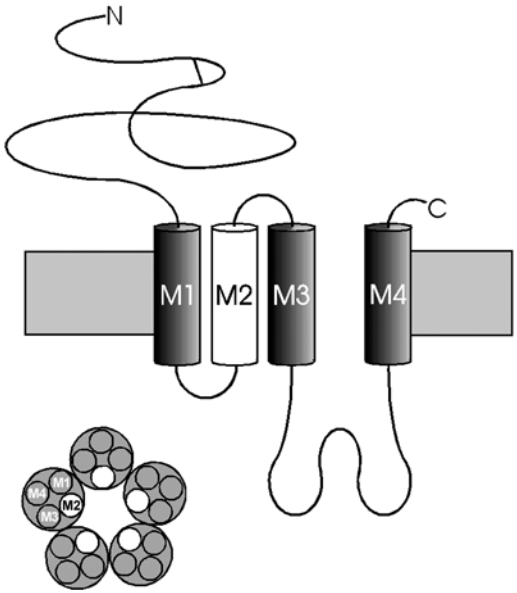

Fig. 1. Schematic representation of a typical Cys-loop ligand-gated ion channel subunit. The diagram at the lower left is a cross-section of the channel shown from above and demonstrates the association of the five subunits within the membrane. Attention is drawn to M2 (white circle), which has been shown to line the pore.

subunit sequences reveal overall identities of only 27% and 31% with the 5-HT$_{3A}$ subunit, respectively (Fig. 2).

Only the 5-HT$_{3A}$ subunit is able to form functional homomeric receptors, but some of their electrical properties, including channel rectification, desensitization, and single-channel conductance, do not resemble those of some native 5-HT$_3$ receptors. Studies of expressed heteromeric complexes of 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits reveal a single-channel conductance that more closely resembles the conductance displayed by some native receptors *(25,27,28)*. In the absence of the B-subunit, 5-HT$_3$ responses have a conductance that is typically less than 1 pS *(28,29)*, but when the B-subunit is introduced, the conductance rises to around 20 pS. Specific amino acids that are responsible for this change in conductance have been identified *(28)*. In addition to low single-channel conductance, there are some differences in pharmacology between homomeric and heteromeric receptors. For example, heteromeric receptors have been shown to be less sensitive to *d*-tubocurarine *(25)*, picrotoxin *(30)*, alcohol *(105)*, and volatile anesthetics *(106)*. Additional biophysical differences include a relatively low permeability of the heteromeric 5-HT$_3$ receptor to calcium ions *(31)*, a linear

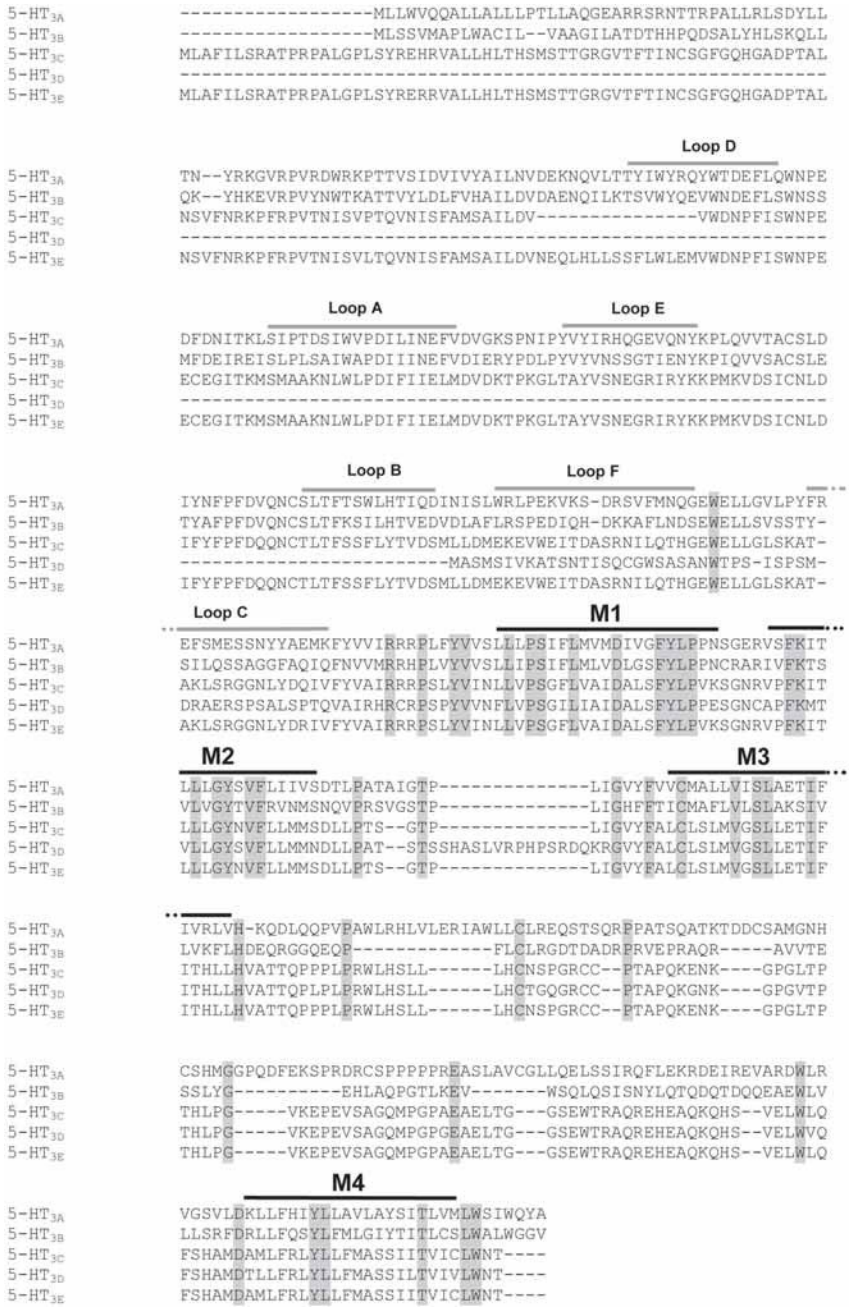

Fig. 2. Alignment of human 5-HT_3 receptor subunits. The binding loops and transmembrane (M1–M4) regions are highlighted by horizontal lines above the text. Conserved residues are highlighted in gray. Accession numbers for the alignment are as follows: 5-HT_{3A}, P46098; 5-HT_{3B}, O95264; 5-HT_{3C}, Q6V706. 5-HT_{3D} and $5HT_{3E}$ were taken from ref. *26*.

current–voltage relationship *(25)*, and complex changes in receptor desensitization *(32)*. The function of the 5-HT$_{3C}$, 5-HT$_{3D}$, and 5-HT$_{3E}$ subunits remains unknown, as they have not yet been characterized.

Structural heterogeneity resulting from alternative splicing has also been identified. A short form of the murine 5-HT$_{3A}$ receptors has been isolated in which five or six amino acid residues within the M3–M4 loop are absent *(33)*. The distribution of this short splice variant varies depending on its location in the adult animal *(34)* and during stages of embryonic development *(35)*, and there are some functional differences when compared to the long variant *(36–39)*. In humans, the splice acceptor site that is responsible for the long form of the receptor is missing *(36,40)* and, consequently, the long variant that is found in rodents is not expressed. However, in humans, a truncated (h5-HT$_{3AT}$) and an alternative long (h5-HT$_{3AL}$) form have been identified *(36)*. The truncated version consists of 238 amino acids and contains only a single transmembrane (M1) region, whereas the long form contains an additional 32 amino acids in the M2–M3 loop. Although these two subunit variants cannot form functional homomeric receptors when expressed alone, they are able to coassemble with 5-HT$_{3A}$ subunits and modulate the 5-HT response. Thus, the presence of splice variants might, in part, explain the functional variation seen in nature, but they are unlikely to account for all the functional diversity of native 5-HT$_3$ receptors.

3. Distribution

The 5-HT$_{3A}$ subunits have been found to be located in many brain areas, including the cortex, hippocampus, nucleus accumbens, substantia nigra, and ventral tegmental area, although the highest levels are in the brainstem, especially the nucleus tractus solitarius and area postrema *(34,41,42)*. Localization of 5-HT$_{3A}$ receptors in cholecystokinin (CCK) and GABA-containing interneurons is consistent with their involvement in the regulation of GABA and CCK neurotransmission *(43)*. 5-HT$_3$ receptors have also been found to colocalize with the CB1 cannabinoid receptor in rat brain neurons *(44,45)*, and a high proportion of 5-HT$_{3A}$/CB1-expressing neurons contained the inhibitory neurotransmitter GABA, indicating a possible interactions between the CB1 and 5-HT$_{3A}$ receptors and their contributable roles to the regulation of GABA neurotransmission in the brain. At the subcellular level, there is strong evidence for differential subcellular localization of presynaptic and/or postsynaptic 5-HT$_{3A}$ receptors within different central regions, depending on the nature of the neurons containing 5-HT$_{3A}$ receptors *(46)*. For instance, 5-HT$_{3A}$ receptor immunoreactivity was most abundant in postsynaptic dendritic sites in the hippocampus, but it was primarily associated with presynaptic nerve endings in the amygdala *(34,46)*. There is also some evidence that 5-HT$_{3A}$ receptor subunits might coexpress with subunits from other ligand-gated ion channels such as the nACh α_4-subunit *(47)*.

Studies have described 5-HT_{3B} receptor mRNA and immunoreactivity being detected in human brain *(48)* and in rat hippocampal neurons respectively *(49)*, but recent evidence has strongly suggested that 5-HT_{3B} subunits are restricted to the peripheral nervous system *(50)*. Thus, if these subunits are in the CNS, it is likely that they are in low abundance and/or in small subpopulations of cells *(26,51)*.

4. 5-HT_3 Receptor Pharmacology

The 5-HT_3 receptor is unusual among ligand-gated ion channels in that there are many highly selective and potent compounds that act at this receptor. Early studies categorized the receptor using the nonselective compounds morphine and cocaine *(2)*, but using 5-HT as the origin, bemesitron and tropisetron were formulated, and it was also found that 2-methyl-5-HT was a more potent agonist than 5-HT. Later compounds that were developed include ondansetron, granisetron, and zacopride, which act at nanomolar concentrations, and there are now a wide range of similarly potent compounds. Although the range of selective antagonists is well represented, the number of selective agonists is more limited. These include 1-phenylbiguanide and chlorophenylbiguanide (mCPBG), which is the most potent 5-HT_3 agonist developed to date *(52)*.

Research into the design of novel compounds has allowed the development of pharmacophore models for the receptor (Fig. 3). 5-HT_3 receptor agonists have a common basic amine, an aromatic ring, a hydrophobic group, and two hydrogen-bond acceptors *(53)*. 5-HT_3 receptor antagonists share a basic amine, a rigid aromatic or heteroaromatic ring system, and a carbonyl group (or isosteric equivalent) that is coplanar to the aromatic system *(54–57)*, and here there are slightly longer distances between the aromatic and amine group when compared to the agonist pharmacophore. Further work has shown that the 5-HT_3 receptor can only accommodate small substituents on the charged amine, and a methyl group here appears to be optimal *(57)*. Most of the potent antagonists of 5-HT_3 receptors have 6.5 heterocyclic rings, and the most potent compounds contain an aromatic six-membered ring. The species differences in 5-HT_3 receptor pharmacology have identified the roles of particular amino acids and/or regions of sequence (discussed in more detail below), for example, a number of residues in the C loop are strongly implicated to interact with *d*-tubocurarine *(75)*. Docking of a range of antagonists into a model of the 5-HT_3 receptor-binding site shows reasonably good agreement with the pharmacophore model and the details that differ between species *(58)*.

5. 5-HT_3 Receptor Structure: The N-Terminal, Extracellular Domain

There are currently no high-resolution structural data available for the 5-HT_3 receptor or, indeed, any ligand-gated ion channel, but the extracellular N-terminal

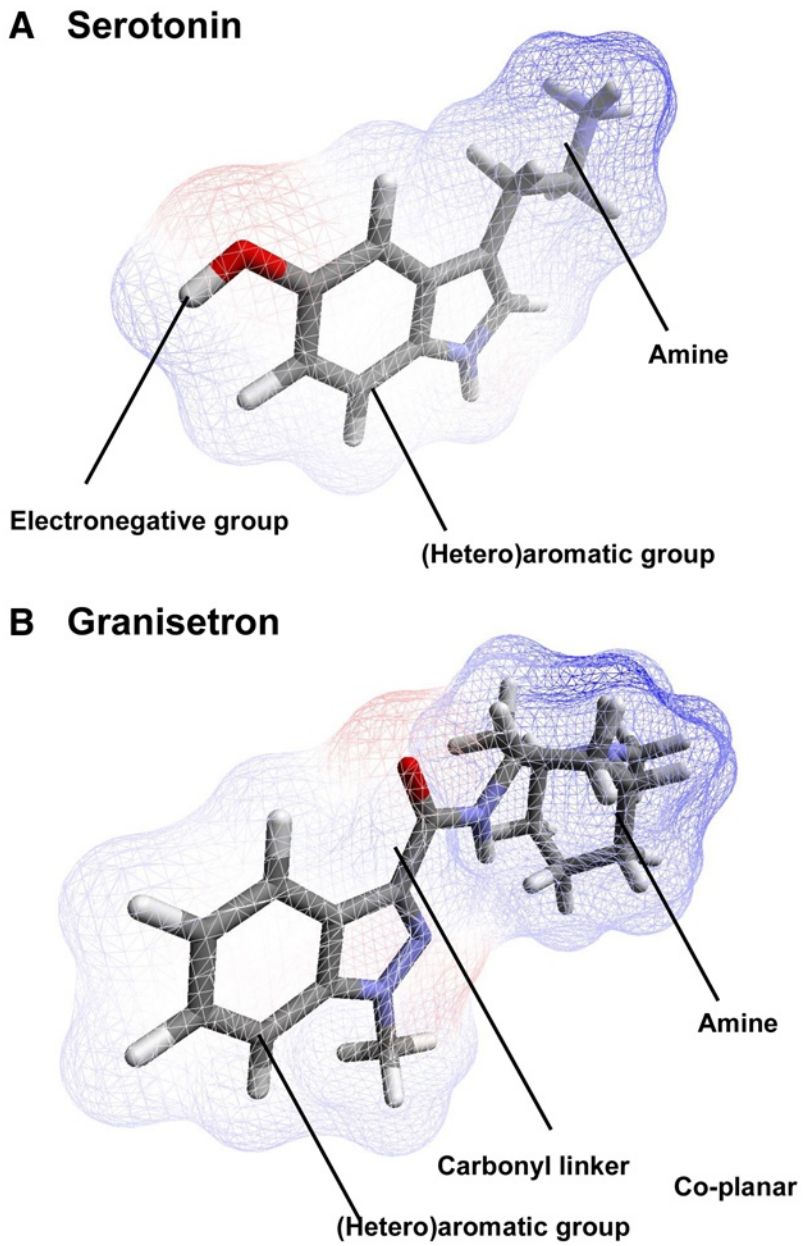

Fig. 3. Examples of 5-HT$_3$ agonist and antagonist pharmacophores. Serotonin **(A)** and granisetron **(B)** are shown as examples of 5-HT$_3$ receptor agonists and antagonists. Both molecules are shown as stick models. Electrostatic potential is displayed in wire frame. Attention has been drawn to the important features of each pharmacophore.

domain of these receptors is homologous to the acetylcholine-binding protein (AChBP), whose structure has been resolved to 2.1 Å *(59)*. The molecular details obtained from AChBP have been used to create homology models of the extracellular domains of a range of Cys-loop receptors, including the 5-HT_3 receptor. Although some structural details of specific regions appear to differ from those of AChBP *(60)*, the creation of a functional chimaeric receptor containing AChBP and the transmembrane domain of the 5-HT_3 receptor demonstrates that there is considerable structural and functional similarity between AChBP and the extracellular domain of the 5-HT_3 receptor. *(61)*.

The homology model of the homomeric 5-HT_{3A} receptor (Fig. 4) shows the ligand-binding site that lies between the faces of two adjacent subunits (as in AChBP) and is formed by three loops (A–C) from the "principal" subunit and three (D–F) from the adjacent or "complementary" subunit. The residues identified as being less than 5 Å from 5-HT are shown in Fig. 4B, and the proposed orientation of 5-HT in this binding pocket in Fig. 4C. As in all Cys-loop receptors, the binding pocket contains a large proportion of aromatic residues, many of which have roles in the binding of agonists or antagonists and/or in receptor gating. These are discussed in more detail below.

The model proposed by Reeves et al. *(62)* suggests that Asn128 is the only loop A residue that is within 5 Å of 5-HT. There are currently no studies to confirm this, but mutation of neighboring residues Glu129 and Phe130 significantly alters the binding efficiency of 5-HT_3 antagonists *(63,64)*. In addition, changing Phe130 to tyrosine resulted in the receptor being activated by ACh *(64)*. This residue is homologous to the AChBP residue Tyr89 that has been identified as a key binding residue *(19)*. There might also be a role of loop A in the structure and/or assembly of the receptor; mutation of residues Trp121 and Pro123 results in receptors that are expressed but no longer reach the cell surface *(65,66)*.

Trp183, in loop B, is critical for both ligand binding and function and has been shown to form a cation–π bond with the primary amine of 5-HT *(67)*. This residue is equivalent to Trp149 in the nACh α_1-subunit, which also forms a cation-π interaction with acetylcholine *(68)*. The equivalent tryptophan residue in AChBP has been found to be a key component of binding of both nicotine and carbamylcholine *(59)*. Equivalent aromatic residues in $GABA_A$ and glycine receptors have also been shown to be vital for ligand binding *(69–71)*.

Fig. 4. (A) 5-HT_3 homology model based on the AChBP crystal structure (1I9B) showing the binding loops within two of the five subunits that make up the extracellular domain of the 5-HT_3 receptor. The binding site is formed from loops A–C within the principal subunit and loops D–F in the complementary subunit. **(B)** Locations of the amino acids that are proposed to be within 5 Å of the ligand-binding site.

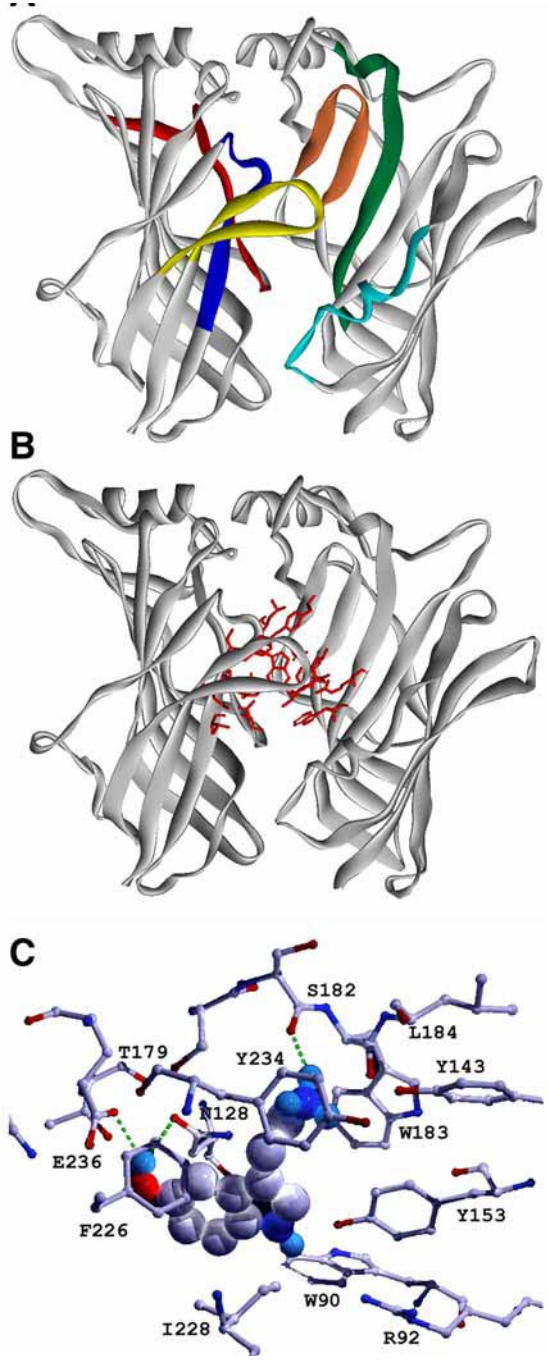

Fig. 4. *(continued)* Residues (black) are superimposed upon two adjacent subunits from the extracellular domain. Data from ref. *62*. **(C)** The proposed orientation of 5-HT in the 5-HT_3 receptor ligand-binding site. Modified from ref. *62*.

The loop C region has been extensively investigated by a number of groups. Studies have revealed that the aromatic group of Tyr234 is essential for both binding and function, whereas the hydroxyl group, or indeed any group at the 4-position, is required for efficient function *(72)*. Glu236 is also an important ligand-binding residue and might form a hydrogen bond with the agonist *(73)*. The C loop has been found to be critical in controlling the potency of both the 5-HT_3 receptor agonist mCPBG *(74)* and the antagonist *d*-tubocurarine *(75)*. However, a single amino acid responsible for the difference could not be identified, indicating that a number of other residues and/or binding regions might also play a role.

A considerable number of residues in loop D have been implicated in binding, showing that this region is important for agonist and antagonist interactions. An aromatic residue is required at both Trp90 and Trp95; Trp90 has a role in ligand binding, whereas Trp95 controls localization of the receptor to the cell surface *(76)*. Aromatic contacts have been demonstrated in AChBP between the residue equivalent to Trp90 (Trp53) and nicotine, and similar contacts probably occur in the 5-HT_3 receptor. There is also evidence that equivalent residues in nACh and $GABA_A$ receptors are important in binding, indicating that this position is functionally similar among many members of the ligand-gated ion channel family *(76)*. Other amino acids that have been studied include Tyr91, Arg92, and Tyr94. Mutation of these residues to alanine altered antagonist-binding affinity depending on the antagonist studied, indicating that different ligands have different points of interaction with the binding site *(77)*.

Loops E and F are considerably more varied than the above-described loops, and interestingly, the sequence variability seen between subunits of the same family suggests that the structures in these regions might differ according to the stoichiometry of the receptor. Scanning alanine mutagenesis of the E loop have revealed that Tyr143, Gly148, Glu149, Val150, Gln151, Asn152, Tyr153, and Lys154 might be important for granisetron binding *(78)*. In particular, mutation of Gly148 and Val150 completely abolished radioligand binding, although it is currently difficult to assign a particular role to these residues. Tyr143 and Tyr153 have been further studied using unnatural amino acid mutagenesis *(72,79)*. These studies have shown that whereas Tyr153 is involved in both binding and gating, the role of Tyr143 is primarily in gating, and it has been proposed to be involved in initiating the conformational changes that lead to channel opening *(79)*.

The role of loop F residues have yet to be elucidated. In the AChBP crystal structure, the loop F region was poorly resolved *(19)*; thus, its current location on the homology model on the 5-HT_3 receptor is only tentative. We await further studies to reveal the importance of this region.

6. 5-HT$_3$ Receptor Structure: The Transmembrane Region

The transmembrane region of the 5-HT$_3$ receptor consists of four transmembrane-spanning segments (M1–M4) that are linked by loops (Fig. 1). The structure of the M1–M4 segments is believed to be similar to that of the nACh receptor, which has been resolved to a resolution of 4 Å *(60)*. Consequently, the 5-HT$_3$ transmembrane segments are thought to be α-helical, an observation that is in agreement with predictions using hydrophobicity analysis, infrared spectroscopy, and circular dichroism *(1,80)*.

The M2 region of the 5-HT$_3$ receptor has been extensively explored using mutagenesis. Studies using the substituted cysteine scanning method (SCAM) have identified residues that run along the ion-accessible inner face of the channel *(83,89)*. These residues are predominantly nonpolar except for rings of charged amino acids. Surprisingly, scanning histidine accessibility mutagenesis (SHAM) on the 5-HT$_3$ receptor has suggested some differences in these water-accessible residues *(81),* which cannot yet be explained. SCAM analysis has also shown that movement in the center of M2 coincides with channel activation, indicating that this is the location of the channel gate *(82)*.

The part of M2 that controls ion selectivity, however, appears to be quite distinct and might involve several regions. Studies on the α7 nACh receptor *(83)* showed that changing only three amino acids could convert this channel from cationic to anionic, albeit with a substantial change in receptor properties. Comparable mutations in the 5-HT$_3$ *(84)*, glycine *(85),* and GABA ρ1 *(86)* receptors demonstrated that changing the equivalent amino acids in these channels also resulted in a change in ion selectivity, although, again, the mutant receptors had significant changes in some of their properties. These changes might be the result of insertion or removal of a proline residue, which was considered essential *(87)*. However, more recent work on the 5-HT$_3$ receptor indicates that this is not the case: Here, neutralizing a single charged ring at the cytoplasmic end of the pore yields a nonselective receptor, which can then be converted to an anion-preferring channel by insertion of a positively charged ring at the extracellular end *(88)*. These changes in selectivity were made without any changes in other channel properties and, therefore, suggest that ion selectivity is largely controlled by the presence of charged amino acids at one or both ends of M2.

7. Posttranslational Modulation of 5-HT$_3$ Receptors

The function of the 5-HT$_3$ receptors has been shown to be regulated by various protein kinases, probably via its large cytoplasmic domain, which contains a cluster of potential phosphorylation sites *(1)*. Activation of protein kinase A (PKA) substantially accelerates desensitization kinetics of 5-HT$_3$

receptors *(38,90,91)*, and activators of protein kinase C (PKC) increase the amplitude of 5-HT-activated currents *(92,93)*. In addition, the PKC activator PMA is reported to regulate the probability of occurrence of certain conductance levels of 5-HT-activated single-channel currents in N1E-115 cells *(94)* and there is some evidence that modulation of receptor responses by casine kinase II and tyrosine kinases might also occur *(95)*. A serine (S409) in the large intracellular loop of 5-HT_{3A} receptor has been found to be critical for PKA-induced phosphorylation of the receptor protein expressed in HEK293 cells *(96)*, but identifying a PKC phosphorylation site has proved more elusive. Neither single mutations or combinations of known sites significantly affected the sensitivity of the mutant receptors to PKC activation *(93,95)*.

There is, however, increasing evidence that PKC modulates 5-HT_3 receptor trafficking. Activation of PKC rapidly increases surface expression of 5-HT_{3A} receptors, which might occur via an F-actin-dependent mechanism; 5-HT_{3A} receptors are colocalized and coclustered with F-actin-rich membrane domains such as lamellipodia and microspikes *(93,97–100)*. In addition, preapplication of phalloidin, which stabilizes the actin polymerization, significantly inhibited PMA potentiation of 5-HT-activated responses, and latrunculin-A, which disrupts F-actin cytoskeletons, altered the topology and the size of 5-HT_{3A} receptor clusters *(97)*. Given that neurotransmitter release can be regulated through an actin-dependent mechanism in the CNS *(101)* and that 5-HT_{3A} receptors can modulate the release of dopamine and GABA in some important brain areas, it is possible that the enhancement of 5-HT_3 receptor function and trafficking by PKC activation might play an important role in modulating the efficacy of serotonergic synaptic troansmission.

Other processes of posttranslational modulation such as protein glycosylation and palmitorylation have also been described in the studies of 5-HT_{3A} receptors *(102–104)*. The exact roles of these processes in the regulation of 5-HT_{3A} receptor assembly, targeting, and trafficking is yet to be determined.

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15

5-HT_3 and 5-HT_4 Receptors as Targets for Drug Discovery for Dementia

Frank Lezoualc'h and Magali Berthouze

Summary

Serotonin (5-hydroxytryptamine) 5-HT_3 and 5-HT_4 receptors are widely distributed in central and peripheral nervous systems, where they participate in many physiological functions. With the availability of selective 5-HT_3 and 5-HT_4 receptor ligands and the molecular identification of 5-HT_3 and 5-HT_4 receptors, the involvement of these receptors in cognitive processes has been largely suggested. This chapter reviews recent data on the brain distribution and pharmacological properties of 5-HT_3 receptor subunits and 5-HT_4 receptor C-terminal splice variants. The potential use of 5-HT_3 and 5-HT_4 receptor ligands as therapeutical agents influencing the cholinergic system and the amyloid precursor protein processing for the treatment of Alzheimer's disease and other dementia are discussed.

Key Words: Alzheimer's disease; 5-HT_4 ligands; amyloid; cAMP; memory; small G proteins.

1. Introduction

Alzheimer's disease (AD) along with vascular and mixed dementia is the commonest form of dementia affecting older people and accounts for 60–65% of dementia cases, whereas vascular dementia and mixed dementia account for 15–20% of the cases each *(1)*. The Brain of individuals with AD manifest two characteristic lesions: senile plaques and intracellular neurofibrillary tangles of the hyperphosphorylated tau protein *(2)*. The amyloid ß-protein (Aß) is the principal component of the senile plaques. It is a peptide of 39–43 amino acids, derived from a larger precursor, the amyloid precursor protein (APP) (Fig. 1).

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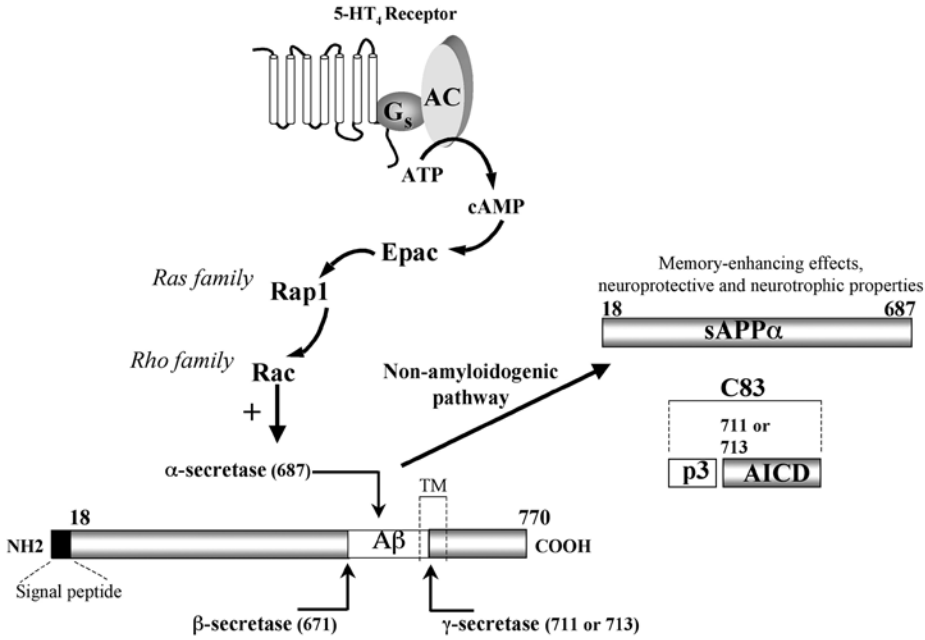

Fig. 1. The 5-HT_4 receptor is a stimulator of APP processing (APP770). The largest form of APP containing 770 amino acids is depicted. A 17-residue signal peptide (black square box) occurs at the N-terminus of the molecule. The transmembrane domain is located between amino acids 700 and 723. Activation of the 5-HT_4 receptor increases cAMP production, which binds and activates the guanine nucleotide exchange factor (GEF) Epac. This GEF stimulates the nonamyloidogenic pathway of APP through Rap1 and Rac and induces the extracellular release of sAPPα and retention of the 83-residue C-terminal fragment (C83) in the membrane. C83 can undergo cleavage by the γ-secretase at residue 711 or 713 to release the p3 peptides and the APP intracellular domain. sAPPα has potent memory-enhancing effects and display neuroprotective and neurotrophic properties *(92)*. In the case of the h5-$HT_{4(e/g)}$ receptor isoform, this signaling cascade has no effect on Aβ production *(100)*. AC, adenylyl cyclase; Aβ, amyloid-β protein; AICD, APP intracellular domain; APP, amyloid precursor protein; GEF, guanine nucleotide exchange factor; PKA, protein kinase A; TM, transmembrane domain.

Epidemiological studies have shown that mutations in APP and presenilin genes are linked to rare familial and early-onset forms of AD *(2)*. This observation led to the "amyloid cascade hypothesis" suggesting that excessive Aß production is the primary cause of the disease.

The brain of AD patients is also characterized by the degeneration of basal forebrain cholinergic neurons, which innervates central regions involved in cognitive functions such as the cortex amygdala and hippocampus *(1,3)*. The decline of cortical cholinergic activity as measured in postmortem brains

correlates with the severity of AD symptoms and the intellectual deterioration *(4)*. Cortical cholinergic dysfunction in AD is also found in other neurodegenerative disorders such as Parkinson's disease (PD). Therefore, the cholinergic hypothesis of memory has led to multiple studies attempting cholinergic replacement therapy in these neurodegenerative diseases, and restoration of cholinergic function remains a major objective in the development of pharmacological approaches to the treatment of cognitive dysfunctions associated with aging and dementia *(5)*. In addition to the cholinergic system, loss of other neurotransmitters such as glutamate and serotonin (5-hydroxytryptamine or 5-HT) have been reported in AD *(6,7)*. The serotoninergic system is of particular interest because it interacts with many other neurotransmitter systems such as glutaminergic, GABAergic, and cholinergic pathways and is involved in learning and memory processes *(8,9)*. Many neurochemical and anatomical studies have indeed shown a functional interaction between serotonergic and cholinergic systems to cooperate in the regulation of cognitive processes. In addition, several lines of evidence indicate that Aβ can negatively regulate various steps in the synthesis and release of acetycholine (ACh), suggesting a link between amyloid burden and cholinergic impairment in AD *(10)*.

With the availability of selective 5-HT_3 and 5-HT_4 ligands and the molecular identification of 5-HT_3 and 5-HT_4 receptors, the involvement of these receptors in learning and memory has been largely suggested. The present chapter reviews recent data showing the potential use of 5-HT_3 and 5-HT_4 ligands as therapeutical agents influencing APP processing and the cholinergic system for the treatment of AD and other dementia.

2. Distribution of 5-HT_3 and 5-HT_4 Receptors in the CNS

Of the seven classes of serotonin receptors, the 5-HT_3 receptor is the only ligand-gated ion channel receptor for 5-HT in vertebrates and its activation results in the influx of Na^+ and Ca^{2+} *(11)*. Only two subunits, 3A and 3B, are currently known. The 5-HT_{3A} subunit contains four transmembrane domains, and two splice variants differing by six amino acids in their putative large intracellular loop have been cloned in different species except human *(12–14)*. So far, the physiological relevance of the existence of these two 5-HT_{3A} receptor isoforms are unknown. The structure of the 5-HT_{3B} receptor is more complex because its second transmembrane domain lacks any structural homology with other ligand-gated ion channels *(15)*. Native 5-HT_3 receptors show distinct functional properties from homopentameric 5-HT_3 receptors *(16)*. In contrast to the 5-HT_{3A} subunit, expression of a recombinant 5-HT_{3B} alone does not produce any functional effect and its participation in the formation of functional native 5-HT_3 receptors is still an open question *(17)*.

In situ hybridization histochemistry and reverse transcriptase–polymerase chain reaction (RT-PCR) experiments demonstrated that 5-HT_{3A} subunit transcripts are expressed in central and peripheral neurons, whereas 5-HT_{3B} subunit mRNA seems to be restricted to peripheral neurons *(18)*. In general, mRNA and radioligand-binding sites follow the same distribution. Central 5-HT_3 receptor-binding sites have been mapped using various radioligands such as [^{3}H]-zacopride and [^{3}H]-tropositron. Briefly, the highest levels of 5-HT_3 receptor-binding sites are located within the dorsal vagal complex in the brainstem (Table 1). To the contrary, lower levels are found in the hippocampus, amygdala, and superficial area of the cerebral cortex *(19)*. However, some specie differences might exist. For instance, levels of 5-HT_3 receptors expressed in human caudate nucleus and putamen are higher than those found in the rat. In the different species studied so far, the hippocampus expresses the highest density of 5-HT_3 receptors compared to other forebrain regions *(20)*. The presence of 5-HT_3 receptors in the hippocampus and amygdala has suggested their possible role in memory and learning.

Several groups have isolated and sequenced C-terminal splice variants of the 5-HT_4 receptor in different species. Eight C-terminal isoforms and one internal splice variant have been currently identified in the human and named h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, h5-$HT_{4(d)}$, h5-$HT_{4(e)}$, h5-$HT_{4(f)}$, h5-$HT_{4(g)}$, h5-$HT_{4(n)}$, and h5-$HT_{4(hb)}$ *(21–25)*. The h5-$HT_{4(e)}$ will be called h5-$HT_{4(e/g)}$ in this chapter, as this isoform was later renamed h5-$HT_{4(g)}$ *(23)* and replaced by another h5-$HT_{4(e)}$ isoform that is more homologous to the mouse 5-$HT_{4(e)}$ receptor (*22*). All of the h5-HT_4 receptor isoforms belong to the seven-transmembrane-spanning G protein–coupled family of receptors (GPCRs) and are generated by alternative splicing from a common gene that spans more than 700 kb and is divided in at least 38 exons *(23,26)*. Whatever the animal species (mouse, rat, or human), the different splice variants have an identical sequence up to Leu^{358}, whereas the length and the composition of the rest of C-terminus tail is specific for each splice variant. Tissue distribution studies revealed that most of the h5-HT_4 receptor isoforms are expressed throughout the brain and the functional relevance of the expression of multiple 5-HT_4 receptor C-terminal splice variants is still an open question (Table 1) *(27)*. All of the 5-HT_4 receptor isoforms are positively coupled to adenylyl cyclase, leading to cAMP production (*see* below) *(28)*.

The distribution of 5-HT_4 receptors has been extensively studied using the selective labeled 5-HT_4 antagonists [^{3}H]GR113808 and [^{125}I]SB207710 in several species such as the rat, pig, and human *(29–33)*. In the rat and guinea pig central nervous system (CNS), the 5-HT_4 receptor is highly expressed in the mesolimbic system as well as in the extrapyramidal motor system *(29,31)*. In the human, the 5-HT_4 receptor is located in the basal ganglia, hippocampus, frontoparietal and entorhinal cortex, and substantia nigra *(29–32)*. These binding

Table 1
Distribution and Expression of 5-HT$_3$ and 5-HT$_4$ Receptors in Postmortem Human Brain

Brain region	5-HT$_3$ Receptors	5-HT$_4$ Receptors		
	Specific [^{3}H]-(S)-zacopride binding sites (from ref. *36*)	Specific [^{3}H]GR113808 binding sites (from ref. *30*)	*In situ* hybridization (from ref. *32*)	Quantitative PCR (from ref. *27*)
Cortex				
Frontal	+	++	nd	nd
Parietal	0	++	nd	nd
Occipital	nd	nd	nd	nd
Temporal	+	nd	nd	++
Basal ganglia				
Caudate-putamen	+	+++	+++	+++
Nucleus accubens	+	nd	+++	+
Globus pallidus (ext-int)	0	nd	0	nd
Substantia nigra	+	+++	0	0
Hippocampus				++
Hippocampus CA1	++	+++	+++	
Hippocampus CA2	+	nd	+++	
Hippocampus CA3	+	++	+++	
Hippocampus CA4	+	nd	nd	
Subiculum	+	nd	++	
Entorhinal cortex	+	++	++	
Dentate gyrus	+	++	+++	
Amygdala	+	nd	nd	++
Thalamus	nd	nd	nd	+/–
Dorsal vagal complex				
Dorso-medial nucleus tractus solitarius	+++	nd	nd	nd
Area postrema	++	nd	nd	nd
Nucleus and tractus solitarus	++	nd	nd	nd
Dorsal motor nucleus of the vagus nerve	++	nd	nd	nd

Abbreviations: nd, not determinated; 0, not detected; +/–, low level; +, moderate level; ++, high level; +++, very high level.

experiments paralleled those recently obtained by *in situ* hybridization and quantitative RT-PCR in the human because the highest levels of expression were also found in the basal ganglia and the hippocampal formation (Table 1) *(27,32)*. Interestingly, real-time quantitative PCR has demonstrated that the h5-$HT_{4(b)}$ is the most predominant form in the CNS and peripheral tissues *(27)*. A consistent finding across the species studied is the relative high expression of the 5-HT_4 receptor in the limbic system, particularly in the septo-hippocampal region, which suggests a role for this 5-HT receptor in learning and memory.

3. Pharmacology of 5-HT_3 and 5-HT_4 Receptors

There are many highly potent 5-HT_3 receptor antagonists. Their nonspecific effects appear to occur at concentrations in excess of those required to antagonize 5-HT_3 receptors by at least 100-fold *(34)*. The nonselective effects of 5-HT_3 receptor antagonists are observed at 5-HT_4 receptors, where they behave as agonists *(28)*. They also include local anestheticlike block of ligand- and voltage-gated ion channels *(35)*. In addition to the recognition site for 5-HT, the 5-HT_3 receptor possesses additional sites that interact with anesthetic agents and alcohols *(36)*.

The pharmacological binding profile determined in competition studies revealed no major differences in the affinity of 5-HT_4 ligands for the recombinant 5-HT_4 receptor splice variants transfected in mamalian cell lines *(28)*. It is also noteworthy that the different h5-HT_4 receptor variants displayed the same binding profile of the 5-HT_4 receptors previously observed in native tissues in different species *(22,23,25,37)*.

As mentioned earlier, all h5-HT_4 receptor isoforms are positively coupled to adenylyl cyclase in vitro. However, in contrast to binding studies, very striking differences in cAMP functional studies were observed between the pharmacological profiles of the h5-HT_4 receptor isoforms *(24,37–39)*. For instance, the h5-$HT_{4(e/g)}$ receptor isoform is less potent than the h5-$HT_{4(d)}$ receptor to increase cAMP accumulation in response to 5-HT_4 agonists in Chinese hamster ovary (CHO) cells *(37)*. In addition, the efficacy of a given 5-HT_4 ligand might also differ from one isoform to another *(37)*. This is illustrated by the pharmacological behavior of the benzamide derivative renzapride at the d- and e/g-isoforms. Renzapride displays full agonist properties at the h5-$HT_{4(d)}$ receptor, whereas it behaves as a partial agonist at the h5-$HT_{4(e/g)}$ receptor *(37)*. These data show that structural differences in the C-terminal tails of h5-HT_4 receptors might influence and contribute to the specificity of their functional pharmacological profile.

These differences in 5-HT_4 receptor pharmacological profile might explain the pharmacological differences of 5-HT_4 compounds observed between the different tissues. For instance, differences observed between the 5-HT_4 receptors

from neurons (full response to renzapride) and cardiac cells (weak response to renzapride) might well be interpreted by the functional dominance of a given receptor subtype in these tissues. Therefore, one could speculate that a given 5-HT_4 receptor isoform might regulate a specific cellular function. Alternatively, several 5-HT_4 receptor isoforms expressed in a given tissue could form heterodimers with distinct functional pharmacological properties. This molecular mechanism has been recently described for other GPCRs such as the opioid receptors *(40)*. Yet, tissue-specific proteins could interact with the intracellular C-terminal tails of h5-HT_4 receptors and influence their intracellular signaling. It is interesting to note that the h5-$HT_{4(a)}$, h5-$HT_{4(e/g)}$ receptor splice variant displays in its C-terminal end a consensus motif for PDZ-domain-containing proteins *(22,24,41)*. These proteins with PDZ domains mediate direct interaction with GPCRs to influence their localization and intracellular events *(41,42)*. As detailed earlier, functional differences between the 5-HT_4 splice variants reside in the pharmacology of their cAMP response. Recent evidence also indicates that 5-HT_4 receptor isoforms are able to couple to distinct heterotrimeric G proteins and might trigger several second-messenger signaling pathways. Ponimaskin and colleagues *(43)* reported that the h5-$HT_{4(a)}$ receptor isoform is functionally coupled both to G_s and G_{13} proteins in Sf.9 cells. When stably expressed in HEK293 cells, the h5-$HT_{4(b)}$ receptor is able to activate both to $G_{i/o}$ and G_s proteins, whereas the h5-$HT_{4(a)}$ receptor is only coupled to G_s proteins *(39)*. Although these data provide additional evidence for a functional diversity of 5-HT_4 receptor splice variants, the physiological relevance of these different signaling pathways remain to be determined.

4. Expression of 5-HT_3 and 5-HT_4 Receptors in Neurodegenerative Disorders

Apparently, expression of 5-HT_3 receptors are conserved in AD *(44)*. However, it is clear that further studies are required to draw any definitive conclusion on their level of expression in degenerative disorders. Regarding 5-HT_4 receptor-binding sites, Wong and colleagues *(45)* have determined the amount of binding for the labeled 5-HT_4 antagonists [^{3}H]-GR113808 in the postmortem brain from patients with AD, PD, and Huntington's disease. A decrease in binding of 67%, 30%, and 26% in the hippocampus, temporal, and prefrontal cortex, respectively, was observed from AD brains. No significant deficits were found in the motor frontal cortex of AD subjects or in the putamen and substantia nigra of PD patients, whereas 5-HT_4 receptor density was decreased by 50% in the putamen of patients with Huntington's disease. In contrast to this study, a recent report did not show any significant difference in 5-HT_4 receptor density between AD and control patients *(46)*. The reasons for this discrepancy

could reside, for instance, in the experimental protocol employed by the authors to quantify the receptors as well as the number and clinical status of the patients *(46)*. Interestingly, the radiolabeled specific 5-HT_4 antagonist [^{125}I]SB 207710 has been shown to be an effective radioligand for single photon emission tomography (SPET) studies in vivo in the monkey brain *(6)*. This study, combined with the recent observation that SB 207710 can be successfully used for the detailed anatomical localization of 5-HT_4 receptors in the human brain whole-hemisphere autoradiography, suggests that this ligand might provide an important complement to in vivo studies using SPET *(47)*.

5. Cognitive Performance

Serotonin 5-HT_3 receptor antagonists such as ondansetron, granisetron, tropisetron, itasetron, and WAY100579 have been shown to display precognitive effects and counteract the deficits in learning associated with dysfunction of central cholinergic neurons during aging (Table 2) *(8)*. For instance, cognitive deficits induced by muscarinic receptor blockade *(48,49)* and or lesions of nucleus basalis magnocellularis *(50)* are ameliorated with ondansetron. However, it has also been reported that 5-HT_3 antagonists did not always attenuate memory dysfunctions *(51,52)*. This is illustrated by ondansetron, which failed to attenuate the scopolamine-induced impairment in a Stone maze task *(51)*. In addition, ondansetron has been abandoned because of the lack of efficiency in clinical trials *(53)*. It has been shown that the effective action of 5-HT_3 antagonists might be task dependent, suggesting that they might be helpful in treating a subset of cognitive functions *(54)*. Also, repeated treatments might be required, as has been shown for DAU 6215 *(55)*. In addition to their effects on cognition, 5-HT_3 receptor antagonists have also been shown to be useful in the treatment of noncognitive disorders such as anxiety that occur frequently in AD *(56)*.

Several behavioral studies have demonstrated that 5-HT_4 receptor agonists can be considered as cognitive and function enhancers because they modulate several aspects of memory performance (Table 3) *(33,57)*. Initial studies were performed with nonselective 5-HT_4 agonists such as BIMU-1, BIMU-8, and RS-66331. Activation of 5-HT_4 receptors with these drugs improved social learning, prevented amnesia, and reversed deficits in learning and memory following hypercapnia and hypoxia *(58–61)*. However, because these compounds display antagonist properties for the 5-HT_3 receptor, more selective 5-HT_4 receptor agonists were subsequently used. For instance, the potent and selective hydrophobic 5-HT_4 receptor agonist RS-67333 was shown to reverse the atropine-induced deficit performance in the Morris water maze and this effect was inhibited by a potent 5-HT_4 receptor antagonist, GR113808 *(62)*. In addition, RS-67333 enhanced the rate of learning acquisition in the normal rat *(63)* and reversed the

Table 2
Cognitive Properties of Two 5-HT$_3$ Receptor Antagonists

5-HT$_3$ Receptor antagonists	Cognitive properties	Ref.
Ondansetron	Improved acquisition of a visual object discrimination at very low doses in aged rhesus monkeys and marmosets. No improvement was found on the delayed response or fine motor tasks.	*54*, *112*
	Reversed the memory deficits as a result of scopolamine treatment but failed to attenuate the scopolamine-induced impairment in a Stone maze task.	*51*, *52*
	Scopolamine-induced impairment of learning and retention in the water maze was fully prevented by ondansetron when given in combination with a cholinesterase inhibitor, tacrine. Combined treatment with ondansetron and flumazenil was able to significantly increase ACh release in situations of cholinergic hypoactivity.	*73*, *110*
Tropizetron	Counteracted the learning and memory impairments resulting from scopolamine treatment	*52*

deficit in the acquisition of olfactory associative memory induced by a 5-HT$_4$ antagonist, RS-67532, in the rat *(64)*. Selective 5-HT$_4$ receptor agonists were also able to improve social learning in rats *(58)*, increased memory performances in delayed matching tasks in both young and old macaques *(65)*, and enhanced place and object recognition in young adult rats *(66)*. Taken together, these results suggest that 5-HT$_4$ receptor agonists might be relevant for the treatment of memory dysfunction in patients suffering from AD.

6. Modulation of ACh Release

The memory-enhancing properties of 5-HT$_3$ receptor antagonists has been suggested to involve the release of cortical ACh *(67)*. Indeed, the 5-HT$_3$ receptor antagonist ondansetron produced an enhancement in ACh release in slices from the rat cerebral cortex. This effect was potentiated by GABA$_A$ receptor antagonists *(68,69)*, suggesting that the mechanism for 5-HT$_3$ antagonists-induced ACh release would be a blockade of an inhibitory influence of GABA on cholinergic neurons. Accordingly, it was further reported that GABA$_A$ receptor antagonists, such as bicuculline and and flumazenil, enhanced ACh release induced by 5-HT$_3$ blockade in freely moving rats *(70)*. These data are

Table 3
Cognitive Properties of Some 5-HT$_4$ Receptor Agonists

5-HT$_4$ Receptor agonists	Cognitive properties	Ref.
BIMU-1 (5-HT$_4$ receptor agonists/5-HT$_3$ receptor antagonists)	Active on the social olfactory recognition test and on the olfactory associative test. Theses effects were antagonized by a selective 5-HT$_4$ antagonist, GR125487.	*58*, *59*
	Reversed spontaneous alternation deficits induced by scopolamine in the mouse.	*113*
	Prevented the amnesia induced not only by 5-HT$_4$ receptor antagonists, but also by antimuscarinic drugs and exposure to a hypoxic environment.	*61*
RS67333 (hydrophobic 5-HT$_4$ receptor agonist)	Reversed the atropine-induced deficit performance in the Morris water maze and enhanced place and object recognition memory in young adult and old rats.	*62*, *66*
	Reversed spontaneous alternation deficits induced by scopolamine in the mouse.	*113*
	Partially reversed impairment of learning and memory performance induced by a selective of 5-HT$_4$ receptor antagonist, RS67532.	*64*
	Accelerated learning acquisition in the rat.	*63*
	At doses ineffective alone, it enhanced place and object recognition in young adult and old rats when coinjected with galanthaminium bromide, a cholinesterase inhibitor.	*109*
RS17017 (hydrophilic 5-HT$_4$ receptor agonist)	Enhanced delayed matching performance in younger and older macaques.	*65*
SL65.0155	Currently under clinical trial (phase II). SL65.0155 and a cholinesterase inhibitor, rivastigmine, had a synergic effect in the object recognition task and in linear maze performances in aged rats.	*108*

consistent with the findings that GABAergic neurones express 5-HT$_3$ receptors *(71)* as well as electrophysiological studies showing a facilitation of GABA release upon 5-HT$_3$ receptor activation *(72)*. Moreover, when evaluated for their effects on cognition, a full reversal of the learning impairment induced by scopolamine was found after the combined treatment with ondansetron and flumazenil *(73)*. Thus, the concomitant blockade of 5-HT$_3$ and GABA$_A$

receptors could contribute to partially restore a loss of cholinergic function, which might obviously have implications for the treatment of associated neurodegenerative disorders such as AD.

The mechanism underlying 5-HT_4-induced cognitive improvement is likely to involve increased neuronal excitability and an increase in neurotransmitter release. In the CA1 pyramidal neurons of hippocampus, 5-HT_4 receptors increase cAMP production, leading to protein kinase A (PKA), which inactivates calcium-activated and voltage-sensitive potassium channels. This process would reduce the afterhyperpolarization that follows action potentials and increase neuronal excitability *(57,74,75)*. This is supported by electrophysiological evidence demonstrating that the 5-HT_4 agonists, zacopride, prucalopride, as well as SL65.0155 can enhance the population spike recorded in the CA1 region of the hippocampus *(76,77)*. Interestingly, a 5-HT_4 receptor-increased population spike amplitude is still observed in a transgenic mice model of AD that overexpress Aβ *(77)*. These results indicate that 5-HT_4 receptors are functional in an animal model of AD because they are still capable of inducing significant enhancement of neuronal excitability despite the excessive amounts of Aβ and dystrophic neurones.

In vivo neurochemical studies have shown an increased ACh release in the rat frontal cortex following intracerebroventricular injection of 5-HT_4 agonists *(78,79)*. Such regulation of 5-HT_4 ligands on cholinergic neurons was also observed in rat hippocampus *(80)* because the benzamide derivative SC 53116 enhanced in a dose-dependent manner the extracellular levels of ACh in the hippocampus of freely moving rats. This effect of SC 53116 on ACh release was prevented by the coperfusion of a specific 5-HT_4 antagonist, GR113808. This latter did not influence ACh release under basal conditions, indicating that the 5-HT_4 receptor does not exert any tonic effect on the cholinergic neuronal system in rat hippocampus *(80)*. Finally, SC 53116 enhanced the amplitude of long-term potentiation (LTP), a phenomenon commonly believed to be important for the mechanisms of learning and memory *(80)*.

7. 5-HT_4 Receptors Influence APP Processing

Whereas drugs are currently available that might slightly ameliorate late-stage symptoms such as cognitive deficits, no drugs are on the market that specifically target the cellular mechanisms of AD, namely the generation of the neurotoxic Aß peptide from APP. APP is an integral membrane glycoprotein constitutively expressed in many types of mammalian cell. APP is first cleaved by the ß-secretase BACE1 at the N-terminus of the Aß domain *(81)*. This cleavage generates the soluble sAPPß and a C-terminal fragment, which undergoes a second cleavage by a protease called γ-secretase (Fig. 1). Since the

combined action of ß- and γ-secretase leads to Aß peptide generation (Fig. 1), the inhibition of their activity is considered to be a highly promising approach to treat AD *(82)*. However, the ß- and γ-secretases might be implicated in the processing of other proteins having specific functions and, consequently, the use of inhibitors might have unwanted side effects *(83,84)*.

In contrast, cleavage of APP by α-secretase occurs in the Aβ sequence at residue 687 (of the 770 isoform) and releases a large soluble N-terminal ectodomain named sAPPα into the extracellular space (Fig. 1). This process is called the nonamyloidogenic pathway because it occurs in the Aβ sequence and, in theory, precludes the formation of Aβ peptide (Fig. 1). Three members of the ADAM family (ADAM stands for a disintegrin and metalloprotease), ADAM-9, ADAM-10, and TACE (tumor necrosis factor-α-converting enzyme, ADAM17), have been identified as candidates for the α-secretase activity of APP *(85–88)*. These metalloproteases are membrane-anchored proteins involved in the proteolytic cleavage or "shedding" of many transmembrane protein ectodomains such as certain growth factors, adhesion molecules, and enzymes *(89)*.

The α cleavage is of physiological and therapeutical interest because secreted sAPPα has putative neurotrophic and neuroprotective properties *(90–92)*. In addition, sAPPα stimulates neurite outgrowth, regulates neuronal excitability and has potent memory-enhancing effects *(92–95)*. APP processing is highly regulated by neurotransmitters and their associated receptors *(96)*. Therefore, considerable emphasis is being placed on the pharmacological modulation of APP processing, which aims to enhance cleavage of APP by α-secretase and reduce Aβ formation. An important observation toward the understanding of the pharmacological regulation of APP metabolism comes from the fact that through a complex network of second messengers, GPCRs might regulate the α-cleavage of APP *(92)*. A pioneering work in this field was the study of Nitsch et al. *(97)*, who showed that the muscarinic acetylcholine M1 and M3 receptor subtypes stably transfected into HEK cells increased extracellular release of sAPPα with a concomitant reduction in the levels of Aβ *(98,99)*. Because strong evidence supports beneficial effects of 5-HT_4 agonists in memory and learning, we investigated the potential involvement of the 5-HT_4 receptor in APP processing *(100)*. Activation of the h5-$HT_{4(e/g)}$ receptor stimulates the secretion of sAPPα. The increase is inhibited by the selective 5-HT_4 receptor antagonist, GR113808. The regulation by the 5-HT_4 receptor of APP processing is also observed in the primary culture of embryonic mouse cortical neurons and in IMR32 human neuroblastoma cells *(100–102)*. Furthermore, secretion of sAPPα induced by the h5-$HT_{4(e)}$ receptor seems not to be the result of a general boost of the constitutive secretory pathway, but, rather, to its specific effect on α-secretases (Robert and Lezoualc'h, unpublished observation). As mentioned earlier, to date none of

the pharmacological studies performed at the cloned 5-HT_4 receptor splice variants has revealed any difference in their binding profile. However, structural differences in the C-terminal tails of 5-HT_4 receptors are known to influence and contribute to the specificity of their cAMP functional response. At present, we still do not know whether such functional differences between the splice variants might also influence the metabolism of APP and the cholinergic functions both in vitro and in vivo. Therefore, a major challenge for the future will be to determine which central 5-HT_4 receptor isoform is the best pharmacological target for the treatment of AD.

An interesting observation is that the effect of the 5-HT_4 receptor on sAPPα release is independent of PKA *(102)*. In addition, the signaling pathway that couples the 5-HT_4 receptor to sAPPα secretion involves small GTPases of the Ras and Rho families. Small G proteins are involved in many cellular processes and are activated by guanine nucleotide exchange factor (GEF), which catalyzes exchange of GDP for GTP *(103)*. The effects of the 5-HT_4 receptor on APP processing are mediated by the cAMP regulated GEF epac. Activation of epac stimulates extracellular release of sAPPα through the small GTPases Rap1 and Rac in a cAMP-dependent but PKA-independent manner (Fig. 1) *(33,102)*. The finding that the cAMP GEF epac and the small GTPase Rac are involved in this process might open new avenues for the identification of new targets for the regulation of APP processing.

8. Conclusion and Perspectives

The above-discussed studies indicate that 5-HT_3 antagonists and 5-HT_4 agonists display potential therapeutic properties for the treatment of cognitive disorders. These drugs could overcome the cholinergic deficit and the overproduction of Aβ protein observed in AD. However, the bioavailability of specific compounds able to cross the blood barrier must be improved. In addition, one major drawback for the use of 5-HT ligands in the treatment of memory disorders resides in their putative second side effects, especially for 5-HT_4 receptor agonists, which display many functional effects at the periphery *(28)*. In the gastrointestinal system, stimulation of 5-HT_4 receptors has a pronounced effect on smooth muscle tone, mucosal electrolyte secretion, and the peristaltic reflex *(104)*. In the adrenal cortex, they increase steroid hormone release, such as aldosterone and corticosterone *(105)*. Second side effects of 5-HT_4 agonists can occur in the bladder, where they induce polyuria and urinary incontinence *(106)*. In addition, there is in vivo, in vitro, and clinical evidence that stimulation of 5-HT_4 receptors can trigger atria arrhythmias, including atria fibrillation *(107)*. Thus, lowering the dose of 5-HT_4 ligands could attenuate their adverse effects. In this matter, a bitherapeutic strategy has been proposed not only to

decrease the dose-dependent effects of the drugs but also to obtain a synergic action of the compounds *(108,109)*. The recent work of Lamirault and colleagues *(109)* nicely supports this idea. In their study, the authors investigated the combination of a new acetylcholinesterase inhibitor, galanthaminium bromide, and the 5-HT_4 receptor agonist RS-67333 at doses that were ineffective alone in a recognition memory task. In old rats, the combined treatment improved both acquisition and consolidation processes in the object recognition task *(109)*. Promising results were obtained with the 5-HT_4 agonist SL65.0155 *(108)*. This 5-HT_4 agonist displayed almost no second side effects and could act synergistically with the cholinesterase inhibitor rivastigmine. SL65.0155 is currently under clinical investigation (phase II).

To date, there are no clinical data to substantiate the utility of 5-HT_3 receptor antagonists for the treatment of cognitive disorders. The recent study of Diez-Ariza and colleagues *(110)* has open new hope for the use of 5-HT_3 receptor antagonists as memory-enhancing agents. They showed that the scopolamine-induced impairment of learning and retention in the water maze is fully prevented by ondansetron when given in combination with a cholinesterase inhibitor, tacrine. Because tacrine cause serious adverse effects with only a very limited therapeutic benefit *(111)*, these approaches of combination therapy might not only improve the effects of the drugs on cognitive performance but also reduce their second side effects.

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16

Electrophysiological Properties of $G\alpha_s$-Coupled 5-HT Receptors (5-HT_4, 5-HT_6, 5-HT_7)

Rodrigo Andrade

Summary

Serotonin receptors of the 5-HT_4 and 5-HT_7 subtypes couple to heterotrimeric G proteins of the $G\alpha_s$ type and signal their electrophysiological effects by stimulating adenylate cyclase, increasing intracellular cAMP, and activating protein kinase A (PKA). These receptors, like many other receptors coupling to $G\alpha_s$, modulate three classic currents in excitable tissues: the hyperpolarization-activated cation current I_h, a calcium-activated potassium current generally known as I_{sAHP}, and the L-type calcium current. 5-HT_4 and 5-HT_7 receptors inhibit I_{sAHP} and facilitate L-type calcium currents by increasing cAMP and activating PKA. However, these receptors facilitate I_h by a direct effect of cAMP that is independent of PKA. Other currents might also contribute to the postsynaptic effects signaled by these receptors in specific neuronal cell types. Little is known at the present time regarding the electrophysiological responses signaled by the activation of 5-HT_6 receptors. A key remaining question is how these receptors and currents are regulated by synaptically released serotonin in a physiological context.

Key Words: Serotonin; 5-HT receptor subtypes; cAMP; protein kinase A; $G\alpha_s$.

1. Introduction

The discovery of serotonin in the 1950s and the realization of its potential role in the pathophysiology of mental disorders launched a sustained effort to

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understand the specific mechanisms by which serotonin regulates neuronal activity in the brain. In this context, electrophysiologists attempted to contribute to this program by identifying how serotonin modulates neuronal excitability and thus regulates the function of neuronal networks. This effort was greatly facilitated by work in the later part of the 20th century that identified a superfamily of serotonin receptor subtypes *(1)*. Hand in hand with these conceptual developments, synthetic chemistry approaches led to the development of pharmacological tools capable of selectively targeting specific 5-HT receptor subtypes. These conceptual and technical developments made it possible for electrophysiologists to begin to identify with confidence the specific 5-HT receptor subtype mediating the physiological responses they encountered in central and peripheral neurons.

Electrophysiological work started during the 1980s led to the realization that specific 5-hydroxytryptamine (5-HT) receptor subtypes tended to activate specific ion currents and that this linkage was highly conserved across cell types. Furthermore, it revealed that the linkage between 5-HT receptor subtypes and ion channels was predictable based on the ability of the receptor to couple to specific G proteins. A corollary of these conceptual breakthroughs was the realization that the highly pleomorphic effects of serotonin on membrane excitability that had plagued earlier studies in fact could be explained by the expression of multiple 5-HT receptor subtypes within single neurons and across cell populations. Our understanding of the electrophysiology of 5-HT_4 and 5-HT_7 receptors falls squarely within this historical context.

2. 5-HT, 5-HT Receptors, and cAMP

The realization that some of the effects of serotonin could be signaled through the activation of adenylate cyclase and the increase in intracellular cAMP can be traced to some of the earliest work on neuronal signaling in *Aplysia (2)*. As such, it is present at the birth of our current understanding of the mechanisms by which G protein–coupled receptors regulate neuronal function. Subsequent work extended this work to mammalian neurons and led to the identification of several signature effects of 5-HT on specific ion currents that appeared signaled through cAMP and protein kinase A (PKA). However, it was not until the cloning of 5-HT receptor subtypes coupling to $G\alpha_s$ that a specific linkage could be established between 5-HT receptor subtypes and the regulation of specific ion currents. In this chapter, the work leading to our current understanding of the specific electrophysiological effects signaled by 5-HT_4 and 5-HT_7 receptors in neurons and heart cells is reviewed.

2.1. Regulation of Calcium-Activated Potassium Currents by 5-HT$_4$ and 5-HT$_7$ Receptors in the Hippocampus

Many neurons in the central and peripheral nervous system display pronounced calcium-dependent afterhyperpolarizations (AHP) that play a key role in sculpturing neuronal responses to excitatory stimuli *(3)*. These AHPs are mechanistically complex and are mediated by the activation of at least three types of calcium-activated potassium current *(3,4)*. One of the classical effects signaled via cAMP and PKA in the central nervous system is the inhibition of the current responsible for the slow component of the AHP (sAHP). Because the sAHP curtails sustained spiking in response to excitatory stimuli, the net effect of inhibiting the current underlying this afterpotential is to increase neuronal excitability *(5)* (Fig. 1A).

Early work on the pharmacology of 5-HT receptors in the hippocampus identified an orphan receptor capable of inhibiting the sAHP in pyramidal cells of the CA1 region *(6)* (Fig. 1B), and subsequent work identified this receptor as belonging to the 5-HT$_4$ subtype *(7,8)*. In hippocampal slices, it was possible to show that the ability of 5-HT$_4$ receptors to inhibit the sAHP was mimicked by cAMP, potentiated by the phosphodiesterase inhibitor IBMX, and inhibited by PKA inhibitors (Fig. 1C), indicating that 5-HT$_4$ receptors regulated I_{sAHP} (a calcium-activated potassium current) by activating adenylate cyclase, increasing intracellular cAMP, and activating PKA *(9)*.

The precise mechanism by which 5-HT$_4$ receptors, and PKA in general, regulate the sAHP is only partly understood. 5-HT$_4$ receptors inhibit the sAHP through a mechanism that relies in part on the inhibition of calcium-induced calcium release *(10)*. However, this effect alone cannot account for the complete suppression of the sAHP seen after robust activations of PKA, indicating that other mechanisms must also be at play. Unfortunately, the molecular basis underlying I_{sAHP} *(11)* remain unresolved, and this has hampered attempts to understand the regulation of this current by cAMP and PKA. As a result, a mechanistic understanding of its regulation by 5-HT$_4$ receptors remains an elusive goal.

The above-outlined results seemed to identify the 5-HT$_4$ receptor as the subtype responsible for the serotonin-induced suppression of the sAHP. However, subsequent experiments conducted in the CA3 region of the hippocampus indicated that saturating concentrations of the 5-HT$_4$ antagonist GR-113808 could not completely suppress the 5-HT–induced reduction in the AHP. Equally surprising, the administration of 5-CT, which displays little affinity for 5-HT$_4$ receptors, mimicked the effect of serotonin on the sAHP *(12)*. These results pointed out to the involvement of a second 5-HT receptor subtype in the modulation of the sAHP. A careful pharmacological analysis of this response, including the use of the then newly developed selective 5-HT$_7$ receptor antagonist

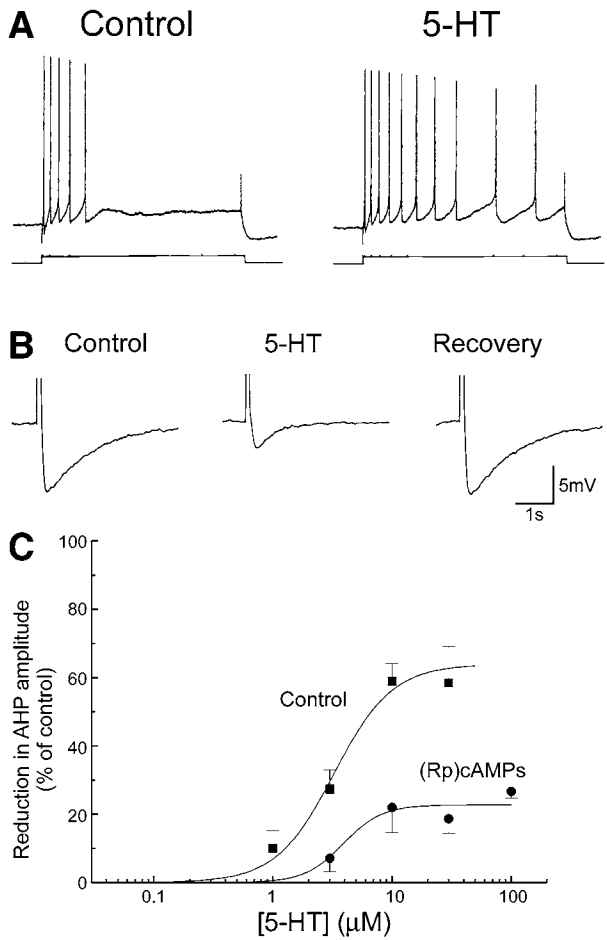

Fig. 1. 5-HT_4 receptors inhibit the sAHP in pyramidal cells of the CA1 region. **(A)** Administration of 5-HT elicits a decrease in spike frequency accommodation. **(B)** This effect is a mediated by a reduction in the sAHP, which curtails repetitive firing in these cells. The serotonin-induced reduction in the sAHP is mediated by 5-HT_4 receptors (see text). The 5-HT_4 receptor-mediated reduction of I_{sAHP} is suppressed by the selective PKA inhibitor (Rp)cAMPs.

SB-269770, indicated that this additional receptor belonged to the 5-HT_7 subtype *(12)*. A similar, albeit weaker, 5-HT_7 receptor-mediated inhibition of the AHP has been reported in the CA1 region *(13)*.

Combined, the above-outlined results indicate that both 5-HT_4 and 5-HT_7 receptors can regulate membrane excitability in the central nervous system (CNS) by targeting the calcium-activated potassium channels mediating I_{sAHP}, the

membrane current responsible for the sAHP. Although there is little reason to doubt the receptor identification underlying these conclusions, the redundant functionality implied by these results is puzzling. One possibility is that this redundancy reflects the testing situation rather than the physiological condition. For example, it is possible that the structure of any cAMP signaling microdomains *(14)* could have been compromised by the bulk applications of 5-HT in the experiments outlined earlier. Future studies will be needed to explore this issue.

2.1.1. Effect of 5-HT$_4$ and 5-HT$_7$ Receptors on the Activity of Hippocampal Neuronal Networks

Pharmacologically induced epileptiform activity in hippocampus slices can be used to assess the effect of 5-HT receptor-subtype-selective agonists and antagonist on network activity. To date, two studies have used this approach to understand how 5-HT$_4$ and 5-HT$_7$ could regulate hippocampal network activity *(15,16)*. Activation of either 5-HT$_4$ *(15)* or 5-HT$_7$ *(16)* receptors increased coordinated epileptiform activity in this preparation, whereas activation of 5-HT$_{1A}$ receptors, which are also expressed in the hippocampus, suppressed it. The increases elicited by 5-HT$_4$ and 5-HT$_7$ receptors are consistent with those that could be predicted from the ability of these receptors to inhibit the sAHP in pyramidal cells *(15,16)*.

2.2. 5-HT$_4$ Receptor Regulation of Voltage-Dependent Potassium Channels: Possible Roles in the Regulation of Neurotransmitter Release and Synaptic Plasticity

The 5-HT$_4$ receptors were first identified by their ability to stimulate adenylate cyclase in mouse collicular neurons maintained in primary culture *(17)*. Subsequent electrophysiological work demonstrated that the resulting increase in intracellular cAMP inhibited a voltage-activated potassium current in this cells by activating PKA *(18)*. Although the specific potassium current regulated by 5-HT$_4$ receptors in these cells was not determined, this effect is consistent with previous work demonstrating regulation of voltage-gated potassium channels by cAMP and PKA.

An inhibition of the voltage-dependent potassium channels located in presynaptic terminals can be expected to lead to spike broadening, an increase in calcium influx, and facilitation of neurotransmitter release *(19)*. Thus, one potential functional role for 5-HT$_4$ receptors could be the facilitation of synaptic transmission in the CNS. Although evidence for such an effect remains equivocal in CNS neurons, activation of 5-HT$_4$ receptors has been reported to increase synaptic transmission in the myenteric nervous system *(20)*. Such an effect might contribute to the prokinetic activity of benzamide 5-HT$_4$ receptor agonists in the gastrointestinal tract.

One of the most interesting aspects of this 5-HT_4 receptor-mediated regulation of potassium currents in cultured collicular neurons is its duration, which greatly outlasted the agonist application *(19)*. Long-lasting regulation of membrane properties is the hallmark of neuronal plasticity; therefore, these results suggest a possible role for 5-HT_4 receptors in the regulation of synaptic plasticity in the CNS *(18,19)*. In fact, given the well-established role for cAMP in the regulation of synaptic plasticity in a wide range of species and cell types *(21)*, a potential role for 5-HT_4 receptors in at least some forms of memory and learning seems likely. Consistent with this idea, several studies have reported effects of selective 5-HT_4 receptor agonists and antagonists on synaptic plasticity and learning in intact animals (e.g., refs. *19* and *22–24*).

2.3. 5-HT_7 Receptor Regulation of HCN Channels

One of the classical cAMP-mediated physiological responses is the ability of β-adrenergic receptors to signal increases in heart rate. Although this effect was initially attributed to an inhibition of potassium channels *(25)*, work by DiFrancesco and collaborators established that this response was in fact mediated by the regulation of the nonselective cation current now generally known as I_h *(26)*. In the sinoatrial node, I_h is activated by the hyperpolarization that follows an action potential and is responsible for the interspike depolarization that leads to the firing of the next action potential. As such, this current is often referred to as a pacemaker current *(26,27)*. Beta-adrenergic stimulation accelerates the beating of the heart by shifting the voltage dependence of I_h, such that the net amount of inward (depolarizing) current contributed by these channels following a heart action potential is increased.

Cloning work has shown that the ion channels responsible for I_h belong to the voltage-dependent potassium channel family. Four different channel subunits (HCN1–HCN4) have been found to date, and these HCN subunits are capable of assembling into ion channels that differ in terms of their voltage dependence, gating, and cAMP sensitivity *(28)*. The ability of cAMP to shift the voltage dependence of these channels is mediated by a direct binding of cAMP to a nucleotide-binding domain located along the carboxy tail of each HCN subunit *(26,27)*.

Shortly after the discovery of I_h in the heart, a very similar current was identified in the brain *(29)*, and not long thereafter, two simultaneous works *(30,31)* reported that serotonin facilitated I_h in neurons of the thalamus and the nucleus prepositus hypoglossi. Like the effect of norepinephrine in the heart, this effect of serotonin was mediated via cAMP and involved a shift in the voltage dependence of I_h. The net effect of this shift in voltage dependence is an increase in the net amount of inward current contributed by HCN channels at a given voltage and thus results in a slow depolarization of the resting membrane potential.

Similar effects were subsequently reported in several other brain regions *(32–39),* indicating that this represented a widespread action of 5-HT in the nervous system.

With the discovery of 5-HT receptor subtypes capable of coupling to Gα_s and increasing intracellular cAMP, it became possible to try to identify the specific receptor subtype(s) mediating the serotonin facilitation of I_h. In dorsal root ganglia neurons, a cell type in which 5-HT can facilitate I_h though cAMP, an initial pharmacological analysis suggested the involvement of 5-HT$_7$ receptors, but it could not definitively exclude other receptor subtypes *(35).* In an attempt to resolve this issue, Chapin and Andrade *(37,40)* focused on neurons of the anterior thalamus, a brain region where *in situ* hybridization studies had located high levels of 5-HT$_7$ receptor *(41)* and HCN channel *(42)* mRNA. In these cells, as in other thalamic neurons, 5-HT elicits a robust depolarization/inward current (Fig. 2A). This effect of serotonin was mimicked by 5-CT as well as 8-OH-DPAT, which acted as a partial agonist and were also blocked by ritanserin and mesulergine but not by cyanopindodol. Most importantly, the serotonin-induced inward current was blocked by the then newly developed selective 5-HT$_7$ receptor antagonist SB-269770. These results identified the receptor mediating the 5-HT-induced depolarization in the anterior thalamus as belonging to the 5-HT$_7$ subtype *(37).* In these same thalamic neurons, activation of 5-HT$_7$ receptor activation also facilitated I_h by shifting its voltage dependence (Fig. 2B) through a cAMP-dependent but PKA-independent mechanism. Furthermore, administration of the I_h channel blocker ZD 7288 greatly suppressed the 5-HT-induced depolarization (Fig. 2C). These results indicated that 5-HT$_7$ receptors depolarize anterior thalamic neurons by facilitating I_h through a cAMP-dependent mechanism. As such, they identified 5-HT$_7$ receptors as a key 5-HT receptor subtype mediating the regulation of HCN channels in the CNS.

What is the physiological significance of the regulation of I_h by 5-HT$_7$ receptors? One potential functional role for I_h is in setting the resting membrane potential. The voltage dependence of I_h makes this current well suited for this purpose, as its depolarizing effect is self-limiting *(27).* Specifically, 5-HT$_7$ receptors, by shifting the voltage dependence of I_h, can regulate the subthreshold voltage range at which neurons operate. Multiple ionic currents activate and inactivate in the subthreshold range, and the state of these currents determines how cell integrate incoming inputs. In addition, at least in pyramidal cells, HCN channels appear to be concentrated in dendrites, where they play an important role in determining the cell's dendritic electrotonic structure (reviewed in ref. *27*). Thus, it is tempting to speculate that by regulating I_h, 5-HT might be able to regulate how neurons integrate synaptic activity.

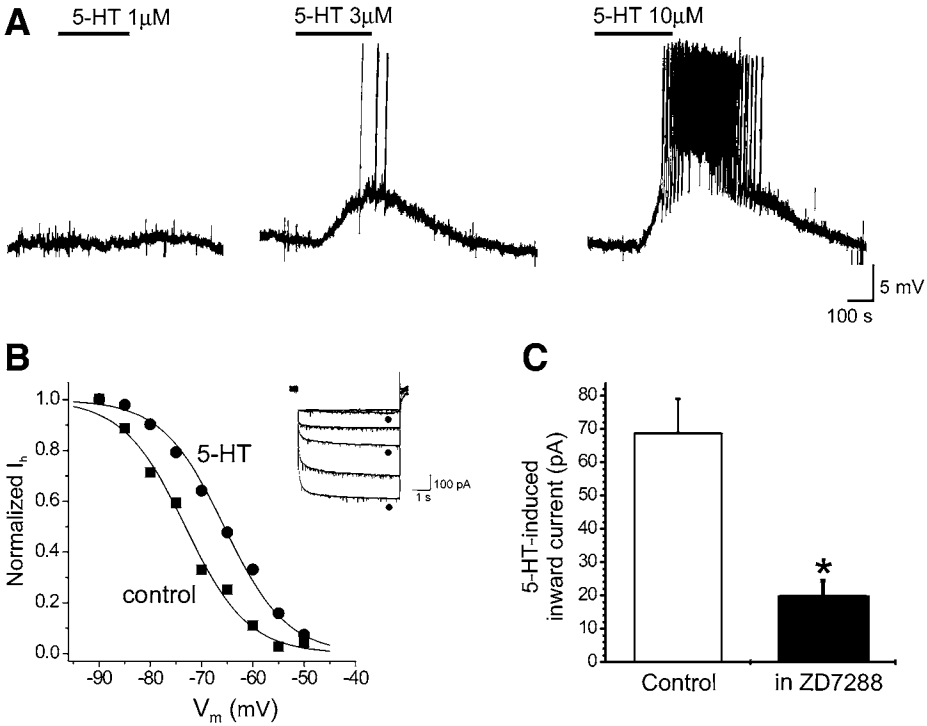

Fig. 2. 5-HT_7 receptors depolarize thalamic neurons by facilitating I_h. **(A)** Administration of 5-HT depolarizes a neuron of the anterodorsal thalamic nucleus. This effect is mediated by the activation of 5-HT_7 receptors (*see* text). **(B)** In these same cells, 5-HT shifts the voltage dependence of I_h in the depolarizing direction. **(C)** The serotonin-induced inward current, which mediates the depolarization, is inhibited by the I_h blocker ZD 7288.

A second tantalizing possibility is that 5-HT_7 receptors might be able to influence neurotransmitter release via their effects on I_h. This possibility is suggested by the observation that serotonin facilitates synaptic transmission via I_h in the crayfish neuromuscular junction *(43)*. However, enthusiasm for this idea must be tempered by results obtained in the calyx of Held, an experimentally accessible giant mammalian synapse. Work therein has shown that facilitation of I_h by cAMP within these presynaptic terminals is without effect on neurotransmitter release *(44)*. Thus, further work will be needed to assess the physiological significance of this possibility.

2.4. 5-HT_4 Receptor-Mediated Depolarization of CA1 Pyramidal Cells

Activation of 5-HT_4 receptors depolarizes pyramidal cells of the CA1 region of the hippocampus *(45,46)* through a mechanism that involves cAMP but is

independent of PKA *(45)*. As such, these properties would appear to point to the involvement of I_h in these responses. Surprisingly, however, pharmacological experiments indicate that this depolarization is at least partly independent of I_h. In pyramidal cells of the CA1 region derived from adult animals, the 5-HT$_4$ receptor-induced depolarization appears to be completely independent of I_h *(45)*, consistent with the predominant expression HCN1 at this developmental stage. HCN1-containing channels tend to produce a fast I_h that is relatively insensitive to cAMP *(47,48)*. A similar situation is seen in CA1 pyramidal neurons derived from younger animals, although in this case, a I_h may make a small contribution to the inward current *(46)*. Thus, both of these studies converge in indicating that 5-HT is capable of depolarizing central neurons through a mechanism that involves cAMP, is independent of PKA, but might not involve I_h. Cyclic nucleotide-gated channels exhibit the properties necessary to mediate this response *(45)*, although better pharmacological or molecular biological tools will be needed to convincingly test this idea. The contribution by I_h early in development might reflect the more robust expression of HCN2 and HCN4 at this developmental stage *(47)*.

3. 5-HT$_4$ Regulation of Ion Currents in Heart Tissue

Serotonin can elicit positive inotropic and chronotropic effects on the heart by acting through 5-HT$_4$ receptors. These effects are mediated by the activation of adenylate cyclase *(49)* and are similar to the well-established effects signaled by β-adrenergic receptors in this tissue. The inotropic effect signaled by 5-HT$_4$ receptors is mediated at least in part by a facilitation of L-type calcium currents and is secondary to the activation of PKA *(49)*. The positive chronotropic effect is most likely the result of an effect on I_h, as 5-HT$_4$ receptors facilitate this current in atria by shifting the voltage dependence of this current *(50)*. Interestingly, multiple alternatively spliced variants of the 5-HT$_4$ receptor appear to be expressed in human atria *(51,52)*. However, the precise role of each of these variants in mediating these 5-HT$_4$ responses remains frustratingly undefined.

4. 5-HT$_7$-Receptor-Induced Phase Shift in Suprachiasmatic Nucleus Circadian Activity

Administration of 8-OH-DPAT during the subjective day results in a phase shift in the pattern of activity of suprachiasmatic nucleus neurons, the cellular correlate of the organism's circadian clock. Given the effectiveness of 8-OH-DPAT, it is perhaps not surprising that this effect would be initially attributed to the activation of 5-HT$_{1A}$ receptors. However, more recent work, including experiments using selective 5-HT$_7$ and 5-HT$_{1A}$ antagonists in vitro, leave little doubt that this response is in fact mediated by the activation of 5-HT$_7$

receptors *(53,54)*. The mechanism by which 5-HT_7 receptors shift the phase of suprachiasmatic nucleus activity is, unfortunately, only partly understood. The 5-HT_7 receptor-induced phase shift cannot be easily explained by a simple regulation of ion channel activity. Therefore, one possibility is that it might involve changes in gene transcription or translation. Consistent with this idea, the 5-HT_7 receptor-induced phase shift has been shown to be blocked by inhibitors of protein synthesis as well as by an inhibitor of transcriptional activity *(55)*. These results suggest that the 5-HT_7 receptor-induced phase shift in suprachiasmatic nucleus activity reflects an effect on gene expression that manifests itself in terms of electrophysiological activity.

5. Electrophysiology of 5-HT_6 Receptors

The 5-HT_6 receptor mRNA and protein are expressed at relatively high levels in the nucleus accumbens, olfactory tubercle, striatum, hippocampus, and anterior cortex *(56)*. However, in spite of numerous electrophysiological studies examining the effects of serotonin in most of these areas, no 5-HT_6 receptor-mediated electrophysiological responses have yet been identified. This absence is particularly striking because the coupling of these receptors to $G\alpha_s$ predicts they should elicit important effects on membrane excitability (*see* above). The recent development of a selective 5-HT_6 receptor antagonist (SB-271046) might help resolve this issue. To date, however, only a single electrophysiological study has examined the effect of SB-271046 in the CNS. This in vivo study found complex effects on the dopaminergic neuronal firing pattern and the number of active cells in the ventral tegmental area *(57)*.

6. Conclusion

From the considerations outlined earlier, it seems clear that 5-HT_4 and 5-HT_7 receptors act as conventional $G\alpha_s$-coupled receptors and signal their effects through adenylate cyclase, an increase in intracellular cAMP, and the activation of PKA. These signals, in turn, regulate a variety of ion currents, most of which represent well-identified targets for cAMP or PKA modulation. The challenge ahead is to build on this foundation and answer two key, and partly interacting, questions that need to be answered before we can understand the role played by 5-HT_4 and 5-HT_7 receptors in the brain. First, kinetic and spatial considerations are probably central to understanding how these receptors control the integrative properties of neurons. Therefore, we need to understand how these receptors are activated by synaptically released serotonin under physiological conditions. Second, there is a growing understanding that signaling by G protein–coupled receptors is shaped and constrained by a variety of regulatory and scaffolding proteins. We need to define and understand the role that these proteins play in

orchestrating signaling by 5-HT$_4$ and 5-HT$_7$ receptors. Although answering these questions will be neither speedy nor trivial, a satisfactory solution will likely be needed before we can truly understand how these serotonin receptors regulate neuronal activity in the brain.

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17

5-HT_6 Receptors as Targets for the Treatment of Cognitive Deficits in Schizophrenia

Rudy Schreiber, Andrew Sleight, and Marie Woolley

Summary

Cloned in the human in 1996 and localized to human chromosome 1p35-p36 *(1)*, the 5-hydroxytryptamine$_6$ 5-ht$_6$ receptor was initially discovered in the rat central nervous system (CNS) using methods from molecular biology *(2,3)*. A 440-amino-acid polypeptide, the human receptor exhibits 89% homology to that of the resequenced rat receptor *(1)*. The almost exclusive distribution of the receptor in the rat CNS (but *see* refs. *3* and *4*), the high abundance of receptor expression in limbic, cortical, and striatal brain regions *(5–7)*, as well as the high affinity of tricyclic antidepressants and atypical antipsychotic drugs for this receptor *(8)* have prompted extensive investigation into the role of this receptor in psychiatric disorders. To date, the receptor has been implicated in affective disorder *(8–10)*, anxiety *(11,12)*, epilepsy *(13)*, and regulation of food consumption *(14,15)*, with the most compelling evidence suggesting a role in cognitive function. Since the role in cognitive function with respect to Alzheimer's disease has received the most attention (reviewed in ref. *16*), herein we have chosen to focus more specifically on the biological rationale for the treatment of cognitive deficits in schizophrenia and we provide recommendations for the evaluation of the potential of 5-ht$_6$ receptor antagonists in this indication.

Key Words: 5-HT_6 receptors; Alzheimer disease; animal models; cognition; schizophrenia.

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1. Introduction: Neurotransmitter Systems Affected in Schizophrenia

The underlying pathology of cognitive deficits in schizophrenia is associated with alterations in multiple neurotransmitter systems. Dopamine, noradrenaline, serotonin, glutamate, GABA, and acetylcholine are all altered in a region-specific manner, with a role for the prefrontal cortex (PFC) and temporal cortex of particular relevance in this disease. The relationship between PFC dopamine and working memory function appears to be that of a U-shaped curve, reflecting the necessity for optimal dopamine levels for working memory performance with hypodopaminergic or hyperdopaminergic states both leading to impaired function *(17–20)*. However, accumulating evidence suggests that elevated dopamine function in the PFC might improve cognitive deficits in schizophrenic patients (for a review, *see* ref. *21*). Similarly, noradrenaline has been proposed to play an important role in cognitive function in schizophrenic patients. Stimulation of postsynaptic α_{2A}-adrenoceptors in the PFC is beneficial in preclinical models of schizophrenia (for a review, *see* ref. *22*). Serotonin in both the PFC and hippocampus is also important because 5-HT_{2A} receptors are downregulated in the two aforementioned regions in schizophrenic brains, with many atypical antipsychotics exhibiting higher affinity for 5-HT_{2A} blockade than dopamine D_2 receptor blockade (for a review, *see* ref. *23*). With respect to the excitatory amino acids, clinical and preclinical data suggest that impaired NMDA receptor function might cause cognitive deficits in schizophrenia and lie upstream of alterations in dopamine function *(24–28)*, whereas GABA dysfunction in cingulate cortex *(29)* and reduced levels of GAD67 (the enzyme responsible for GABA synthesis) in the PFC of schizophrenic patients *(30)* has also been described (for a review, *see* ref. *31*). Reduced cholinergic function is reflected by a decrease in muscarinic M_1 receptors and nicotinic receptors in the forebrain and hippocampus of schizophrenic patients (for reviews, *see* refs. *32* and *33*).

Taken together, the distribution of the 5-ht_6 receptor in corticolimbic regions *(2,5–7)* and the high affinity of several atypical antipsychotic agents for the receptor *(8)* support the 5-ht_6 receptor as a potential target for the treatment of cognitive deficits in schizophrenia. Indeed, accumulating evidence suggests interactions between the 5-ht_6 receptor and several neurotransmitter systems known to be involved in cognitive function.

2. 5-ht_6 Receptors Affect Neurotransmitters Implicated in Cognition

2.1. Acetylcholine, Glutamate, and GABA

The 5-ht_6 receptor antagonists RO65-7199, RO65-7674, and RO04-6790 *(34,36–38)* increase acetylcholine levels in the hippocampus and the cortex *(35)*.

The antagonists SB-271046, RO04-6790, and RO4378554 increase extracellular glutamate levels in the hippocampus (*39–41*; Bonhaus et al., unpublished findings). SB-271046 increased glutamate levels also in the frontal cortex (FCT). However, the mechanism underlying an increase in acetylcholine and glutamate levels by blockade of a postsynaptic *(5)*, stimulatory [coupled to G_s (*42,43*)] receptor is unclear. Preliminary data from dual-labeled immunohistochemistry studies suggest that 5-ht$_6$ receptors are colocalized on GABAergic interneurons throughout the brain, including the hippocampus and FCT, which, in turn, might serve to inhibit glutamatergic and cholinergic transmission in these areas. Accordingly, blockade of the 5-ht$_6$ receptor with selective antagonists might lead to an increase in glutamate and acetylcholine release in the hippocampus and cortex by disinhibiting GABAergic inhibition, as outlined in Woolley et al. *(16)*. Consistent with this hypothesis, preliminary data using in vivo microdialysis described an increase in GABA release in the PFC and dorsal hippocampus of rats following acute and chronic treatment with a selective 5-ht$_6$ receptor agonist, WAY-466, an effect that was blocked following the application of the 5-ht$_6$ receptor antagonist SB-271046 *(44)*.

2.2. *Monoamines*

Although most work has suggested limited interaction of the 5-ht$_6$ receptor with monoaminergic systems [*(15,39,40,45–47)*; but *see also (11,48,49)*], such effects are likely to be region-specific. Indeed, recent in vivo microdialysis experiments with SB-271046 have revealed an increase in dopaminergic and noradrenergic, but not serotonergic, neurotransmission in the medial prefrontal cortex (mPFC), an area critical for cognitive function in schizophrenia *(50)*. This is a particularly interesting finding, as elevated dopamine function in the PFC might improve cognitive deficits in schizophrenic patients (for a review, *see* ref. *21*). This consists of a different mechanism than the elevation in cholinergic activity by acetylcholinesterase inhibitors for the treatment of cognitive deficits in Alzheimer's disease.

Another interesting and recent observation with potential consequences for the clinical use of 5-ht$_6$ receptor antagonists is that they augment the neurochemical and behavioral effects of amphetamine. SB-258510A potentiated the effects of amphetamine on the release of dopamine in the FCT and nucleus accumbens, as well as its locomotor-activating and reinforcing effects *(51)*. SB-271046 potentiated the amphetamine-induced release of dopamine (and serotonin) in the striatum *(52)*, and a novel 5-ht$_6$ receptor antagonist, MS-245, augmented the discriminative stimulus effects of amphetamine in a two-lever drug discrimination task *(53)*. The general lack of effect of 5-ht$_6$ receptor antagonism on the serotonergic system is consistent with a postsynaptic location of the 5-ht$_6$ receptor *(5)*, but recent behavioral studies suggest that the 5-ht$_6$

receptor might serve to indirectly modulate 5-HT release *(52,54)*. These recent findings indicate that 5-ht$_6$ receptors might have a more modulatory role in the control of monoaminergic neurotransmission. A future challenge will be to identify how such a modulatory function might translate into potential clinical applications that involve modulation of dopaminergic and, potentially, serotonergic neurotransmission. For example, the combination of 5-ht$_6$ receptor antagonists with dopaminergic agonists such as levodopa/carbidopa in the treatment of Parkinson's disease might help to lower the dose of the agonist, which, in turn, might result in fewer side effects *(53)*. Furthermore, an add-on therapy of a 5-ht$_6$ receptor antagonist with a dopamine-enhancing agent could augment the cognition-enhancing effects of the latter *(55)*. Although further studies are required to address the precise interactions between 5-ht$_6$ receptors and the aforementioned neurotransmitter systems, with mechanisms undoubtedly proving to be region-specific, preclinical data support a role for the 5-ht$_6$ receptor in cognitive function.

3. Potential of 5-ht$_6$ Receptor Antagonists for the Treatment of Cognitive Deficits in Schizophrenia

The neurocognitive deficits in schizophrenia are considered a separate domain of the illness that is relatively independent of psychotic symptoms and closely related to functional outcome. Following the MATRICS initiative (http://www.matrics.ucla.edu), interest in this field recently increased. A description of the background and rationale for this initiative can be found in ref. *56*. The following separable cognitive domains in schizophrenia were identified:

1. Working memory
2. Attention/vigilance
3. Verbal learning and memory
4. Visual learning and memory
5. Reasoning and problem-solving
6. Speed of processing

Social recognition was added as a seventh domain at a MATRICS meeting in 2004. The cognitive domains summarized in Table 1 to a large extent represent the cognitive domains affected in schizophrenia. Obviously, domains that cannot be modeled in animals, such as verbal learning and memory, have been omitted. It is proposed to test 5-ht$_6$ receptor antagonists in these models in order to evaluate their potential against cognitive disorders in schizophrenia. Herein, we will review in what models these compounds have been tested and make recommendations for future studies.

Table 1
Effects of 5-ht$_6$ Receptor Antagonists on Cognitive Domains Affected in Schizophrenia

Cognitive domain	Suggested animal tests	Effects of 5-ht$_6$ antagonists	Ref.
Preattentive and attention processes	Mismatch negativity	—	
	P50 ERP gating	—	
	Prepulse inhibition	NA	*58,60*
	P300 ERP	—	
	Latent inhibition	NA	*58*
	Continuous performance	—	
	Occulomotor antisacchade	—	
Executive functioning	Attentional set shifting	A	*84*
	Serial reversal discrimination	—	
	Switching tasks	—	
	ED/ID shift in NHP	—	
Sustained attention	Five-choice serial reaction time task	NA?	Roche, in house
Working memory	Operant or T-maze D(N)MTP	NA?	Roche, in house
	Radial arm maze	A	*94*
	NHP:		
	DMTP	—	
	Spatial delayed response	—	
Learning and memory	Morris water maze	A	*65,96,99, 102,103*
		NA	*100,101*
	Novel object recognition	A	*47,54,95,103, 104,113*
	Fear conditioning	NA?	*101*
	Passive avoidance	A	*37,101*
	Autoshaping	A	*34,35,102*
		NA	*103*
		NA	*101,104,105*
Motor function/ speed of processing	Operant tasks	A	
	Locomotor activity	A	
	Simple reaction time task	—	
Social cognition	Social recognition	A	*62*

Abbreviations: A, active; D(N)MTP, delayed (non) match to position; ED, extradimensional; ERP, event-related potential; ID, intradimensional; NA, not active; NHP, nonhuman primates.

Source: Adapted from J. Hagan (http://www.matrics.ucla.edu).

3.1. Preattentive and Attention Processes

Schizophrenic patients exhibit sensorimotor gating deficits that can be modeled in rodents and nonhuman primates (NHP) as deficits in acoustic startle prepulse inhibition *(57)*. Prepulse inhibition (PPI) of the startle response refers to the phenomenon that presentation of a subthreshold stimulus (prepulse) shortly before the presentation of an intense startling stimulus (pulse) results in activation of an inhibitory process that attenuates ("gates") the startle response. To date, 5-ht$_6$ receptor antagonists have shown little beneficial effect in reversing sensorimotor gating deficits induced with the psychostimulants amphetamine and MK801 *(58–60)*. RO4368554 failed to reverse PPI deficits seen in a rodent neurodevelopmental model of schizophrenia (i.e., the neonatal ventral hippocampal lesioned rat) *(61,62)*. Latent inhibition involves more attentional processes and is also disrupted in schizophrenic patients *(63)*. Latent inhibition refers to the phenomenon that prior preexposure to a nonreinforced stimulus leads to subsequent retardation of conditioning to that stimulus compared to nonpreexposure in controls (for a review, *see* ref. *64*). RO04-6790 and RO65-7199 did not improve latent inhibition utilising a conditioned lick suppression procedure *(58)*.

The following studies are suggested to address whether 5-ht$_6$ receptor antagonists improve preattentive/attention processes (*see also* next subsections on attentional set shifting and five-choice serial reaction time tasks). First, investigation of the effect of 5-ht$_6$ receptor antagonists on PPI performance in rodents exhibiting poor baseline sensorimotor gating, such as DBA2 mice *(66)*, Brattleboro rats *(67)*, or Brown-Norway rats *(68)*, as well as in models that induce PPI deficits by different means such as social isolation *(69)* or repeated treatment with amphetamine *(70,71)*. A word of caution with regard to mouse testing: given the disparity between the affinity of several 5-ht$_6$ receptor antagonists for the rat and mouse receptor *(72)*, it will be necessary to identify compounds exhibiting sufficiently high affinity for the receptor before performing mouse studies. Second, to test 5-ht$_6$ receptor antagonists in further latent inhibition studies using drug-induced deficits, such as (sub)chronic PCP or amphetamine treatment (*64* and references therein) and/or other behavioral read-outs such as conditioned emotional response. Development of a neonatal hippocampal lesion model to enable testing of 5-ht$_6$ receptor antagonists in a neurodevelopmental deficit model of latent inhibition might consist of another option worthwhile to explore further. Third, test these compounds in further measures for preattentional and attention-dependent cognitive deficits in schizophrenia, such as mismatch negativity, P50 event-related potential suppression, P300 event-related potential, continuous performance task performance, and oculomotor antisaccade performance, especially because these measures typically

have dissociable neural substrates *(57,73)*. Mismatch negativity might be of special interest in light of the association with poor functioning in schizophrenia (Light and Braff, unpublished data).

3.2. Executive Functioning: Attentional Set Shifting

Patients with frontal lesions *(74,75)*, including schizophrenics *(76,77)* as well as Alzheimer's disease (AD) patients *(78,79)*, are impaired in their ability to inhibit previously learned responses and, as a result, are unable to shift their attention when required to a new relevant stimulus dimension. Adapted for the rat *(80,81)*, this task is directly comparable to the human *(82)* and monkey *(83)* version of the task as used in the CANTAB test battery for the assessment of cognitive deficits as a result of frontal lobe dysfunction. In this task, rats must progress through the test by satisfying a criterion of learning at each stage. In the simple discrimination phase, rats have to identify which exemplar is correct (e.g., odor or digging media). In the compound discrimination phase, a second dimension is introduced and one exemplar from each dimension is paired to form the compound stimulus. To succeed, rats have to continue to respond to the correct exemplar of the previous stage. During the intradimensional shift (IDS), rats are presented with new pairs of exemplars, but the relevant dimension for a correct response remains unchanged (i.e., if odor was the correct dimension, it continued to be). Thus, the IDS involves the transfer of a rule within the same stimulus dimension. During the extradimensional shift (EDS), new exemplars are introduced and rats are required to respond to the previously irrelevant dimension (e.g., digging media rather than odor). Thus, the EDS requires a transfer of attention across different stimulus dimensions and is related to the ability of the rat to be aware of the conceptual category within which they are responding (for a detailed description, *see* ref. *80*). Work by Hatcher et al. *(84)* showed that in normal adult rats, subchronic treatment with two 5-ht$_6$ receptor antagonists, SB-399885T and SB-271046-A, significantly improved reversal and EDS performance when compared with vehicle-treated controls. This shows that, in normal rats, 5-ht$_6$ receptor antagonists improve attentional set shifting.

Further work should aim to substantiate the findings obtained in unimpaired rats with findings in *models* of impaired function representative of the human condition. For example, lesions of the mPFC and subchronic PCP both impair EDS in the attentional set shifting task *(80,85)*. In addition, the risk with extrapolations made from animals to humans could be reduced if the profile of action of 5-ht$_6$ receptor antagonists in the rat attentional set-shifting task would be reproduced in a ID/ED task in NHP *(83)*, because in a CANTAB battery of neuropsychological tests *(86)*, a functional magnetic resonance imaging (fMRI)

study showed that performance of the same attentional set-shift task recruited homologous regions of the ventral–lateral PFC in NHP and humans *(87)*. Assessment of these compounds in alternative tests of executive function such as serial reversal discrimination and switching tasks will also prove interesting.

3.3. Sustained Attention: Five-Choice Serial Reaction Time Task

Schizophrenic patients show an overall performance decrement on the continuous performance test (CPT), and this attentional deficit is associated with frontal dysfunction. The five-choice serial reaction time task (5-CSRTT) is a test of visual–spatial attention that involves functioning of the mPFC and consists of a rat analog of the human CPT *(88,89)*. The task is conducted in an operant chamber with multiple response locations in which brief visual stimuli are presented. Performance is maintained using food reinforcers to criterion levels of accuracy (*see* ref. *88* for a full description of this task). In-house studies suggest that the 5-ht_6 receptor antagonist RO65-7674 did not improve percentage correct responding and correct latency in unimpaired rats and "poor performers" (Higgins, unpublished results). In view of the finding that systemic SB-271046 increased dopamine release in the mPFC *(50)* and that direct infusion of dopamine agonists into this brain area enhanced response accuracy in animals with low baselines *(18)*, it seems worthwhile to further investigate the effects of 5-ht_6 receptor antagonists in low-baseline performers. Another option is to test against the NMDA antagonist MK801, which increased premature responding, reduced choice accuracy, and increased errors of omission *(90)*. Finally, drug testing in an extension of the 5-CSRTT, the so-called combined attention–memory test *(91)*, might be useful to assess drug effects on attention and working memory in the same task.

3.4. Working Memory

Working memory deficits are among the most consistently observed symptoms in schizophrenic patients, and as working memory is critical for the integrity for the thought process, it has been postulated that a deficient working memory might underlie the myriad of cognitive deficits and associated thought disorder in schizophrenia *(92,93)*. Accordingly, drug candidates for the treatment of the cognitive dysfunction in schizophrenia should be broadly tested for their potential to improve working memory. So far, only results from two preliminary studies published in abstracts are available. Several 5-ht_6 receptor antagonists from Lilly enhanced spatial learning in an eight-arm radial maze *(94,95)* and SB-271046 improved performance of aged rats in an operant delayed-alternation procedure *(96)*.

This is clearly an area where much more work is needed. For rodent studies, further testing in neurodevelopment models, such as lesions of the ventral

hippocampus in neonates, and pharmacological models, such as (subchronic) treatment with NMDA antagonists, are suggested. These treatments cause deficits in delayed alternation, radial arm maze, Morris water maze, and delayed match-to-position tasks (*see* ref. *93* for references and a discussion of the challenges with the pharmacological deficit models). In rodents, a potential concern is that the circuitry involved in working memory tasks might differ as a function of the extent to which the hippocampus is recruited by the task. Therefore, it has been proposed to use NHP to test the same mechanisms of working memory that operate in humans, because the underlying circuitry can be fully determined and the working memory tasks are identical between both species *(93)*. The proposed tasks consist of a spatial response task for spatial working memory and a delayed-match-to-sample task for object working memory potentially impairing with acute and/or subchronic PCP treatment and/or amphetamine sensitization *(97)*.

3.5. Learning and Memory

Episodic long-term memory is also impaired in schizophrenia and—as for AD—this might also involve a cholinergic mechanism. This is supported by the finding that the dual acetylcholinesterase inhibitor and allosteric potentiator of nicotinic receptors, galantamine, improved cognitive function in schizophrenic patients *(32,98)*. Because 5-ht$_6$ receptor antagonists might increase acetylcholine release in the FCT *(35)*, these compounds could have promise for the treatment of episodic memory deficits in schizophrenia.

3.5.1. Morris Water Maze (Spatial Learning and Reference Memory)

Using very similar protocols, downregulation of the 5-ht$_6$ receptor using 5-ht$_6$-directed antisense oligonucleotides, as well as blockade of the receptor with RO04-6790, SB-271046-A, and SB-357134-A, improved the retention of a previously learned platform position in the Morris water maze in nonimpaired rats, with no effect on acquisition of the task *(14,96,99)*. Thus, antisense-treated rats showed no improvement during acquisition of the task (five trials per day, 2 d) but improved probe test performance 1 d following the cessation of acquisition. RO04-6790-treated rats showed no improvement during the acquisition of the task (three trials per day, 3 d) but improved probe test performance 4, 7, and 10 d following the cessation of acquisition, whereas SB-271046- and SB-357134-treated rats showed also no improvement during acquisition of the task (28 trials over 5 d, 4 trials on day 1, followed by 6 trials daily) but improved retention of the learned platform position 7 d following the cessation of training. However, it should be noted that Russel and Dias *(100)* and Lindner et al. *(101)* were unable to replicate these findings with either RO04-6790 or SB-271046 when using similar protocols. The reasons for this are not clear.

In light of their findings and the fact that repeated probe tests, an example of an extinction procedure, were used in the aforementioned studies, Russel and Dias *(100)* proposed that the results seen might simply reflect behavioral inflexibility rather than facilitation in spatial reference memory and that further studies employing a working memory or reversal learning version of this task might help to resolve this issue. Although as yet this has not been addressed in the water maze paradigm, Hatcher et al. *(84)* examined the effect of SB-271046 in the rat attentional set-shifting task and showed a reduction in the number of trials and errors to criteria during the reversal learning part of the task, suggesting that 5-ht_6 receptor antagonism does not cause behavioral inflexibility. Consistent with a role for the 5-ht_6 receptor in cognition, Stean et al. *(65)* went on to show that, in contrast to acute treatment, subchronic treatment with SB-357134 (twice daily, 10 d) improved both acquisition of the task (spatial learning) and recall of the target quadrant (memory) in nonimpaired rats.

Of course, one caveat for all the aforementioned studies is that they were performed in nonimpaired animals. However, RO65-7199 improves scopolamine-induced deficits in this task *(37)* and recent data from Foley et al. *(102)* showed that chronic treatment with SB-271046 (once daily, 40 d) improved the age-related impairment in spatial learning and memory in 20-mo-old Wistar rats, an effect also seen in aged Fisher 344 rats *(103)*. Further testing of 5-ht_6 receptor antagonists in glutamate-deficiency models and neurodevelopment-deficiency models is suggested.

3.5.2. Passive Avoidance, Autoshaping, and Fear Conditioning

In both step-down and step-through passive avoidance tasks, rats learn to avoid a compartment previously associated with an aversive stimulus. In the step-down version, animals are placed on a platform with an adjacent grid electrified. As soon as the rat steps off the platform onto the grid, it automatically receives a foot shock and typically jumps back rapidly onto the platform. During retention testing, stepping down voluntarily onto the grid floor or failing to resist being pushed gently onto the grid floor is judged as passive avoidance failure. The more widely used step-through version is typically performed in shuttle cages with a brightly lit "safe" compartment and a dark "unsafe" compartment where a foot shock has been delivered during training. Consistent with a 5-ht_6–cholinergic interaction, several structurally distinct antagonists reversed a scopolamine-induced deficit in the passive avoidance task: RO65-7199 *(34,37)*, SB-271046 *(102,103)*, and a series of aryl pyridyl sulfone 5-ht_6 antagonists *(35)*. SB-271046 was also effective in reversing an age-related deficit in Fisher 344 rats in this task (*103*; but *see* ref. *100*).

In an autoshaping task, rats are trained in an operant box where a right or left lever is presented and if the animal presses the lever, the lever is retracted

and the rat receives one reward pellet. The increase or decrease in these responses is considered an enhancement or impairment in learning, respectively. Testing takes place 24 h after the initial training day and follows a similar procedure as training. Several 5-ht$_6$ receptor antagonists improved memory consolidation with respect to control groups when assessed during memory consolidation of conditioned responses in an autoshaping Pavlovian/instrumental learning task (*105*; but *see also* ref. *101*). Moreover, structurally diverse 5-ht$_6$ receptor antagonists, RO04-6790, SB-357134, and SB-399885, have all been shown to reverse deficits in learning consolidation produced by scopolamine and the NMDA receptor antagonist MK801 *(104,105)*.

During fear conditioning, animals are resubmitted to a chamber where they previously received a foot shock and time freezing is measured as a measure for contextual memory. Testing occurs in the absence of a footshock. Neither RO04-6790 nor SB-271046 reversed a scopolamine-induced deficit in this task *(101)*.

As passive avoidance, autoshaping, and fear-conditioning tasks are typically much less laborious than most of the other cognition tasks, their value might be in their use as early screening models, where a lower validity can be acceptable when there is a concomitant gain in drug throughput. Their value for characterizing the cognitive profile of advanced compounds appears limited, as analogous tasks in humans are absent or less feasible, and it is not always clear whether the cognitive domains and underlying neural circuits in these tasks are also affected in cognitive deficits in schizophrenia.

3.6. Novel Object Recognition (Visual Recognition)

Object recognition is an attractive task for testing compounds for their potential against cognitive deficits in schizophrenia. It is a relatively simple task, with the additional benefit that it can be performed across species. Although the literature for the neural substrates involved in this task remains controversial, there does seem to be some overlap in the neural circuits that mediate object recognition in rats, primates, and humans and that contain medial temporal lobe structures *(106–113)*.

Preliminary data show that the 5-ht$_6$ antagonist LY483518 improved baseline novel object recognition (NOR) performance in adult rats *(95)*. Moreover, several structurally distinct 5-ht$_6$ receptor antagonists have shown improvements in function across different models of impaired function in this task. Both RO04-6790 and SB-271046 reversed a temporal deficit (i.e., forgetting at a 4-h and 48-h retention interval) *(103,113)*. It should be noted that RO4368554 failed to reverse a deficit following a 24-h retention interval in a modified version of the NOR task *(54)*. SB-271046 also reversed an age-related impairment in Fisher 344 rats in the NOR task *(103)*. Although the

underlying mechanism for the improvement in this model is unknown, several studies suggest 5-ht$_6$ receptor regulation of cholinergic, glutamatergic, and serotonergic transmission. Consistent with a role for the 5-ht$_6$ receptor in the regulation of cholinergic transmission, the selective 5-ht$_6$ receptor antagonist RO04-6790 effectively reversed a scopolamine-induced deficit in this task *(47)*, an effect recently replicated with alternative 5-ht$_6$ receptor antagonists RO4368554 *(54)* and PMDT *(114)*. Consistent with a 5ht$_6$-glutamatergic interaction, King et al. *(113)* recently showed that the RO04-6790-induced reversal of forgetting at a 4-h retention interval was abolished by the NMDA receptor antagonist MK-801. Notably, despite much evidence to suggest limited 5-ht$_6$–serotonin interactions, Lieben et al. *(54)* showed that RO4368554 reversed a deficit in NOR produced by tryptophan depletion, which, given the postsynaptic location of the 5-ht$_6$ receptor, is proposed to most likely represent an indirect mechanism of action. Future studies should focus on rodent neurodevelopment-deficiency models and NHP studies.

3.7. Social Recognition

Social cognition has been defined as "mental operations underlying social and emotional interactions, including the human ability and capacity to perceive the intentions and dispositions of others" and is closely related to functional outcome in schizophrenics *(56)*. A possible rodent task for social cognition might consist of the social recognition task. During the acquisition phase of this task, an unfamiliar juvenile and an adult rat are placed together for a few minutes. Following a retention time, both rats are again placed together and the time the adult rat spends investigating (sniffing, grooming, closely following) the juvenile is recorded during the test session. An adult rat recognizes the juvenile as familiar at short retention intervals (typically around 2 h), as indicated by a decrease in the duration of social investigatory behavior during testing. The 5-ht$_6$ receptor antagonist RO4368554 improves social recognition *(62)*.

Because social cognition in humans appears more complex than simple recognition alone, a rodent social recognition task might not adequately model this condition in humans. Instead, the richer repertoire of social behaviors in NHP (e.g., ref. *115*) might lend itself better to address drug effects on social cognition.

3.8. Motor Function/Speed of Processing

A challenge in the development of novel drugs is to obtain a sufficient margin between their therapeutic effects and their side effects. There are at least two aspects to this concern. First, it is desirable to know whether a good margin exists between efficacious plasma exposures and exposures that correspond with side effects. This information helps to guide dose selection for

testing in humans. For this aim, side effects are often assessed in separate studies using automated locomotor activity assays, lever responding in operant boxes employing variable interval or fixed ratio schedules of reinforcement, or simple reaction time tests. No studies have been published with 5-ht$_6$ receptor antagonists in such tasks. Second, side effects can potentially confound drug effects on cognition. The preferred way to address this is to build parameters into the cognition task that allow the detection of side effects. Indeed, most of the tasks mentioned previously have such parameters, and 5-ht$_6$ receptor antagonists typically improve cognition at doses that do not induce side effects, such as improved NOR in the absence of any alteration in exploratory activity *(47)* or reduced escape latency and path length in the absence of reduced swim speed in the Morris water maze *(65)*.

4. Concluding Comments

Because 5-ht$_6$ receptor antagonists have been mostly tested in Morris water maze and NOR tasks, both within the domain of learning and memory models, the existing data package is encouraging and "hypothesis generating," rather than proof of concept for the potential of these compounds in the treatment of cognitive deficits in schizophrenia. Further studies need to be performed for the other cognitive domains affected in schizophrenia (Table 1) and it needs to be addressed which animal cognition data package would support phase II clinical testing of a 5-ht$_6$ receptor antagonist against cognitive disorders in schizophrenia.

Because a working memory deficit is a hallmark cognitive impairment in schizophrenia, the focus should be on the generation of a dataset that unequivocally supports the action that a drug improves this cognitive domain. In addition, a cognitive enhancer should improve functioning of at least one of the other cognitive domains. Additional activity in models for executive functioning/attention processes is preferred. For each domain, evidence should be obtained from at least two different models. This is especially relevant for an area where there is an increased risk for false-positive or false-negative findings because of a lack of clinically efficacious "gold standards" for model validation (*see* ref. *116* for an analysis of 24 clinical studies).

It is preferred to use comparable cross-species tasks of cognition function such as delayed-(non)match-to-position(sample), 5-CSRTT, and attentional set shifting such that common neural circuits are involved across species, something that can be confirmed by running imaging methods, such as magnetic resonance imaging, in parallel. Ideally, the activity in these neural circuits would be similarly affected by 5-ht$_6$ receptor antagonists. If such a converging dataset were obtained, generalizations about the underlying processes would

be facilitated and a more valid extrapolation of data from animals to humans could be made.

If the animal experimentation is concluded and a drug indeed reaches the patient, a final caveat needs to be addressed. As many atypical antipsychotic drugs are also 5-ht_6 receptor antagonists, the risk exists that add-on therapy with a 5-ht_6 receptor antagonist might not further improve cognition in treated schizophrenics. Therefore it is suggested to preferentially combine a 5-ht_6 receptor antagonist with a drug with low 5-ht_6 antagonism such as risperidone or aripiprazole *(23)*.

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18

5-HT_7 Receptors as Favorable Pharmacological Targets for Drug Discovery

Peter B. Hedlund and J. Gregor Sutcliffe

Summary

The 5-hydroxytryptamine$_7$ (5-HT_7) receptor was among a group of 5-HT receptors discovered through targeted cloning strategies 13 yr ago. It is a seven-transmembrane-domain G protein–coupled receptor positively linked to adenylyl cyclase. The distributions of 5-HT_7 receptor mRNA, immunolabeling, and radioligand binding show strong similarities, with the highest densities found in the thalamus and hypothalamus, but with significant presence also in the hippocampus and cortex. The recent availability of selective antagonists and knockout mice strains has dramatically increased our knowledge about this receptor. Together with unselective agonists, these new tools have revealed the 5-HT_7 receptor distribution and function in more detail. Important functional roles have been established for the 5-HT_7 receptor in thermoregulation, circadian rhythm, learning and memory, hippocampal signaling, and sleep. The 5-HT_7 receptor is possibly also involved in other psychiatric and neurological disorders, such as schizophrenia, epilepsy, and migraine. Hypotheses driving current research strongly indicate an involvement in mood regulation, suggesting the 5-HT_7 receptor as a putative target in the treatment of depression.

Key Words: 5-Hydroxytryptamine; serotonin; receptor; antagonist; knockout; autoradiography; thermoregulation; hippocampus; behavior; electrophysiology; depression; oleamide.

1. Introduction

Traditional pharmacological techniques had revealed the existence of multiple receptor subtypes for 5-hydroxytryptamine (5-HT), but it was not until after

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the first cDNA cloning of a serotonin receptor that it became clear how large the family of 5-HT receptors is *(1)*. In a large set of studies performed slightly more than a decade ago using targeted analysis of cDNA libraries based on conserved sequences in the known receptors, the number of identified 5-HT receptors was greatly expanded. No fewer than 14 different receptor subtypes for 5-HT have been described *(1)*, not including the multiple proteins generated by alternative splicing of the transcripts of single genes. Thus, of all known neurotransmitters, 5-HT acts on the most diverse group of receptors. Given the amount of time that has passed and our increased knowledge of the genome since the last of the novel 5-HT receptors was reported, it is likely that the list is now complete. Although 5-HT is synthesized by only a small group of neurons within the raphae nuclei of the brainstem, these cells send both ascending and descending projections to large parts of the central nervous system *(2)*. Because of this widespread innervation, 5-HT has been implicated in numerous important physiological and pathophysiological phenomena, including sleep-wakefulness and several psychiatric disorders *(1)*. The large number of receptor subtypes underscores the importance of 5-HT and the need for fine-tuning its actions.

The 5-HT_7 receptor was added to the list of 5-HT receptors in 1993, when it was independently discovered by several laboratories *(3–6)*. The receptor protein was readily expressed in transfected cells and found to stimulate cyclic AMP (cAMP) production *(3–5,7)*. Using Northern blots *(3–5)* and *in situ* hybridization *(4,5)*, 5-HT_7 receptor mRNA was found in the brain, mainly in the hypothalamus, thalamus, hippocampus, and cortex, and in the periphery, mainly in blood vessels and the intestines. Initial pharmacological characterization showed that the 5-HT_7 receptor had high affinity for 5-carboxytryptamine (5-CT) and 5-HT, and relatively high affinity for 8-OH-DPAT *(3–5)*. Ritanserin, but not pindolol, showed antagonistic properties *(4)*. Since its discovery, the 5-HT_7 receptor has been found in numerous species, including humans *(8–11)*. Recent additions to the list of species include *Caenorhabditis elegans (12)* and *Aedes aegypti (13)*. Different splice variants have been detected in rats and humans *(14,15)*. Two splice variants correspond to each other in the rat and human, whereas both species have distinct third variants that are generated by the retention of different exon cassettes. The different splice products result in different C-terminal ends of the receptor proteins, but as far as is known, they do not seem to possess any functional differences and are pharmacologically indistinguishable *(16)*. In humans, a pseudogene for the 5-HT_7 receptor has also been described *(17,18)*. It is transcribed, but contains an in-frame stop codon in the fifth trans-membrane region *(18)*. The pseudogene is also present in rhesus monkeys, but not in rats and mice *(18)*.

One consequence of the vast expansion of the number of known receptors for 5-HT is that it casts confusion on studies that were interpreted before the extent of the list was appreciated. However, much progress has been made in deconvoluting the contributions of these several receptors to serotonin signaling. For the 5-HT$_7$ receptor, this is true especially because of the recent availabilities of selective antagonists and knockout mice. Recent studies have pointed to its functional roles in thermoregulation, circadian rhythm, learning and memory, hippocampal signaling, and sleep. Hypotheses driving current research indicate a possible involvement in mood regulation, suggesting the 5-HT$_7$ receptor as a potential target for the treatment of depression.

2. 5-HT$_7$ Receptor Pharmacology

2.1. Agonists

In order to elucidate the characteristics and function of a receptor, the availability of selective ligands is of utmost importance. As described earlier, both 5-CT and 8-OH-DPAT act as agonists at the 5-HT$_7$ receptor. Especially the fact that 8-OH-DPAT, previously considered the standard selective agonist for the 5-HT$_{1A}$ receptor, has high affinity for the 5-HT$_7$ receptor contributes to the confusion regarding which subtype is involved in previously observed phenomena. Unfortunately, more selective agonists for the 5-HT$_7$ receptor are not yet readily available. A problem seems to be the ability to discriminate between the 5-HT$_{1A}$ and 5-HT$_7$ receptor *(19)*. This might, however, change soon, as recent reports have described compounds with agonistic or partial agonistic properties from several chemical classes *(20,21)*.

2.2. Antagonists

The availability of selective antagonists for the 5-HT$_7$ receptor has led to several important discoveries. Compounds belonging to two chemically distinct groups have been described *(22,23)*, the first group being represented by SB-258719 [(*R*)-3,(*N*)-dimethyl-*N*-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]-benzene-sulfonamide] *(22)* and SB-269970 [(*R*)-3-(2-(2-(4-methylpiperidin-1-yl)-ethyl)pyrrolidine-1-sulfonyl)phenol] *(24)* and the second group by the tetrahydrobenzindole DR4004 [2*a*-(4-(4-phenyl-1,2,3,6-tetrahydropyridyl)-butyl)-2*a*,3,4,5-tetrahydrobenzo[*cd*]indol-2(1*H*)-one] *(23)*. Work to characterize additional compounds *(20,25,26)* and the chemical properties required for optimal antagonism continues *(27,28)*. Using the antagonists SB-266970 and SB-656104, it has been possible to demonstrate that blockade of the 5-HT$_7$ receptor leads both to inhibition of 5-HT-mediated hypothermia and to an increased latency to onset of rapid eye movement sleep (REM) with less time spent in REM sleep.

2.3. Inverse Agonists

Constitutive activity of G protein–coupled receptors is a relatively new notion mainly described in recombinant receptor systems *(29)*, but also in living animals *(30)*. Such activity has been described for the 5-HT_7 receptor when expressed in cell lines *(24,31,32)*. A drug that inhibits constitutive activity is called an inverse agonist, and it has been reported that the selective antagonists SB-258719, SB-258741, and SB-269970 act as inverse agonists to a varying degree in vitro. It remains to be determined if constitutive activity is of relevance for the 5-HT_7 receptor in vivo.

2.4. Allosteric Modulation by Oleamide

The endogenous amidated lipid oleamide has been described as a modulator of 5-HT_7 receptor affinity in vitro *(33)*. Oleamide acts at an apparent allosteric site on the receptor protein to modulate its function. Oleamide is a member of a family of amidated lipids found in the plasma and cerebrospinal fluid of mammals, including humans *(34–36)*. These lipids have diverse bioregulatory properties, including neuromodulatory effects, direct receptor-mediated mechanisms, and high biological activities *(37,38)*. Included in this family are two endogenous ligands for the cannabinoid receptor, anandamide and palmitoylethanolamine, which have been shown to have not only direct actions on cannabinoid receptors but also a variety of modulatory properties *(39–41)*. In vivo, oleamide has been demonstrated to induce sleep after intraperitoneal injections in rats *(36)* and to exhibit long-lasting hypothermic effects *(42)*. In vitro, oleamide has been shown to modulate the signaling of several 5-HT receptor subtypes, including 5-HT_{1A}, 5-$HT_{2A/2C}$, and 5-HT_7 *(38,43,44)*. These effects involve potentiation and/or inhibition of cAMP and inositol phosphate pathways. Studies have indicated that oleamide acts at an apparent allosteric site on the 5-HT_7 receptor to regulate cAMP formation *(44)*, because oleamide induced a concentration-dependent increase in cAMP formation that could not be inhibited by clozapine, suggesting that it acted at a site distinct from the primary 5-HT-binding site. However, in the presence of 5-HT, oleamide had the opposite effect, antagonizing the cAMP-stimulating effect of 5-HT *(44)*. Oleamide has also been shown to activate 5-HT_7 neurons in the mouse thalamus and hypothalamus, as indicated by c-*fos* induction after intraperitoneal administration, further supporting the notion of serotonergic mechanisms in oleamide function *(45)*. In another study, oleamide was shown to modulate 5-HT_7 receptor affinity by inducing a decrease in the affinity of the 5-HT_7 receptor for [^{3}H]5-HT, without affecting maximal binding. With increasing concentrations of oleamide, a plateau value was reached where the shift in K_D could not be further increased. In addition, a maximally effective concentration of oleamide caused only a 40% displacement of [^{3}H]5-HT binding, indicating a noncompetitive mechanism of

action. Taken together, these findings support the hypothesis that oleamide acts via an allosteric site on the 5-HT$_7$ receptor, at which it induces a decrease in receptor affinity without influencing the number of binding sites.

3. 5-HT$_7$ Receptor Signal Transduction

When the 5-HT$_7$ receptor was discovered, it was found to stimulate cAMP formation *(3–5)*. Interestingly, it has been demonstrated in cell lines that the 5-HT$_7$ receptor not only activates adenylyl cyclase-5 (AC) normally linked to G_s, but also AC1 and AC8, which are activated by intracellular calcium *(46)*. Coexpression of the 5-HT$_7$ receptor, AC1, and AC8 is present in the hippocampus and might thus be important for hippocampal function. At least when expressed in cell lines, the receptor is tightly associated with the G protein, regardless of agonist binding *(47)*. This suggests the existence of a complex between inactive receptor and the G protein. The 5-HT$_7$ receptor has been found to activate the extracellular signal-related kinase (ERK) *(48)* through a mechanism dependent on a Ras G protein *(49)*. This activation of ERK might also of importance for hippocampal function and depression. Although cAMP activation is normally believed to be mediated through protein kinase A (PKA), it has been demonstrated that the 5-HT$_7$ receptor can stimulate ERK through a PKA-independent pathway, possibly by using the cAMP–guanine nucleotide exchange factor Epac *(50)*.

4. Distribution of the 5-HT$_7$ Receptor

Although the distribution of 5-HT receptors is described elsewhere in this volume, it is of interest to discuss the regional expression of 5-HT$_7$ receptors in the brain in relation to proposed receptor functions. The distribution of 5-HT$_7$ receptors has been determined using *in situ* hybridization, immunohistochemistry, and radioligand binding.

4.1. In Situ *Hybridization*

Both early and more recent studies have used *in situ* hybridization to determine with increasing resolution where the 5-HT$_7$ receptor gene is expressed *(4,51–53)*. The studies consistently show that 5-HT$_7$ receptor mRNA is most abundantly present in the thalamus, hippocampus, and hypothalamus. It should especially be noted that receptor mRNA is present in all of the CA fields of the hippocampus and in the suprachiasmatic nucleus (SCN) of the hypothalamus. *In situ* hybridization has also shown that, exclusively within the ventral CA3 region of the rat hippocampus, the 5-HT$_7$ mRNA levels decrease by 30% between young and middle-aged animals, with no further decrease between middle and old age *(54)*.

4.2. Immunohistochemistry

A detailed mapping of the receptor protein distribution using immunohistochemistry has, to our knowledge, not been performed. Nevertheless, a growing number of studies have been published describing the distribution of the 5-HT_7 receptor protein *(53,55–58)*. Two of these studies have shown that the protein distribution is very similar to that of the mRNA, with the highest abundance in the thalamus, hypothalamus, and hippocampus. As with *in situ* hybridization, low levels of immunoreactivity have been detected in the striatum *(53,58)*. In the hippocampus, CA1 pyramidal neurons have been shown to be 5-HT_7 receptor immunoreactive *(56)*. Within the mouse SCN, the 5-HT_7 receptor is located in both dendrites and axon terminals of neuronal elements containing GABA, vasoactive intestinal polypeptide, or vasopressin *(55)*. In these neurons, most of the receptors seem to be in the plasma membrane, but outside the active zone of the synapse. Within the cerebellum, it has been found that the 5-HT_7 receptor protein is located exclusively in the Purkinje cells *(57)*.

4.3. Radioligand Binding

The availability of selective antagonists for the 5-HT_7 receptor have made them the logical candidates for receptor-binding experiments to study distribution. Indeed, studies using radiolabeled [^{3}H]SB-266970 have been published, most performed using tissue membrane preparations *(59,60)*, but also one brief report using tissue sections of the human brain *(61)*. [^{3}H]SB-266970 binding was found to be most abundant within the anterior thalamus and the dentate gyrus. Significant binding was also detected in the hypothalamus, anterior cingulate gyrus, hippocampus, and amygdala *(61)*. All other reports have used nonselective ligands in combination with indirect methods to determine 5-HT_7 receptor-binding distribution. These studies have used either unlabeled antagonists or knockout mice or a combination of both to discriminate 5-HT_7 receptors from other receptor subtypes. Two reports have used 5-HT_{1A}, 5-HT_{1B}, and 5-HT_7 receptor knockout mice in combination with [^{3}H]5-CT and [^{3}H]8-OH-DPAT to obtain a detailed map of 5-HT_7 receptor-binding distribution *(62,63)*. Because both of these ligands bind to both 5-HT_{1A} and 5-HT_7 receptors, the knockout mice were used in combination with selective antagonists (SB-269970 for 5-HT_7; WAY-100135 and pindolol for 5-HT_{1A}) to discriminate between the two receptor subtypes. In summary, these studies confirm the distribution pattern seen also with *in situ* hybridization and immunhistochemistry, showing the highest binding densities in the thalamus, hypothalamus, and hippocampus. A noteworthy observation in one of the studies is that 8-OH-DPAT not only binds to 5-HT_{1A} and 5-HT_7 receptors but also to a significant degree to α_{2A} receptors *(63)*. A similar binding pattern, but with some notable differences, was found in rats, guinea pigs, and humans

when using [^{3}H]mesulergine as the radioligand *(64)*. The most striking difference was the observation of relatively high binding density in the caudate-putamen. Although other studies have not reported binding in this region, it is in agreement with the immunohistochemistry findings.

4.4. Receptor Distribution and Function

When attempting to correlate 5-HT$_7$ receptor distribution with function, it becomes evident that there is a significant agreement between the localization of 5-HT$_7$ receptors in the brain and the functions in which they are implicated. Its presence in the hypothalamus correlates with involvement in circadian rhythm, thermoregulation, and endocrine regulation. Thalamic and cortical 5-HT$_7$ receptors might be of importance for sleep and mood regulation. It has also been shown that thalamic 5-HT$_7$ receptors might be of importance in epilepsy *(65)*. Finally, 5-HT$_7$ receptors in the hippocampus are of interest for learning and memory.

5. The 5-HT$_7$ Receptor and Thermoregulation

That 5-HT is involved in thermoregulation is a well-known phenomenon. Injection of 5-CT or 8-OH-DPAT will induce hypothermia. The effect is similar regardless of whether the drug is administered systemically or directly into the brain, suggesting a central mechanism of action. As 5-CT and 8-OH-DPAT are both 5-HT$_{1A}$ receptor agonists, this receptor was generally considered the main mediator of hypothermia, although some reports had suggested involvement of other receptor subtypes. The first indication that the 5-HT$_7$ receptor is of importance for 5-HT-induced hypothermia came when it was reported that the effect of 5-CT on body temperature could be blocked by SB-269970 *(24)*. Such an antagonistic effect has also been observed for SB-656104, another selective 5-HT$_7$ receptor antagonist *(66)*. Furthermore, the hypothermic effect of 5-CT could not be blocked by the 5-HT$_{1A}$ receptor antagonist WAY-100635 or the 5-HT$_{1B/D}$ antagonist GR127935 *(67)*. In contrast, the hypothermic effect of 8-OH-DPAT could be inhibited by a 5-HT$_{1A}$ antagonist (WAY-100135), but only partially by SB-269970 *(68)*. The importance of the 5-HT$_7$ receptor was further emphasized when it was observed that 5-HT and 5-CT failed to induce hypothermia in 5-HT$_7$ receptor knockout mice *(67,69)*. When including 8-OH-DPAT in the analysis in combination with selective antagonists and knockout mice to discriminate between 5-HT$_{1A}$ and 5-HT$_7$ receptors, it was revealed that both receptor subtypes are involved in 5-HT-mediated hypothermia *(68)*. This would explain the results obtained with antagonists and also why 8-OH-DPAT fails to induce hypothermia in 5-HT$_{1A}$ receptor knockout mice *(70)*. Interestingly, the 5-HT$_7$ receptor seems to be most important at low agonist concentrations, thus contributing to the fine-tuning of temperature homeostasis,

whereas the 5-HT_{1A} receptor comes into play at higher agonist concentrations, possibly providing a defense against hyperthermia *(68)*.

6. The 5-HT_7 Receptor in Learning and Memory

The first attempt to assess the role of the 5-HT_7 receptor in various behavioral paradigms used antisense oligonucleotides to inhibit receptor synthesis *(71)*. This report found no effects of the treatment in feeding, locomotor activity, or anxiety-like behavior using an elevated plus maze. A more comprehensive study used 5-HT_7 receptor knockout mice to evaluate the role of this receptor in various behavioral and learning tasks *(72)*. It was found that knockout mice had a specific impairment in contextual fear conditioning. Contextual fear conditioning is generally believed to be a hippocampus-dependent learning, as are other types of place learning. It is therefore of interest that a Barnes maze test showed no difference between wild-type and knockout mice *(72)*. There was also no difference in three hippocampus-independent learning tasks: cued fear conditioning, operant food conditioning, and motor learning (rotarod) *(72)*. The impairment seen in contextual fear conditioning was not the result of alterations in motor skills, visual acuity, or anxiety level *(72)*. The finding that impaired contextual fear conditioning is an early phenomenon in models of Alzheimer's disease opens up the intriguing possibility for an involvment of the 5-HT_7 receptor in this disorder *(73,74)*. The 5-HT_7 receptor has also been implicated in memory formation in a Pavlovian learning test, as the selective antagonists SB-269970 and DR4004 could reverse amnesia induced by scopolamine and dizocilpine *(75)*. Furthermore, in 5-HT_7 receptor knockout mice, there is a reduced ability to induce long-term potential (LTP) in CA1 *(72)*.

7. The 5-HT_7 Receptor in Neuropsychiatric and Neurological Disorders

7.1. Central Nervous System Development

The developing cortex receives a dense serotonergic innervation with 5-HT acting on several different receptor subtypes. Recent studies have shown that the 5-HT_7 receptor is involved in the postnatal formation of synaptic connectivity in the prefrontal cortex, where early postnatally there is a high expression of 5-HT_7 receptors that later declines and is replaced by an increase in 5-HT_{1A} receptor expression *(76,77)*, Furthermore, serotonergic projections from the dorsal raphe have been shown to modulate cholinergic neurons within the ventral pallidum *(78)*. These studies were made in slices from immature animals and the findings might be of importance for the etiology of psychiatric disorders *(78)*. In the developing rat brain, 5-HT_7 receptor immunoreactivity has been observed in a cytoplasmic inclusion termed *stigmoid body (58)*. In neonatal

animals, these immunopositive inclusions were most prominent within the hypothalamus. The stigmoid bodies have been linked to the development of sexual dimorphism, which might be of interest in relation to the involvement of 5-HT$_7$ receptors in endocrine regulation (*see* below) *(58)*.

7.2. Circadian Rhythms

Since the discovery of the 5-HT$_7$ receptor, at which time it was shown that 8-OH-DPAT-induced phase resetting within the SCN was inhibited by ritanserin but not pindolol, a pharmacology congruent only with this receptor, it has been implicated in circadian rhythm regulation *(4)*. A series of recent studies have provided additional evidence for the importance of the 5-HT$_7$ receptor in SCN function *(79–84)*. That the phase shifting induced by 8-OH-DPAT is mediated by the 5-HT$_7$ receptor is supported by the finding that the shift can be inhibited by SB-269970 *(84)* and DR4004 *(81)*. There is evidence to suggest that the phase shifting induced by 8-OH-DPAT directly involves regulating the SCN clock gene *Period (80)*. Shifting the SCN pacemaker neurons with 8-OH-DPAT is a nonphotic stimulus involving serotonergic input from the dorsal and median raphe nuclei. In these nuclei, 5-HT$_7$ receptors have been shown to modulate SCN phase resetting *(85)*. The 5-HT$_7$ receptor is probably also involved in photic regulation of the SCN. It has been demonstrated using pharmacological profiling with unselective drugs *(79)* and with DR4004 *(81)* that 5-HT-mediated reduction of photic stimulation of SCN neurons is most likely mediated by the 5-HT$_7$ receptor. The inhibition of 8-OH-DPAT on spontaneous SCN activity is also mediated by the 5-HT$_7$ receptor *(82)*. The above studies were performed in either rats or hamsters. In the mouse, however, there is evidence to suggest that the effects of 8-OH-DPAT and, hence, the 5-HT$_7$ receptor on SCN function are not as pronounced as in other species *(83)*.

7.3. Sleep

That the 5-HT$_7$ receptor is directly involved in sleep regulation has been shown using selective antagonists and knockout mice. Both SB-269970 and SB-656104, when administered at the beginning of the sleep phase, increased the latency to REM sleep and decreased the amount of time spent in REM sleep *(66)*. Other sleep parameters were not affected. Similar changes has been observed in 5-HT$_7$ receptor knockout mice because these mice spend less time in REM sleep without changes in wakefulness or slow-wave sleep *(86)*. The knockout mice also had longer, but less frequent REM episodes than wild-type mice. Untreated knockout mice did not have increased latency to REM, but when treated with citalopram, a selective serotonin reuptake inhibitor, the REM latency induced by citalopram was potentiated in the knockouts *(86)*. Overall, the changes in sleep

pattern seen after 5-HT_7 receptor blockade or inactivation are directly opposite those seen in depressed patients.

7.4. Mood Disorders

More recently, it has also been shown that antidepressants might exert at least some of its function through the 5-HT_7 receptor *(87)*. Several antidepressants, both tricyclics and selective serotonin reuptake inhibitors, induced *cFos* expression in a way consistent with 5-HT_7 receptor activation within the SCN *(87)*. The effect on cFos expression was attenuated after chronic treatment with antidepressants. Furthermore, chronic drug treatment led to a downregulation of 5-HT_7 receptor binding *(87)*. Even more interesting, it has recently been shown that mice lacking the 5-HT_7 receptor show an "antidepressant-like" phenotype in two behavioral models for depression: the forced swim test and the tail suspension test *(86)*. Thus, unmedicated 5-$HT_7^{-/-}$ mice showed decreased immobility in both tests and the selective 5-HT_7 antagonist SB-269970 decreased immobility in 5-$HT_7^{+/+}$ mice. The selective serotonin reuptake inhibitor citalopram, a widely used antidepressant, decreased immobility in both 5-$HT_7^{+/+}$ and 5-$HT_7^{-/-}$ mice in the tail suspension test, suggesting that it utilizes an independent mechanism.

Taken together, these findings are compatible with the hypothesis that the 5-HT_7 receptor is of considerable importance for regulating sleep, circadian rhythms, and the overall mood of the individual. The direct actions of antidepressants on the 5-HT_7 receptor, the reversal of sleep disturbances seen in depressed patients by 5-HT_7 receptor blockade, and the "antidepressantlike" behavior of 5-HT_7 receptor knockout mice all suggest that a 5-HT_7 receptor antagonist by itself might be sufficient to treat depression and might have advantages over currently available options.

7.5. Schizophrenia

It was shown early that several antipsychotics had a high affinity for the 5-HT_7 receptor *(88)*. Behavioral studies have also presented results suggesting that the 5-HT_7 receptor might be of importance for schizophrenia *(75,89)*. Specifically, it has been shown that the selective 5-HT_7 receptor antagonist SB-258741 alters prepulse inhibition, a model widely used to assess the antipsychotic activity of a drug and a phenomenon that can also be observed in human schizophrenics *(89)*. Electrophysiological studies from the hippocampus and ventral pallidum also suggest a role for the 5-HT_7 receptor in schizophrenia. One study used pharmacological profiling to show that activation of the 5-HT_7 receptor decreased the amplitude of slow afterhyperpolarizations in CA3 *(90)*. Further evidence that the 5-HT_7 receptor is important in CA3 neuronal activity was provided by the finding that the selective antagonist SB-269970 could inhibit bursting activity induced by 5-CT *(91)*. It was found that the

5-HT$_7$ receptor acts by inhibiting Ca^{2+}-activated K^+ channels. The effect of clozapine to block 5-HT effects on GABAergic neurons in the ventral pallidum might be of importance for its antipsychotic effect *(78)*.

7.6. Stress and Anxiety

It has been suggested that the 5-HT$_7$ receptor might be important for stress regulation because 5-HT$_7$ receptor mRNA is upregulated in the hippocampus after acute, but not chronic, restraint stress *(92)*. The acute restraint stress also caused a reduction in glucocorticoid receptor mRNA *(92)*. In constrast, loss of 5-HT$_7$ receptor function through antisense ologinucleotide treatment *(71)* or targeted gene disruption *(72)* did not cause an altered behavior in tests of anxiety. This is in contrast to 5-HT$_{1A}$ receptor knockout mice, which are generally considered a model for anxiety *(70,93,94)*.

7.7. Epilepsy

Blockade of the 5-HT$_7$ receptor has been shown to reduce epileptic activity in animal models. Audiogenic seizures induced in DBA/2J mice could be prevented by drugs in a rank order of potency corresponding to their affinity for the 5-HT$_7$ receptor *(95)*. The selective 5-HT$_7$ receptor antagonist SB-258719 has been shown to reduce epileptic activity in an animal model for absence epilepsy, the WAG/Rij rat *(65)*. It is believed to do so by modulating the "pacemaker" current I_h within the thalamus *(96,97)*. The 5-HT$_7$ receptor has been demonstrated to mediate depolarization within the anterodorsal thalamus by increasing I_h through a cAMP-dependent, PKA-independent mechanism *(96,97)*.

8. The 5-HT$_7$ Receptor in Endocrine Regulation

There is evidence to suggest a role for the 5-HT$_7$ receptor in both central and peripheral parts of the endocrine system. The 5-HT$_7$ receptor is most likely involved in 5-HT-mediated stimulation of both vasopressin and oxytocin release *(98)*. Furthermore, activation of the 5-HT$_7$ receptor has been shown to stimulate the release of luteinizing hormone-releasing hormone *(99)*. Peripherally, 5-HT$_7$ receptors are present on granulose-lutein cells, where it stimulates progesterone production *(100)*. In addition, in the adrenal, the 5-HT$_7$ receptor has been shown to mediate 5-HT-induced aldosterone release *(101)*. In the hippocampus, 5-HT$_7$ receptors are involved in regulating the effects of glucocorticoids on their receptors *(102,103)*. 5-HT$_7$ receptor-directed antisense oligonucleotide treatment did not alter plasma corticosterone or prolactin levels *(71)*.

9. Peripheral 5-HT$_7$ Receptors

In peripheral tissues the 5-HT$_7$ receptor has mainly been found on smooth muscle cells in blood vessels and other internal organs. In general, the 5-HT$_7$

receptor mediates relaxation of blood vessels, both arteries and veins *(104,105)*. However, one recent study excluded the 5-HT_7 receptor from involvement in the relaxation of human occipital artery *(106)*. Because of its presence in blood vessels of the skull, it has been suggested that the 5-HT_7 receptor is a putative target for migraine treatment *(104)*.

Other recent studies have reported that the 5-HT_7 receptor is involved in mediating the effects of 5-HT on ileum peristalsis *(107)*, the micturition reflex *(108)*, and relaxation of the oviduct *(109)*.

10. Conclusions

The availability of selective antagonists and knockout mice has led to an unprecedented activity in 5-HT_7 receptor research during the last few years. Several studies from many laboratories have contributed to these advances. The distribution and functional coupling of the receptor has been worked out in more detail. Important roles have been suggested for the 5-HT_7 receptor in thermoregulation, learning and memory, hippocampal activity, and endocrine function. An involvement of the 5-HT_7 receptor has also been suggested in several neurological and psychiatric disorders. Most intriguing is the role of the 5-HT_7 receptor in the regulation of sleep, circadian rhythms, and mood. Future studies and pharmaceutical development will answer the question of whether a 5-HT_7 receptor antagonist is suitable for the treatment of depression.

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19

Serotonin System Gene Knockouts

A Story of Mice With Implications for Man

Miles Berger and Laurence H. Tecott

Summary

To better understand the roles of individual serotonin receptor subtypes in modulating the neural circuitry of complex behavior, a number of groups have generated "knockout" mice lacking individual serotonin receptor subtypes over the last decade. Overall, 10 (at least 14) serotonin receptor subtypes have been knocked out in mice, as well as several genes that regulate the development and activity of serotonin neurons (e.g., Pet-1, the serotonin transporter, and tryptophan hydroxylase). We review these studies, discuss their relevance to the pathophysiology of neuropsychiatric disorders, and close with a perspective on where the field may head in the future.

Key Words: Transgenic and knockout mice; neuropsychiatric disorders; anxiety; the neural circuitry of complex behavior; SERT; Pet-1; tryptophan hydroxylase 2.

1. Introduction

Selective serotonin reuptake inhibitors (SSRI's) like Prozac™ are used to treat many psychiatric disorders ranging from intermittent explosive disorder, to obsessive–compulsive disorder, to major depression and panic disorder *(1)*, even though these disorders differ in their behavioral expression. How does one drug class treat these disparate disorders?

One possibility is that the various serotonin receptor subtypes (15 have been cloned to date) selectively regulate neural circuits that underlie specific

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Table 1
5-HT Receptors and the Neural Circuitry of Behavior

Receptor knocked out	Behavioral phenotype	Proposed neural circuit underlying phenotype
5-HT1A receptor	Increased anxiety-like behavior	• Loss of hippocampal 5-HT1A receptors in development • Loss of 5-HT1A autoreceptors and disinhibited 5-HT release
5-HT1B receptor	Decreased immobility in the tail suspension test, only in females	• Loss of 5-HT1B terminal autoreceptors and disinhibited 5-HT release
5-HT2C receptor	Increased food intake	• Altered hypothalamic feeding circuitry • Disinhibition of central dopamine pathways
5-HT3A receptor	Increased fear conditioning	• Loss of 5-HT3A-R-driven GABAergic inhibition of lateral amygdala

Note: These examples are representative, not comprehensive. *See* chapter text for further discussion.

behaviors (Table 1). According to this view, alterations in the activity of different serotonin receptors (or alterations in the activity of a subpopulation of a given receptor) might perturb distinct neural circuits and therefore contribute to the characteristic behavioral dysregulation of a specific psychiatric illness.

To examine the role of individual serotonin receptor subtypes in the neural circuitry of these behavioral abnormalities and in normal behavior, many laboratories have generated "knockout" mice lacking individual serotonin receptor subtypes. Although it is wise to note that mice are not just miniature humans *(2)*, mice and humans do express the same serotonin receptor subtypes (with the exception of the 5-hydroxytryptamine$_{5B}$ [5-HT5B] receptor) with a roughly similar anatomic distribution *(3)*. Moreover, mice and humans share many neurobehavioral processes such as explicit and implicit memory, ingestion of drugs of abuse, aggression, and anxiety-like behavior. Thus, studies of serotonin receptor knockout mice are likely to enhance our understanding of the roles that these receptors play in the neural circuitry of complex behavior in both mouse and man.

Over the last decade, 10 serotonin receptor subtypes have been knocked out, and studies of these knockout animals have confirmed existing hypotheses and revealed new insights into the roles of these receptors. Perhaps the most surprising finding is that none of these "knockouts" causes a lethal central nervous system (CNS) defect. Although many of the knockout lines have intriguing behavioral abnormalities, that none have lethal CNS defects suggests that central serotonergic activity subtly modulates the neural circuitry underlying these behaviors without being absolutely necessary for brain functions or behaviors necessary to support life.

Many of the behavioral abnormalities in serotonin receptor knockout mice enrich our understanding of the specific serotonergic pathways that might be dysregulated in psychiatric disorders. For example, 5-HT1A knockout mice display anxiety-like behavior in numerous behavioral tests *(4)*, and more recent work has begun to elucidate the dysregulated neural pathways that might underlie their anxiety phenotype *(5–7)*. These data suggest possible mechanisms for the pathogenesis of anxiety and depressive disorders, and they imply future directions for therapeutic strategies.

Although the behavioral phenotypes seen in 5-HT-R knockout mice are intriguing, it can be challenging to discern the full range of behavioral processes disrupted by the knockout of a 5-HT-R. For example, a behavioral abnormality in a single test paradigm could arise from many different underlying causes, just as a single symptom such as anxiety appears in the context of many different medical and/or psychiatric illnesses. A mouse that performs poorly in a test of spatial memory might, indeed, have impaired spatial memory or it might have a vision defect or simply a change in overall motor activity. Thus, the more different behavioral phenotypes investigated in a given knockout mouse, the more confident we can be about our interpretation of a given abnormality.

Yet, no matter how confident we can be of the specificity of a behavioral phenotype, another issue with knockout studies is that they may not always reflect the normal adult role of the gene product. Standard knockout models ablate the expression of the knocked-out gene throughout development in all tissues where it would normally be expressed. Therefore it is unclear whether a behavioral phenotype is the result of the absence of the receptor in the adult animal, the absence of the receptor during development, or other compensatory alterations resulting from the chronic loss of the receptor. In addition, identifying which subpopulations of the knocked-out receptor are most responsible for the altered phenotype might be difficult. Newer techniques such as the Cre/loxP and Tet inducible systems *(8)*, RASSLs *(9)*, and chemical genetic approaches *(10)* allow investigators to study these subpopulations of receptors with spatial and/or temporal control and will clearly play an increasingly important role in the future.

The application of mouse genetics to the study of serotonin receptor biology over the last 10 yr has revolutionized our understanding of the roles that these receptors play in the neural circuitry of behavior. Although these studies have confirmed existing ideas about links between serotonergic tone and affective behavior, they have also provided surprising new insights into classic questions *(7)*. Recent studies have also begun to clarify the roles of other genes like Pet-1, tryptophan hydroxylase (TPH2), and the serotonin transporter (SERT) that regulate the development and activity of serotonin neurons; future work is likely to focus on how mutations in these genes might perturb serotonergic function and on developing drugs to modulate their activity. In this chapter, we review these studies, discuss their relevance to the pathophysiology of human psychiatric disorders, and close with a perspective of future directions in this field.

2. 5-HT1A KO Mice and Anxiety- and Depression-Related Behavior

The 5-HT1A receptor is perhaps the most extensively studied of all the serotonin receptors; a recent Medline search for "5-HT1A" found 4505 papers. In accord with this intense interest, three independent groups (including our own) simultaneously generated 5-HT1A receptor knockout (KO) mice *(11–13)*. All three groups reported increased anxiety-like behavior in these mice, although each group maintained the KO allele on a different genetic background, highlighting the robust influence of *5-htr1a* in regulating anxiety-like behavior.

Specifically, these studies found that 5-HT1A-R null animals showed increased anxiety-like behavior in the open field, elevated plus maze, elevated zero maze, and novel object tests. 5-HT1A-R KOs were also less immobile in the forced swim and tail suspension tests, the same response induced in these tests by antidepressant drugs. However, it is unclear whether this decreased immobility truly reflects an antidepressant-like coping reaction mediated by increased 5-HT transmission or whether it simply reflects an increased stress response in these anxiety-prone mutant mice *(12)*.

The striking anxiety-like phenotype of 5-HT1A-R KO mice raises an important question: Does this phenotype reflect the absence of normal receptor activity in the adult animal, indirect compensatory responses to its absence throughout development? Initially, all three groups proposed that the KO phenotype was the result of the absence of the receptor in adult animals, in part because the administration of a 5-HT1A antagonist induced an anxiety-like response in wild-type mice in the open field test similar to the baseline response seen in 5-HT1A-R KOs *(13)*.

The 5-HT1A receptors are expressed widely throughout the cerebral cortex and are densely present in the hippocampus and the brainstem raphe nuclei, which also raises the question of the relative contribution of these 5-HT1A-R subpopulations to the anxiety-like phenotype. In the raphe nuclei, 5-HT1A receptors are expressed on 5-HT neurons and act as inhibitory autoreceptors (5-HT1A-AR) that restrain the firing of these neurons *(14)*. Because increased serotonergic tone has been linked to anxiety (*15*, and references therein), the loss of raphe 5-HT1A-AR could enhance anxiety by disinhibiting 5-HT neurons. In line with this hypothesis, the serotonin neurons of 5-HT1A KO mice fire at a higher rate on average *(16)* although increased basal 5-HT release has been found in 5-HT1A KO mice in only one study *(17–20)*.

Tissue 5-HT content is not increased in the brains of 5-HT1A KO mice *(21)*, and microdialysis studies have produced conflicting data on phenotypic alterations in extracellular 5-HT levels. Furthermore, lowering 5-HT synthesis with the tryptophan hydroxylase inhibitor PCPA was not sufficient to normalize the decreased immobility of 5-HT1A knockouts in the forced swim test *(22)*, indicating that this phenotype might not simply result from the acute loss of 5-HT1A-AR and disinhibited 5-HT release. This finding, along with the fact that 5-HT1A-R antagonists induce some but not all of the anxiogenic responses seen in 5-HT1A KO mice (*13,23*, and supplementary data therein) suggests that much of the 5-HT1A-R KO behavioral phenotype might not simply reflect disinhibited serotonin release as a result of the loss of 5-HT1A-AR. Instead, much of the behavioral phenotype of 5-HT1A-R KO mice could reflect compensatory events distal to the actual loss of the 5-HT1A receptor protein.

Indeed, the loss of hippocampal 5-HT1A receptors disrupts the dendritic maturation of CA1 pyramidal neurons during development *(6)*, and 5-HT1A-R KO mice have impaired hippocampal synaptic transmission and impaired hippocampal-dependent learning and memory *(24)*. Furthermore, forebrain expression of transgenic 5-HT1A receptors in a 5-HT1A-R KO background during early postnatal development (but not in adulthood *[23]*) prevented the appearance of an anxiety-like phenotype in adult animals. This genetic strategy resulted in significant receptor expression in regions of the hippocampus that normally do express the 5-HT1A-R, but it also led to ectopic receptor expression in forebrain regions implicated in the control of fear and anxiety-like behavior *(25)*. Nonetheless, when taken together, these findings suggest that compensatory events resulting from the loss of forebrain receptors during early postnatal development might contribute to the anxiety phenotype of 5-HT1A-R KO mice.

What might these compensatory events be? Recent work shows that 5-HT1A-R KO mice, at least on the Swiss–Webster background, have decreased

expression of the $GABA_A$ receptor subunits α1 and α2 and do not display normal anxiolytic responses to benzodiazepine $GABA_A$ agonists *(5)*. However, these findings were not seen in 5-HT1A-R KO mice on the C57Bl/6J background, indicating that these alterations in the GABA system observed on the Swiss–Webster background are the result of a specific genetic modifier in the Swiss–Webster genome. These data fit nicely with human studies showing that SSRI administration increases cortical GABA release *(26)*, also suggesting that serotonergic pathways enhance GABAergic signaling. Additional studies indicate that the hippocampi of 5-HT1A KO mice display large alterations in the expression of many different genes *(27)*, suggesting that these widespread alterations might perturb hippocampal circuitry and could increase anxiety-related behavior in these mutant mice.

These findings in 5-HT1A-R KO mice are paralleled by human data showing lowered forebrain 5-HT1A-R binding in patients with panic disorder *(28)* and depression *(29)*, suggesting that decreased forebrain 5-HT1A-R signaling might play a role in causing the anxiety and affective symptoms of these patients. The decreases in 5-HT1A-R forebrain binding in patients with panic disorder are also intriguing because these patients are frequently insensitive to benzodiazepines and have decreased $GABA_A$ binding *(30,31)*, similar to 5-HT1A-R KO mice on the Swiss–Webster background *(5)*. This similarity suggests that the decreased 5-HT1A-R binding in these patients might lead to lower $GABA_A$ subunit expression and benzodiazepine resistance, changes that might increase anxiety symptoms.

Because some patients with anxiety disorders and depression display decreased forebrain 5-HT1A-R binding, it is possible that these patients might have altered CA3-CA1 hippocampal circuitry similar to that seen in 5-HT1A-R KO mice *(6)*. Because partial 5-HT1A agonists like buspirone exert anxiolytic and antidepressant effects only after chronic administration *(32)*, it is intriguing to speculate that these drugs ameliorate anxiety symptoms by chronically increasing hippocampal 5-HT1A-R signaling to "rewire" and normalize hippocampal circuitry that might become dysregulated in the absence of normal 5-HT1A-R signaling.

Consistent with this hypothesis, a recent study suggests that the anxiolytic and antidepressant behavioral effects of SSRIs and drugs like buspirone in mice depend on 5-HT1A-dependent increases in hippocampal neurogenesis *(7)*. Whether these drugs work in humans by a similar mechanism is unknown, but indirect evidence suggests that they might: Depressed patients show hippocampal volume loss; antidepressants may protect against this loss *(33)*. In any case, how newly born hippocampal neurons might modulate the neural circuitry that underlies anxiety and affect regulation is an area of active interest.

Meanwhile, another set of studies in mice and humans has focused on the role of 5-HT1A-AR. A line of mice that show a wide variety of depressive-like traits exhibit 5-HT1A-AR overexpression *(34,35)*, and, conversely, a line of rats specifically bred for increased 5-HT1A responsivity also show depressive-like behavior *(36)*. Similarly, 5-HT1A-AR overexpression and/or hyperactivity have been found in imaging studies in depressed patients *(37)* and in postmortem studies of depressed suicide victims *(38)*. A variant allele of *5-htr1a* that increases autoreceptor expression has also been found and is tied to depression and suicide (*39*, although this result was not replicated in ref. *40*), anxiety disorders *(41)*, neuroticism *(42)*, and poor SSRI treatment response *(43)*, and schizophrenia, substance abuse disorder and panic attacks *(44)*. High 5-HT1A-AR expression is also associated with longer latency to treatment response in depressed patients *(45)*, which suggests that desensitizing or downregulating 5-HT1A-ARs might be a rate-limiting step in SSRI treatment response. Given the well-described role of 5-HT1A-AR in restraining the activity of 5-HT neurons *(14)*, these studies suggest that increased 5-HT1A-AR expression might excessively inhibit 5-HT neurons and lead to a hyposerotonergic state associated with depression (as proposed by the monoamine hypothesis), and perhaps with resistance to SSRI treatment response.

Taken together, these studies suggest that drugs specifically modulating the activity or expression of these distinct receptor pools could impact the treatment of anxiety and affective disorders. Further studies with conditional 5-HT1A-R mouse mutants will further our understanding of the role this receptor plays in regulating the neural circuitry of affective behavior.

3. 5-HT1B-R KO Mice, Aggression, and Drug Abuse

The 5-HT1B-R was the first 5-HT receptor to be knocked out, and mice lacking this receptor were initially found to have increased aggression *(46,47)*. Conversely, 5-HT1B-R agonists (termed "serenics") reduce aggression in wild-type mice and this effect can be reversed by 5-HT1B-R antagonists *(48,49)*, suggesting that the aggression phenotype of 5-HT1B KOs might arise directly from the absence of 5-HT1B-R rather than from secondary compensations to its loss.

Other studies found increased prepulse inhibition and decreased startle responses in 5-HT1B-R KOs *(50,51)*. Later work showed that these mice display decreased anxiety-related behavior *(52)*, although another study showed increased autonomic response to novelty in these mice *(53)*. Meanwhile, viral overexpression of 5-HT1B autoreceptors in the raphe nucleus led to increased stress-induced anxiety behavior *(54)*. This finding, together with decreased anxiety-like behavior in the 5-HT1B KOs, suggests that 5-HT1B-R-mediated suppression of serotonergic tone might increase anxiety responses and that inhibiting this 5-HT1B-R

activity might have an anxiolytic effect. This hypothesis is consistent with the anxiolytic activity of serenics *(48)*.

Further work showed that 5-HT1B-R KO mice have enhanced spatial memory and are resistant to age-related declines in spatial learning and memory *(55)*. These cognitive phenotypes might be caused by increased activity at glutamatergic CA1-subiculum terminals lacking 5-HT1B receptors or by increased cholinergic transmission to the hippocampus because of the loss of inhibitory 5-HT1B receptors on medial septal cholinergic terminals (*55*, and references therein). 5-HT1B receptors also regulate serotonergic projections from the median raphe that innervate the forebrain: 5-HT1B KO mice display elevated hippocampal 5-HT release in response to SSRIs *(19)* and have a compensatory increase in 5-HT1A-R activity on these projections *(56)*. 5-HT1B-R KO mice also display sexually dimorphic differences in serotonergic activity: 5-HT1B-R KO females, but not males, display lower tail suspension and forced swim test immobility *(57)*, and this antidepressant-like phenotype can be reversed by depleting serotonin with *p*-chlorophenylalanine. 5-HT1B-R KO females also display an enhanced decrease in immobility time in response to fluoxetine when compared to males *(57)*, further suggesting that the loss of the 5-HT1B-R alters serotonergic activity to a much larger extent in female than male mice. These findings are of considerable interest given the increased prevalence of affective disorders in women; might this increase relate to sexually dimorphic serotonergic activity (perhaps via similar 5-HT1B-related mechanisms)?

The 5-HT1B-R KO mice also displayed increased alcohol consumption and decreased alcohol sensitivity: They consumed more alcohol than control animals, displayed less alcohol-induced ataxia, and developed less tolerance to alcohol *(58)*. However, later studies failed to replicate these findings and suggested that increased ethanol consumption in 5-HT1B-R KOs might be the result of increased size of the KOs and a general increase in fluid consumption *(59,60)*. Alternatively, the increased ethanol consumption seen originally in 5-HT1B-R KOs might reflect genetic drift from wild-type controls (*61*, and references therein); careful breeding schemes can minimize this possibility *(61)*.

Although 5-HT1B-Rs have been implicated in the serotonergic suppression of feeding, an obesity phenotype (increased adiposity) has not been observed in these KOs. One report did find that 5-HT1B-R KO mice weigh, eat, and drink more than littermate controls *(60)*, although this finding has not been consistently replicated. Another recent study suggested that 5-HT1B receptors mediate a tonic satiety signal in brain regions that control food intake *(62)*. 5-HT1B-R KOs also display a reduced hypophagic response to *d*-fenfluramine, although this effect might arise from secondary adaptive changes in 5-HT2C receptor signaling or other pathways rather than the acute absence of the 5-HT1B-R *(63)*.

The 5-HT1B-R KO mice are also resistant to the locomoter effects of 3,4-methylene dioxy methamphetamine (MDMA, also known as Ecstasy), although they still exhibit MDMA-induced hypophagia and MDMA-induced suppression of exploratory behavior *(64,65)*. Other studies have demonstrated increased cocaine self-administration in these mice *(66)* and suggested that loss of the 5-HT1B-R sensitizes mice to the behavioral effects of cocaine *(67)*. However, 5-HT1B-R antagonists do not increase cocaine self-administration in wild-type mice *(65,68)* which implies that this phenotype in 5-HT1B-R KOs is the result of compensatory changes rather than the absence of the 5-HT1B-R itself. Indeed, several studies have demonstrated other alterations in the reward circuitry of 5-HT1B KO mice. These mice have enhanced dopamine release in the nucleus accumbens (NAcc) *(65,69)*, which might result from the loss of the 5-HT1B receptor on GABAergic terminals projecting to the substantia nigra and ventral tegmental area *(70)*. 5-HT1B-R KO mice also have higher striatal expression of deltaFosB and AP-1 *(67)*, two transcription factors whose expression is increased by cocaine. Taken together, these findings suggest that adaptations secondary to the loss of the 5-HT1B-R place animals in a behavioral and neurochemical state at baseline similar to that induced in wild-type mice by cocaine *(67)*.

Additional evidence also suggests that the 5-HT1B-R KO cocaine phenotype is not the result of the actual loss of the 5-HT1B-R itself: overexpression of 5-HT1B-R on serotonergic efferents to the NAcc sensitizes animals to cocaine *(71)*, a phenotype similar to that seen in KO mice lacking the 5-HT1B-R. The 5-HT1B-R KO cocaine phenotype might therefore result from other adaptive changes, just as the 5-HT1A-R KO anxiety-like phenotype might result from adaptive changes rather than the acute loss of the receptor protein. However, the discrepancy between the KO and overexpression phenotypes can also be explained in another way. Because the 5-HT1B-R is expressed so widely, both as an autoreceptor on serotonin neurons and as a heteroreceptor on cholinergic, GABAergic, and glutametergic neurons, different 5-HT1B-R populations might play opposing roles in the regulation of cocaine intake and its behavioral effects. As such, the specific overexpression of 5-HT1B receptors on NAcc efferents could produce the same phenotype as the loss of the receptor on other neuronal populations in the full KO.

Despite the observation of behavioral phenotypes in 5-HT1B-R KOs, numerous studies have failed to show an association between the C129T or C861G *5-htr1b* polymorphisms and suicide or alcoholism, although a recent report did find an association between the 861G allele and major depression and substance abuse disorder *(72)*. Neither of these mutations changes the amino acid sequence of the 5-HT1B-R, but both variants are in linkage disequilibrium with several other mutations in the 5-HT1B promoter that lead to decreased receptor expression. Indeed, an approx 20% decrease in 5-HT1B-R

binding has been found in the brains of individuals with the 861G allele *(73)*, suggesting that clinical disorders associated with the 861G allele might actually be the result of these other closely linked promoter mutations. Future studies directly examining these promoter mutations in patients might lead to a clearer understanding of any possible relationship between 5-HT1B-R activity and psychopathology.

4. 5-HT2A-R KO Mice and Drugs of Abuse

The 5-HT2A-R is a primary molecular target of the atypical antipsychotic drugs *(74)*. As expected, 5-HT2A-R KO mice have reduced cellular responses to hallucinogenic drugs with high affinity to the 5-HT2A-R, like lysergic acid diethylamide (LSD) *(75)* and might display a decrease in anxiety-like behavior *(76)*.

Another set of studies have examined 5-HT2A-R function by using antisense oligonucleotides (ASO) to block expression of this receptor. Intracerebro-ventricular (ICV) anti-5-HT2A-R ASO injection of mice caused a significant decrease in immobility in the forced swim test similar to that seen with antidepressant administration *(77)*, leading the authors to suggest that 5-HT2A-R downregulation might be an important part of antidepressant response. Another report using the same technique in rats found that anti-5-HT2A-R ASO injection into different brain regions had opposing effects on ethanol (ETOH) intake *(78)* and anxiety measures; intra-PFC injection increased ETOH intake, whereas ASO injection into the central nucleus of the amygdala decreased ETOH intake. These studies suggest that different 5-HT2A-R populations might play opposing roles in modulating the circuitry underlying these behaviors. Future studies utilizing careful ASO injections and both traditional and conditional 5-HT2A-R KOs are likely to yield new insights into the role of this important receptor in regulating anxiety, antidepressant responses, ETOH intake, and various other behaviors.

Several studies have examined the potential role of human 5-HT2A-R variants in psychotic behavior and memory. There is a T102C silent polymorphism in the first exon of *5-ht2a*, which might lower 5-HT2A-R expression through its linkage disequilibrium with a –A1438G promoter polymorphism *(79)*, although other reports have not supported this claim *(80,81)*. Nonetheless, the 102C allele has been associated with delusions and agitation/aggression *(82,83)*, visual and auditory hallucinations *(84)*, and psychosis *(85)* in Alzheimer's disease patients, as well as with schizophrenia and other phenotypes (*82,86*, and references therein). These findings are surprising, given that the 102C allele might lower 5-HT2A-R expression, because 5-HT2A-R agonists like LSD produce psychosis in humans, whereas many antipsychotic drugs work by blocking the 5-HT2A-R *(74)*.

A rare polymorphism (H452Y) in the C-terminal tail of the 5-HT2A-R has also been found that may alter agonist-induced receptor activity *(87)*. Carriers of this rare 452Y allele were found to have decreased verbal memory performance in a delayed free-recall test *(88)*, and it will thus be of interest to examine what effect this polymorphism has on other cognitive and emotional processes.

5. 5-HT2B-R KO Mice and Cardiovascular Function

The 5-HT2B-R KO mice have been generated, but approx 50% of homozygous KOs die by the first week of life because of defects in cardiac development *(89)*. Some mutant mice do survive to 6 wk and beyond, although they too have a variety of cardiac defects and display decreased fertility *(89)*. Mice overexpressing cardiac 5-HT2B-Rs exhibit cardiac abnormalities of an opposite type *(90)*. These findings are of interest in particular because of the increased incidence of cardiac disease in patients with depression who also demonstrate alterations in central and peripheral serotonergic activity *(91)*: Might some of this increased risk be attributable to altered 5-HT2B-R activity in the heart?

The 5-HT2B-R is also expressed on the cardiac valve fibroblasts, and the cardiac valvulopathy induced by drugs such as *d*-fenfluramine has been attributed to excessive activation of this population of 5-HT2B receptors *(92,93)*. A similar phenomenon has been observed in Parkinson's disease patients treated with the ergot-derived dopamine agonists pergolide and cabergoline *(94)*, in patients treated with the antimigraine ergot alkaloid ergotamine *(95)*, and in patients with carcinoid tumors that release 5-HT (*96*, and references therein). Pergolide, ergotamine, and MDMA have mitogenic effects on human valvular interstitial cells via the 5-HT2B-R *(97)*, providing a mechanistic explanation for these clinical findings and suggesting that chronic MDMA users might also be at risk for cardiac valvulopathy.

The 5-HT2B receptors are also expressed at low levels in the CNS *(98)* and 5-HT2B-R antagonists have effects on theta activity and sleep/wake states *(99)*. However, to our knowledge, no reports to date have examined the behavior or neurochemistry of 5-HT2B KO mice. This might be because of the confounding effects of the cardiac deficits in these mice, in which case conditional genetic approaches to delete only neural 5-HT2B receptors might be necessary. To our knowledge, there are no known associations between 5-HT2B-R polymorphisms and any clinical disorder.

6. 5-HT2C-R KO Mice, Obesity, Epilepsy, and Drug Abuse

A major contribution of 5-HT2C receptors to the appetite-suppressant actions of central serotonin systems was indicated by a feeding and obesity phenotype in

5-HT2C-R KO mice *(100)*, which were the first line of KO mouse model of obesity. Accordingly, these animals displayed reduced sensitivity to the anorectic effects of the nonselective serotonergic releasing agent dexfenfluramine *(101)*, which fits well with recent work showing that 5-HT2C-R antagonists block the hypophagic and hyperlocomotor responses to MDMA *(64)*. 5-HT2C-R KO mice exhibit a 25–30% increase in food intake, beginning in young adulthood. Interestingly, the obesity phenotype does not develop until the "middle-age" period of the mouse life span (beginning at 5–6 mo of age) *(102)*. Young adult mutants are able to compensate for their increased energy consumption and maintain normal body composition. However, compensatory processes appear to falter later in life, leading to body weight gain and enhanced adiposity. Subsequent studies revealed that progressive increases in the energy efficiency of physical activity might contribute to the late onset of obesity in these animals *(104)*. In addition, a role for 5-HT2C receptors in the central regulation of glucose homeostasis was indicated by the enhanced susceptibility of the mutants to type 2 diabetes mellitus *(102)*. These and other features of this obesity syndrome mimic common forms of human obesity. Partly on the basis of this work, a number of pharmaceutical companies are developing 5-HT2C receptor agonists as potential appetite suppressants. Although 5-HT2C-Rs are expressed in many CNS sites implicated in energy balance, recent attention has focused on the arcuate nucleus of the hypothalamus, where 5-HT2C-Rs activate neurons expressing melanocortins *(105)*.

Initial studies of 5-HT2C receptor mutants also revealed evidence of enhanced neuronal network excitability. Videotape monitoring of mutant mice revealed infrequent and sporadic tonic–clonic seizures and increased susceptibility to seizures induced by the $GABA_A$ receptor antagonist pentamethylenetetrazole *(100)*. In addition, the mutants were found to be highly sensitivity to fatal audiogenic seizures—the first instance in which a gene underlying such a phenotype had been identified *(106)*. Subsequent studies showed that 5-HT2C-R KOs have a globally enhanced sensitivity to a variety of convulsant stimuli, such as electroshock, electrical kindling of the olfactory bulb, and the chemoconvulsant flurothyl *(107)*. These results implicate 5-HT2C receptors in the serotonergic inhibition of neuronal network excitability and indicate a potential direction for anticonvulsant drug development.

Additional studies highlighted a role for 5-HT2C receptors in the actions of cocaine. Mutant mice displayed enhanced locomotor responses to cocaine and elevated cocaine self-administration in an operant progressive ratio paradigm *(108)*. In vivo microdialysis studies revealed enhanced cocaine-induced elevations of extracellular dopamine levels in the nucleus accumbens. These findings were in accord with pharmacological studies, indicating that 5-HT2C receptors mediate tonic inhibition of the mesoaccumbens dopamine projection. In accord

with this, mutants also displayed enhanced novelty-induced locomotion, a behavior associated with mesolimbic dopamine system activation and the susceptibility to self-administer drugs of abuse.

Recent work has also suggested a role for the 5-HT2C-R in modulating responses to antidepressants. 5-HT2C-R KO mice display enhanced antidepressant-like behavioral responses to fluoxetine, as well as enhanced fluoxetine-induced elevations of extracellular serotonin levels *(109)*. No differences in either of these parameters were observed in the absence of fluoxetine. Very similar findings were independently observed in rats treated with SSRIs and 5-HT2C receptor antagonist compounds *(109)*. Taken together, these data suggest that 5-HT2C antagonists might augment the efficacy of SSRIs.

Several other behavioral abnormalities have also been described in 5-HT2C-R KOs. These mice display several types of repetitive behavior that have been likened to compulsions *(110)*. 5-HT2C-R KO mice also display altered stress responses *(111)*, a deficit in maternal behavior (Storm, unpublished data), decreased spatial memory performance in the Morris water maze, and decreased long-term potentiation (LTP) in medial perforant path–dentate gyrus synapses *(112)*.

Indeed, several of the phenotypes seen in 5-HT2C KO mice are related to clinical disorders associated with polymorphic variants of *5-htr2c*. *5-htr2c* promoter variants have been associated with diabetes and obesity *(113)*, whereas 5-HT2C-R agonists cause weight loss and decrease subjective feelings of hunger in people *(114)*. Antipsychotic drugs like clozapine and olanzapine are 5-HT2C-R antagonists *(115)*, which raises the possibility that the significant weight gain caused as a side effect of these drugs might be the result of their action at this receptor. Indeed, the −759T variant allele of the 5-HT2C promoter was reported to protect schizophrenic patients from clozapine-induced weight gain *(116,117)*, although other studies have failed to replicate this association *(118–120)*.

A structural polymorphism has also been found in *5-htr2c*, which results in a cys23serine change in the N-terminus of the receptor. This polymorphism has been associated with major depression and bipolar disorder *(121,122)* and with increased risk of bipolar disorder among women with a family history of mental illness *(123,124)*. This polymorphism has also been associated with increased risk for depression and psychopathology among patients with Alzheimer's disease *(125)*, a more severe clinical course in schizophrenic patients *(126)*, and weight loss and anorexia nervosa among teenage girls *(127)*. Individuals carrying this allele show significantly higher cerebrospinal fluid (CSF) levels of the norepinephrine metabolite 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) *(128)*. Elevated CSF MHPG levels have also been found in alcoholic violent offenders, and although the *5-htr2c* 23ser allele was

not found at an increased rate in this group, this allele has been associated with impulsivity in another study *(129)*. This 23ser variant has also been shown to increase constitutive activity of the 5-HT2C-R while also making it less sensitive to 5-HT and other agonists *(130)*.

The finding that the 23ser allele alters the constitutive activity of the 5-HT2C-R is of interest because the constitutive activity of this receptor is tightly regulated. Five adenosines in the 5-HT2C mRNA are converted to inosines by the enzyme adenosine deaminase, a process known as RNA editing. This mRNA editing process alters the amino acid coding sequence of the receptor protein and the basal activity of the receptor, and the editing pattern shows significant alterations in depressed suicide victims that result in decreased receptor activity in the PFC *(131,132)*. Mouse studies show that this editing process is controlled by serotonergic tone *(133)*, suggesting that the alterations in 5-HT2C-R editing in depressed suicide victims reflect defective editing regulation by serotonin *(134)*.

Taken together, these data from mouse and man suggest that the 5-HT2C-R plays an important role in the regulation of neural circuitry that underlie many behaviors, from drug abuse and satiety to energy balance and affective state. Future studies with conditional KOs and viral rescue strategies are under development to explore the involvement of distinct 5-HT2C-R subpopulations in these processes and to understand how their dysregulation perturbs behavior.

7. 5-HT3A-R KO: Channeling Nociception, Anxiety, and Alcohol Through the Most Unique of 5-HT-Rs

The 5-HT3A-R and 5-HT3B-R are unique among the known 5-HT-Rs because they encode a serotonin-gated ion channel rather than a G protein–coupled receptor. The 5-HT3A-R is sufficient to produce excitatory channel activity on its own as a homopentamer *(135)*, although it also forms heteromeric complexes with and is modulated by the 5-HT3B-R *(136)*. Nonetheless, because the 5-HT3B-R is incapable of reconstituting channel activity on its own, the 5-HT3A-R is probably a core component of all serotonin-gated channels.

The 5-HT3A-R KO mice were recently generated and found to have a variety of phenotypes related to the role of this receptor in both the peripheral nervous system and the CNS. These KOs have no change in acute pain responses to a variety of stimuli, but they do display a significant reduction in tissue injury-induced persistent nociception even though they display normal post-injury edema *(137)*. Furthermore, this phenotype was replicated in control mice treated with a 5-HT3-R antagonist by the intrathecal or intraplantar route, suggesting that the reduction in persistent nociception in the KOs is the result of the acute loss of the receptor rather than secondary adaptations in its absence.

These findings might be explained by the observation that 5-HT3A-R are expressed mostly on myelinated Aδ afferents and a unique population of C fibers, few of which coexpress the vanilloid/capsaicin receptor VR1 or the proedema factor substance P *(137)*. Consistent with this expression pattern, deep dorsal horn neurons in the KOs fire at a lower rate than those of control animals during exposure to nociceptive stimuli.

The 5-HT3A-R KO mice also display decreased nociceptive behavioral responses to the intrathecal admistration of 5-HT, although the injection of a selective 5-HT3-R agonist was insufficient to produce nociceptive behavioral effects itself *(137)*. Taken together, these findings suggest that 5-HT3A-R activation is necessary but not sufficient for the pronociceptive behaviors elicited by 5-HT in the spinal cord and suggest that chronic tissue injury pain responses are partly mediated by both central and peripheral 5-HT3-R populations.

Injection of 5-HT into the spinal cord can also have antinociceptive or analgesic behavioral effects at high doses, although this effect did not differ between KOs and control animals *(137)*. However, antisense oligos against the 5-HT3-R were reported to decrease the analgesic effects of intrathecal 5-HT administration *(138)*. The discrepancy between these findings might reflect a nonspecific effect of the antisense oligos, compensatory changes in the 5-HT3-R KOs, or other differences in experimental paradigms.

The 5-HT3A-R might also play a role in modulating anxiety-like behavior, a possibility previously suggested by pharmacologic studies and now supported by the finding of decreased anxiety-like behavior in 5-HT3A KO mice. Specifically, these mice spent more time in the illuminated zone of a light/dark box and made more entries into the open arms of an elevated plus maze *(139)*. The KO animals also investigated more closely a novel object placed in an open field enclosure *(139)*. A second study also found that the KOs display a significant anxiolyticlike phenotype in the elevated plus maze and found an anxiolyticlike trend in their behavior in the open field and light–dark tests *(140)*. Furthermore, wild-type mice treated with a specific 5-HT3A-R antagonist also displayed decreased anxiety-like behavior in the elevated plus maze *(141)*, suggesting that the anxiolyticlike phenotype of the KO animals is the result of the loss of 5-HT3A-R activity rather than secondary compensations in its chronic absence.

However, 5-HT3A-R KO mice also displayed enhanced freezing behavior in both the conditioning and later phases of a fear-conditioning test *(140)*. This finding suggests that 5-HT3A-R loss enhances fear memory formation, which might be the result of the loss of 5-HT3A-R-driven GABA-ergic inhibition of the lateral amygdala *(142,143)* and/or an upregulation of CRH mRNA in the central nucleus of the amygdala *(140)*, two brain regions thought to play a role in the neural circuitry of fear behavior *(25)*.

Despite this increase in fear-related behavior, the 5-HT3A-R KO mice actually display decreased ACTH release in response to restraint stress or lipopolysaccharide (LPS) injection, although the KOs did not differ from controls in their CRH expression or sensitivity to CRH *(140)*. This decrease in stress-induced ACTH release might thus result from lower arginine vasopressin (AVP) mRNA expression in the paraventricular nucleus (PVN) of 5-HT3A-R KO mice *(140)*, as AVP release in the PVN potentiates CRF-induced ACTH release *(144)*.

The 5-HT3A-R KO females also displayed significantly increased immobility in the forced swim test, and males showed a similar trend *(145)*. Increased immobility in this test is commonly thought to reflect a depressive-like state because antidepressant medications decrease immobility *(146)*. However, another interpretation is that increased behavioral activity (measured as decreased immobility) in the forced swim and tail suspension tests actually reflects increased somatic anxiety *(4)*; according to this view, the increased immobility seen in 5-HT3A-R KO mice might simply be another reflection of decreased anxiety-like behavior in these animals. The 5-HT3A-R KO mice also displayed sex differences in the defensive withdrawal test *(145)*. The finding of sex differences in this test and the forced swim test suggest a sexually dimorphic role for this receptor in the serotonergic modulation of affective behavior, an intriguing finding that merits further study.

A long literature suggests that 5-HT3A-R activation contributes to the reinforcing properties of alcohol, because 5-HT3A-R antagonists decrease alcohol consumption (*147,148*, and references therein). It was surprising to find, then, that 5-HT3A-R KO mice do not display any difference from controls in alcohol intake or preference or in the locomotor response to alcohol *(149)*. This study also replicated previous reports that 5-HT3A-R antagonists decrease alcohol consumption in wild-type mice, but not in 5-HT3A-R KO animals, suggesting that this effect is the result of a specific interaction of these drugs with the 5-HT3A-R *(149)*. Furthermore, the fact that these drugs decrease alcohol intake by specifically blocking the 5-HT3A-R whereas the corresponding KO mice do not drink less alcohol than controls provides behavioral evidence for secondary compensations in the KOs that obfuscate the normal role of the 5-HT3A-R in alcohol consumption. Nonetheless, this study is consistent with the larger literature in suggesting that 5-HT3-R antagonists might be useful clinically in treating alcoholism and related disorders.

Aside from this role of presumably central receptor populations in modulating alcohol intake and affective behavior, peripheral 5-HT3A-Rs might play a role in urinary system function. A line of mice was recently described that contain a val13ser point mutation in the M2 channel lining domain of the 5-HT3A-R, which makes the channel more sensitive to serotonin and increases its constitutive activity when combined with 5-HT3B-R subunits *(150)*. These 5-HT3A

"hypermorph" mice died prematurely from an obstructive uropathy and displayed significant urinary system dysfunction. Heterozygous male mice and homozygous female mice with this mutation live for approx 4–6 mo, future studies could also examine behavioral changes related to changes in central 5-HT3A-R function in these animals.

In addition to this line of 5-HT3-R hypermorph mice, a line of mice has been created that overexpresses the 5-HT3A-R in forebrain regions. These mice display an interesting set of behavioral phenotypes such as altered ethanol responses *(151)*. However, the relevance of these findings to the normal role of the endogenous 5-HT3A-R is limited by the extreme nature of this genetic manipulation: The overexpressing mice express the 5-HT3A-R in the forebrain at a level two orders of magnitude higher than normal *(151)*. These animals display significant ectopic expression of the 5-HT3A-R in brain regions such as amygdala and in cellular populations such as pyramidal neurons that do not normally express this receptor *(151,152)*.

Nonetheless, phenotypic abnormalities observed in 5-HT3A-R KO mice, together with pharmacologic studies and clinical trials using the 5-HT3-R antagonist ondansetron *(153)*, suggest that 5-HT3A-R antagonists could be used to treat chronic pain, anxiety disorders, and alcoholism in addition to their established role as anti-emetics. Further support for a role for this receptor in regulating affective behavior comes from recent studies on a polymorphism (C178T) in the 5′ untranslated region of the 5-HT3A-R mRNA, which might increase 5-HT3A-R expression by improving translational efficiency in vitro *(154)*. This polymorphism was associated with decreased harm avoidance in women in a recent study *(155)* and was replicated in a second group of women. This finding was also corroborated by the finding of higher social desirability and lower indirect aggression scores in the nonconformity section of the Karolinska Scales of Personality test *(155)*. The 178T allele was more common among bipolar patients than controls in another study *(154)*. Future studies will be necessary to replicate these intriguing findings and to study what effect this polymorphism might have on neural 5-HT3A-R expression in vivo; these findings also raise the possibility that similar phenotypes might be seen in 5-HT3A-R KO mice.

8. 5-HT4-R KO Mice, Novelty, and Stress Responses

The 5-HT4-R KO mice were recently generated and characterized *(156)*. These mice have decreased viability (a decreased percentage of homozygous null animals were born from a heterozygote cross than expected), which might be the result of a lower seizure threshold in these mice. 5-HT4-Rs are present on GABAergic interneurons and increase the firing of these cells *(157,158)*,

which raises the possibility that the seizure phenotype of the KO mice is the result of a loss of 5-HT4-R-driven GABA release. The 5-HT4-R is also expressed on rhythm-generating respiratory neurons in the lower brainstem Pre-Boetzinger complex *(159)*, raising the possibility that 5-HT4-R KO animals might have impaired ventilatory regulation.

The 5-HT4-R KO mice gained weight normally, but they displayed a lack of restraint-induced anorexia and weight loss compared to controls *(156)*. Despite this apparent difference in stress sensitivity, the KOs did not display a stress-induced change in corticosterone levels. The 5-HT4-R KO mice also displayed lower activity in an open field during a first exposure but no difference in thigmotaxis or other validated measures of anxiety. These KOs also displayed normal home cage activity. Taken together, these findings suggest that the 5-HT4-R might play a role in neural circuits that underlie stress responses and reactivity to novelty. However, it is unclear whether these phenotypes seen in the KO mice are the result of secondary compensations in its absence.

Few studies have examined potential relationships between the human 5-HT4-R and psychiatric disorders, although *5-htr4* polymorphisms have been linked to both bipolar disorder and schizophrenia *(160,161)*. These findings are of particular interest because *5-htr4* maps to 5q32, a genomic region linked to bipolar disorder in a Costa Rican kindred study *(162,163)* and in another ethnic sample as well *(164)*. Future work will be necessary to corroborate these findings and clarify the potential role of 5-HT4-R polymorphisms in bipolar disorder, but these findings suggest that it would be of interest to examine a wide variety of other affective behaviors in the 5-HT4-R KO mice.

9. 5-HT5A-R KO Mice and Exploratory Activity

The 5-HT5A-R is among the least explored of the serotonin receptors (a Medline search for 5-HT5A yields only 39 papers), yet 5-HT5A-R KO mice display increased exploration in the open field, elevated plus maze, and novel object tests *(165)*. This phenotype appears specific to increased exploratory activity, because the KOs displayed no significant alterations in anxiety-related measures in these tests. The KOs also display a blunted increase in locomotor activity induced by LSD, suggesting that some of the behavioral effects of LSD might result from its interaction with the 5-HT5A-R. It is difficult to know whether these phenotypes reflect the absence of 5-HT5A-R signaling in the adult brain, or indirect compensatory responses in its absence; this question is difficult to answer in part because of the absence of specific 5-HT5A antagonists. Several studies have examined polymorphisms of the human *5-htr5a* gene in schizophrenia or affective disorders, but consistent findings have yet to

emerge *(166–168)*. Mice also express a 5-HT5B-R, but humans lack this receptor because its gene has been interrupted by stop codons *(169)*; to our knowledge, this is the only serotonin receptor expressed in either mouse or man without a functional homolog in the other species.

10. 5-HT6-R KO Mice and Alcohol Insensitivity

Pharmacological and antisense oligonucleotide studies support a role for the 5-HT6-R in fear conditioning and cognition (*170*, and references therein), although similar phenotypes were not found in 5-HT6-R KOs (Bonasera and Tecott, unpublished data). This discrepancy might be the result of species differences, compensatory processes in the KO mice, or limitations of the tools used in the pharmacologic and antisense studies. Although 5-HT6-R KO mice display normal behavior in a wide array of tests, they do display reduced sensitivity to the ataxic and sedative effects of alcohol (Bonasera and Tecott, unpublished data). Several studies have looked for an association between 5-HT6 polymorphisms and various neuropsychiatric disorders, but consistent findings have yet to emerge.

11. 5-HT-7-R KO Mice, Thermal Regulation, and Affective Behavior

The 5-HT7-R KO mice have recently been generated and shown to have decreased contextual fear conditioning, which might be the result of decreased LTP in the CA1 region of their hippocampus *(171)*. Studies with these mice also suggest that the 5-HT7-R might regulate body temperature changes in response to serotonergic drugs *(172)*. These animals also display decreased immobility in both the forced swim and tail suspension test, and they spend less time in rapid eye movement (REM) sleep *(173)*. Taken together, these results suggest that 5-HT7-R antagonists warrant consideration as potential antidepressant agents *(174)*. Several studies have looked for an association between *5-htr7* polymorphisms and various neuropsychiatric disorders, but consistent findings have yet to emerge.

12. Pet-1 KO Mice, the Transcriptional Cascade of Serotonergic Development, and Affective Behavior

Pet-1 is an ETS-domain transcription factor that was first discovered in adrenal chromaffin-derivd PC12 cells, where it was found to induce transcription of the nicotinic acetylcholine β4 receptor *(175)*. Pet-1 is expressed in the adrenal medulla as well as the eye and the brain *(175)*; within the brain, it is expressed exclusively in serotonin neurons *(176,177)*. Pet-1 expression appears

within these neurons approximately one-half day before they express 5-HT itself, and functional Pet-1-binding sites are found in the promoter regions of the human and mouse 5-HT1A receptor, the serotonin transporter, tryptophan hydroxylase, and aromatic L-amino acid decarboxylase *(176)*, suggesting that Pet-1 might coordinately regulate many of the genes necessary for the final differentiation of serotonin neurons.

To evaluate this possibility, Hendricks and colleagues *(178)* then generated Pet-1 KO mice. These mice were subsequently found to lack approx 70% of the normal number of serotonin neurons, and the remaining 30% of serotonin neurons displayed defective expression of tryptophan hydroxylase and the serotonin transporter *(178)*. Furthermore, the Pet-1 KO mice display an 85–90% decrease in both 5-HT and the serotonin metabolite 5-hydroxyindole-acetic acid (5-HIAA) in their cortex, hippocampus, and caudate nucleus, although these animals did not display any noticeable differences in overall cortical or cytoarchitectural structure. Other studies have given further support for this essential role for Pet-1 in the final differentiation of serotonin neurons and suggest that Pet-1 acts in a molecular cascade containing several other transcription factors including Nkx2.2, Lmx1b, and Ascl1/Mash1 *(179–182)*.

Consistent with the neurochemical findings in Pet-1 KO mice and the widely reported inverse correlation between low serotonin levels and affective disorders, these mice also displayed significant anxiety-like behavior in the open field and elevated plus maze. These animals also displayed increased aggressive behavior (when compared to wild-type controls) in the resident–intruder test *(178)*, including an increased number of overall attacks on the intruder mouse. Furthermore, the Pet-1 KO mice frequently attacked the intruder within the first 10 s of encountering it, a striking phenotype that was never observed with wild-type control animals. Given the strong association between low serotonergic activity and depression as well as alcoholism, future studies are likely to evaluate these and other related behaviors in Pet-1 KO mice.

The human Pet-1 gene (also known as FEV) is also expressed exclusively within serotonin neurons in the CNS *(183)*, as well as in megakaryocytes *(184)*. To date, no association studies have examined whether mutations in the Pet-1/FEV gene are associated with neuropsychiatric disorders in humans; however, a linkage study in patients suffering from a severe, recurrent, and early-onset form of major depression did show a significant peak at 2q36 *(185)*, the genomic location of Pet-1/FEV. Future studies are thus likely to focus on the tantalizing possibility that this linkage peak is the result of mutations or polymorphisms in Pet-1/FEV. Indeed, the phenotype of Pet-1 KO mice suggests that deficient Pet-1 activity could profoundly hinder the differentiation of

serotonin neurons and result in a hyposerotonergic state that could lead to affective disorders, as proposed by the monoamine hypothesis.

13. SERT KO Mice, Altered Serotonergic Tone, and Unusual Affective Changes

Serotonin transporter (SERT) KO mice were initially generated by two groups *(186,187)* and were found to display equivocal phenotypes in the tail suspension and forced swim tests *(188)*, but they display reduced novelty-induced exploratory activity and increased anxiety-like behavior in a variety of tests *(189)*. SERT KO mice also display a decrease in 5-HT neuron firing rates *(186,190)* and a 50% reduction in the number of 5-HT neurons *(186)*. Despite this decrease in the firing rate and total number of 5-HT neurons, SERT KO mice have significantly higher baseline extracellular 5-HT levels as measured by microdialysis *(191)*. Taken together, these data suggest a model in which the absence of SERT impairs either the embryonic differentiation or adult viability of 5-HT neurons and lowers the firing rate of the remaining 5-HT neurons; however, 5-HT reuptake in these mice is so profoundly deficient that they still display almost 10-fold more extracellular serotonin than wild-type controls.

One potential explanation for the decreased firing rate of 5-HT neurons in the SERT KO mice is that the additional extracellular serotonin present in these animals excessively activates 5-HT1A-AR, which then hyperpolarize the 5-HT neurons and decrease their firing rate. Consistent with a chronic enhancement of this process, SERT KO mice display alterations in 5-HT1A-AR expression *(192)* and behavioral changes consistent with these 5-HT1A-AR alterations *(189)*. In fact, overexpression of 5-HT1A receptors in specific brain regions of SERT KO mice can rescue some parts of the abnormal stress/anxiety phenotpe of these animals *(193)*, further suggesting that this aspect of the SERT KO phenotype might actually result from altered 5-HT1A receptor expression/activity.

An alternative, although not necessarily mutually exclusive, hypothesis is that the SERT KO anxiety-like behavioral phenotype arises from developmental alterations in these mice. In line with this hypothesis, inhibiting SERT with SSRIs during a transient early postnatal period causes a variety of affective changes later in the resultant adult animals that are generally similar to those seen in SERT KO mice *(194,195)*. Furthermore, the effects of neonatal SSRI exposure are specifically the result of SERT blockade because they are absent in mice genetically deficient in SERT *(196)*.

Why does SERT blockade during development disrupt affective behavior later in life? One possibility is that this manipulation increases postsynaptic serotonin receptor signaling in the forebrain, which then exerts maladaptive compensatory

changes that increase anxiety-like behavior later in life. Another possibility is that neonatal SSRI exposure increases serotonin concentrations in the raphe nucleus, which then excessively activates 5-HT1A-ARs. Excess 5-HT1A-AR activity during development could then impede the growth and differentiation of serotonin neurons, because 5-HT1A-AR might exert a negative control over 5-HT neuron development *(197)*. Consistent with this hypothesis, rats treated neonatally with SSRIs show a persistent reduction in tryptophan hydroxylase expression as adults *(198)*.

In either case, the anxiety-like phenotype of SERT KO mice fits well with data associating human SERT polymorphisms to affective disorders. The human SERT promoter contains a 44-basepair variable repeat sequence that commonly exists in either a short (s) or long (l) form, and the s allele is generally associated with lower SERT expression and activity. Furthermore, the s allele has been tied to increased amygdala reactivity to fearful faces *(199)*, anxiety symptoms *(200)*, and an increased probability of depressive episodes after significant life stressors *(201)*. These findings are similar to those seen in the SERT KO mice; in both cases, lower SERT activity is associated with negative affective behavior.

In the case of both the SERT KO mice and individuals carrying the s allele, the challenge is to understand how blocking SERT with antidepressant drugs generally improves affective symptoms, whereas people or animals with lower SERT expression/activity are at risk for affective disorders. Because individuals with the s allele are likely to have lower SERT expression/activity throughout development and adulthood, whereas SSRIs are usually prescribed only in adolescents or adults (who would have had higher SERT activity prior to taking the SSRI), it is possible that blocking SERT during development has opposite effects to blocking it in adulthood. This hypothesis fits well with the literature on the effects of neonatal antidepressant use in rodents, and future work is likely to focus on clarifying the molecular and cellular mechanisms by which this manipulation perturbs adult behavior and brain function. This work is likely to have an important clinical implication for pregnant women taking SSRIs, as many of them cross the placental barrier and might interact with the developing brain of the fetus.

14. TPH2 Mutant Mice, and Affective Behavior: New Support for the Monoamine Hypothesis?

Until 2 yr ago, it was thought that there was only a single tryptophan hydroxylase (TPH) gene. However, when this TPH gene was knocked out in mice, the investigators were surprised to find normal 5-HT levels in the brain *(202)*. This observation led to the cloning of a second TPH gene (TPH2), which is expressed

predominantly in the brain (whereas the original TPH1 gene is expressed in the gut, pineal gland, spleen, and thymus). A Pro447Arg mutation in TPH2 was then serendipitously found in two commonly used inbred mouse lines (BALB/cJ and DBA/2), whereas two other commonly used inbred lines (C57Bl/6J and 129/SvJ) have the normal proline residue at this position *(203)*. This study also demonstrated that the 447Arg mutant enzyme is associated with an approx 55% decrease in 5-HT synthesis rate, and the inbred lines carrying this mutant allele display a 40–45% decrease in tissue 5-HT content in several brain regions. These data suggest an interesting explanation for the fact that BALB/cJ and DBA/2 mice are sensitive to the effects of fluoxetine in the forced swim test, but C57Bl/6J and 129/SvJ mice are not *(204)*: perhaps only animals with lower 5-HT neurotransmission will respond behaviorally to SSRIs.

The TPH2 Pro447Arg mutation has not been found in humans, but a nearby Arg441His mutation has been found that causes an approx 80% reduction in 5-HT synthesis in in vitro assays *(205)*. This 441His mutation was found in patients with unipolar major depression at 10 times the rate at which it was seen in healthy controls (and it was not found at all in patients with bipolar disorder), suggesting that this functional polymorphism is a significant risk factor for unipolar major depression. Future studies will be necessary to confirm this finding and clarify the inheritance and penetrance pattern of this allele in affective disorders, but a variety of other studies already fit well with this finding. Several studies have found an association between the TPH2 gene and depression *(206)* and between TPH2 and suicidality and/or bipolar disorder *(207–209)*. Furthermore, another study showed significant linkage to a region of chromosome 12 just proximal to the TPH2 locus (at 12q21) among a large number of individuals suffering from depression *(210)*.

Taken together, these findings suggest that TPH2 mutations can lead to affective disorders in humans, and a mouse model similar to these mutations already exists in the inbred mouse strains carrying the 447Arg allele. Further study of these mouse strains might help us understand how decreased serotonin synthesis leads to affective behavioral changes. These mouse strains can also serve as a model to help us evaluate new treatments and early interventions designed to ameliorate or even prevent the negative affective changes that result from chronically impaired 5-HT transmission.

15. Summary

The past 10 yr have been an intensely productive period in 5-HT receptor KO studies: Of the 14 murine 5-HT-Rs that have been cloned to date, 10 have been "knocked out" and behavioral characteristics of these mice have been reported. Additionally, several other genes that regulate the development and

activity of serotonin neurons (like Pet-1, SERT, and TPH) have been studied in mutant mice and human subjects. These studies are all the more remarkable when placed in historical context: Serotonin was not identified as a neurotransmitter until just over 50 yr ago *(211)*, at which time it was not clear that receptors even existed as true physical entities. Pharmacologic and biochemical studies provided evidence for the existence of receptors over the following decades, and these studies were finally molecularly substantiated for the serotonin receptors, as they were cloned over the last 15 yr (*see* Chapter 1). Thus, the field has undergone a metamorphosis, from an initial finding of receptor-like activity in various tissue preparations, down to the focused molecular cloning of these receptors, and back up to the analysis of their roles in complex behavior with pharmacology studies and 5-HT-R KO mice.

These studies are illuminating for both what they have and what they have not found: none of the KOs are lethal as a result of defects in CNS development or function, suggesting that although 5-HT receptors modulate the neural circuitry of diverse behaviors, they are not absolutely necessary for brain functions required to support life. These mutants have provided models to understand the neural circuitry of some complex behaviors with relevance to human diseases such as epilepsy and obesity, anxiety and affective disorders, substance abuse, and nociceptive processing. Careful study has provided evidence of alterations in synaptic connectivity *(6)* and the expression of other genes in some of these KO mice *(5)*, and similar studies are likely to further enrich our understanding of additional roles that 5-HT receptors play beyond the acute regulation of neuronal activity. Because KO mice lack the knocked-out receptor throughout development and adult life (a phenomenon difficult to replicate with pharmacology), it provides a model to investigate other secondary processes that depend on the activity/expression of the missing receptor. Similarly, KO studies have greatly clarified our understanding of how relatively broad-spectrum serotonergic drugs like SSRIs, MDMA, LSD, and *d*-fenfluramine exert particular behavioral effects through their action at specific serotonin receptors.

Despite the benefits of KO studies, none of the 5-HT-R KOs precisely resembles a single human neuropsychiatric disease, just as human genetics studies suggest that no single human neuropsychiatric disease is perfectly correlated with mutations in a single 5-HT receptor gene. Instead, these studies suggest that 5-HT-Rs modulate the neural circuits that underlie complex behavior and that pharmacologic modulation of these receptors might be efficacious in specific neuropsychiatric disorders even if these disorders are not "caused" by a primary dysfunction in a 5-HT-R. Nonetheless, emerging data suggests that SERT, 5-HT1A-R, and TPH2 mutations might influence susceptibility to affective disorders. Given the severe 5-HT deficit in Pet-1 KO mice, it will not be surprising if Pet-1 mutations are also associated with affective dysregulation.

Taken together, these findings suggest the potential of a new horizon in clinical psychiatry, in which the careful diagnosis and specific treatment of mental health disorders might be based on genetic testing and/or neuroimaging in combination with a clinical interview and in which future treatments might be targeted to correct underlying genetic/neuroanatomic lesions.

Our understanding of the roles 5-HT-Rs play in complex behavior is likely to expand dramatically in future years with the increasing availability of conditional genetic mutants that rely on cell type- and tissue-specific promoters, microarrays, and other tools to analyze compensatory changes in genetically modified mice at a genome-wide and proteome-wide level and with new behavioral testing equipment designed to allow high-resolution quantitative analysis of mouse behavior *(212)*. Considering how far the study of these receptors has progressed in the last 50 or even just 10 yr, the next 10 yr and the decades beyond are likely to be a fascinating journey full of unexpected surprises, a story of small mice with big implications for man.

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Effects of Serotonin-Related Gene Deletion on Measures of Anxiety, Depression, and Neurotransmission

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Summary

Recent advances in molecular technology have made possible the generation of mice lacking specific serotonin (5-hydroxytryptamine [5-HT]) receptors or regulatory targets governing 5-HT neurotransmission. The goal of this review is to summarize the effects of deleting serotonin-related genes on certain behavioral and neurochemical outcomes in mice. The influence of various mutations of 5-HT receptors and the 5-HT transporter on behaviors related to anxiety and depression is reviewed. In addition, changes in transmission of 5-HT and other neurotransmitters, measured principally by in vivo microdialysis, produced by genetic mutations of 5-HT targets is summarized. Most of the literature refers to mutations of genes for the 5-HT_{1A} receptor, 5-HT_{1B} receptor, and the 5-HT transporter (5-HTT) and comprise the largest part of this review. However, data regarding other murine knockouts were included where available. Although some phenotypes might be expressed as a result of developmental compensation, comparison with pharmacological antagonists has been helpful in distinguishing a functional role for the absence of the targeted receptor. Advanced techniques, involving evaluation of the effects of inducible gene deletion, gene rescue, or selective topographical expression of receptors in the contribution of 5-HT receptors to mood disorders, are discussed.

Key Words: Serotonin; knockout; anxiety; depression; microdialysis.

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1. Introduction

Through recent advances in molecular technology, it has been possible to generate mice without the genetic machinery for expressing specific serotonin (5-hydroxytryptamine [5-HT]) receptors or regulatory targets governing 5-HT neurotransmission. The first published 5-HT receptor knockout was the 5-HT_{1B} receptor knockout mouse *(1)*, which demonstrated an aggressive phenotype. A number of other mutant lines expressing specific deletion of 5-HT receptor targets have been generated since then. Among the strengths of this approach is the ability to develop a complete and specific deletion of a targeted 5-HT receptor. Initially, the value of genetic deletion studies was justified by comparison with pharmacological receptor antagonists or other tools. It was argued initially that genetic deletion of a particular receptor would provide a degree of selectivity that is usually not possible with receptor antagonists, which are inherently nonselective to some degree. Also, mutant models can provide information about 5-HT receptors for which pharmacological agents were not available. Furthermore, gene deletion models would provide long-lasting or permanent changes in receptor function that are not usually possible given the brief time-course of most pharmacological agents. The phenotype expressed by the mutant mice is evaluated in order to identify changes in function associated with deletion of the targeted receptor. When selective pharmacological agonists are available, the absence of agonist effects in mutant mice can be used to demonstrate that the targeted deletion of a receptor is functional.

The use of mutant mice rapidly demonstrated a number of drawbacks to their initial conceptualization. Because most mutant models are constitutive, the normal gene products are absent throughout development and later life in all tissues and are not subject to normal regulation. Issues created by the constitutive deletion become most problematic when the absence of a receptor results in some form of developmental compensation. In this case, a phenotype in mutant mice is caused by alterations in other systems because of its absence at a critical point early in the life span, rather than the deletion of the receptor at the time of testing. Discordant patterns of effects between pharmacological antagonists in wild-type mice and receptor deletion in mutant phenotypes often suggests the potential contribution of developmental compensation to the mutant phenotype. Also, when conceived as models for evaluating genetic contributions to psychiatric disease, there are clearly major differences between single-nucleotide polymorphisms, which might involve minor structural variations that subtly alter the function of receptor targets, and mouse models involving null mutations that completely delete targeted gene products. In some instances, however, heterozygotes can provide assessment of partial loss of function, a type of gene dose–response curve. More recently, a refined conceptualization of knockout mice has emerged

as targeted developmental models of neuropsychiatric disorders *(2)*, rather than as sophisticated pharmacological antagonists. The behavioral and physiological consequences demonstrated for mouse mutants with targeted gene disruptions of 5-HT receptors might provide unique insight into the vulnerable behavioral consequences that arise from naturally occurring genetic variations within a particular receptor system that impacts human psychopathology *(3)*.

The assessment of behavioral phenotypes in 5-HT receptor mutant mice can be complicated by varying assessment procedures used by different laboratories. Even when specific attempts by investigators were made to control animal supply and behavioral assessment procedures between laboratories, some test results using 5-HT_{1B} receptor mutant mice still varied between sites, although consistency was maintained on some other tests *(4)*. In contrast to this alarming outlook for behavioral testing, another example demonstrated remarkable consistency for robust behavioral phenotypes between independent laboratories that did not even attempt to standardize their testing procedures. 5-HT_{1A} receptor mutants have been reported to demonstrate a phenotype of highly anxious behavior by all three laboratories that generated them *(5–7)*. It is unlikely that behavioral assessment procedures for genetic mutants are any more intrinsically unstable between laboratories than assessment procedures for other phenotypes. Care taken to follow standardized assessment protocols, whenever possible, is a prudent strategy to guide test procedures. The demonstration of convergent findings from multiple measures of the same construct is critical for helping to build confidence in any particular set of conclusions.

The goal of this review is to provide a current synopsis of the effects of deleting serotonin-related genes on certain behavioral and neurochemical outcomes in mice. Specifically, we described the consensus of findings suggesting that these mutations influence behaviors related to anxiety and depression, as well as neurotransmission measured by in vivo microdialysis. The largest bodies of literature in this regard refer to mutations of genes for the 5-HT_{1A} receptor, 5-HT_{1B} receptor, and the 5-HT transporter (5-HTT). They comprise the largest part of this review. However, we have included data regarding other murine knockouts where available. We have attempted to include all of the relevant citations in this venture and apologize for any work that might have been inadvertently omitted.

2. Tests of Anxiety and Exploratory Behavior

Most rodent behavioral tests of anxiety take advantage of natural conflicts that are faced by rodents; that is, rodents are naturally driven to explore a novel environment but also innately to avoid novel environments. These tests are typically referred to as “state” or “normal” anxiety tests in order to emphasize that these tests put animals into a one-time stressful situation where natural *reactions*

are measured *(8)*. For example, in the open field test, animals are placed into a novel arena that is brightly lit. This experience alone is considered anxiety provoking, as rodents ordinarily avoid brightly lit open spaces. Therefore, under normal circumstances, one might expect a rodent to avoid the centermost part of the arena and remain near the walls, a phenomenon called thigmotaxis. In this case, animals are simply expressing a short-term or "state" of anxiety, rather than pathology *per se*. Genetic deletion studies might provide a source of important information on the genetics of the pathophysiology of anxiety *(9)*. In other words, although examined in tests of "state" anxiety, mutant animals might provide important information regarding the physiological substrates underlying chronic and consistent patterns of exaggerated or blunted anxiety responses. Table 1 summarizes findings regarding 5-HT-related knockouts and tests of anxiety and exploratory behavior.

2.1. 5-HT$_{1A}$ Receptors

Deletion of the 5-HT$_{1A}$ receptor gene results in an anxiogenic phenotype. Multiple laboratories have reported that animals lacking the 5-HT$_{1A}$ receptor (5-HT$_{1A}^{-/-}$) showed greater anxiety levels across several behavioral tests. 5-HT$_{1A}^{-/-}$ mice spent less time *(5–7,10)*, made fewer entries *(5,6)*, and were less active *(5,7,10,14)* in the center region of an open field. Likewise, 5-HT$_{1A}^{-/-}$ mice made fewer entries *(7,10)*, spent less time, and made fewer head dips *(5,7)* in the open arms of the elevated plus or zero maze. Similar findings were also reported using the novel object test, where 5-HT$_{1A}^{-/-}$ mice showed increased latency to approach a novel object, less activity exploring the novel object, and increased time in the nesting area *(5)*. 5-HT$_{1A}^{-/-}$ mice also demonstrated an increased latency to feed in a novelty-suppressed feeding test *(10,12)*. Finally, in addition to behavioral tests of anxiety, these mice demonstrated exaggerated autonomic responses to stress as evidenced by greater body temperature and heart rate increases compared to wild-type mice *(13)*.

Taken together, these findings are consistent with the hypothesis that the 5-HT$_{1A}$ receptor might normally serve to reduce anxiety. However, more recent findings have challenged this simple explanation and suggested that it is the role of the 5-HT$_{1A}$ receptor during development that is most crucial for developing anxiety *(10)*. Transgenic mice were developed using the insertion of a tetO site upstream of the coding region for the 5-HT$_{1A}$ receptor, allowing transcription in the presence of tTA. This is prevented by treatment with doxycycline, thus allowing inducible expression of the 5-HT$_{1A}$ receptor (i.e., animals treated with doxycyline remain 5-HT$_{1A}^{-/-}$). These studies showed that when mice were depleted of 5-HT$_{1A}$ receptors as adults, they failed to demonstrate the anxiogenic phenotype *(10)*. Moreover, mice in which 5-HT$_{1A}$ receptors in the forebrain were rescued early in life did not develop the anxiety phenotype. This

suggests that postsynaptic receptors in forebrain regions associated with emotional behaviors might be involved in mediating this anxiety behavioral phenotype *(10)*. Finally, administration of the 5-HT_{1A} receptor antagonist WAY 100635 failed to induce anxiogenic effects in a number of responses (e.g., ref. *14*).

Some findings suggest that impairment of γ-aminobutyric acid $(GABA)_A$ receptor function might be one compensatory change mediating anxiety in 5-$HT_{1A}^{-/-}$ mice from a Swiss–Webster background *(15)*. In particular, diazepam was ineffective in reducing anxiety in 5-$HT_{1A}^{-/-}$ mice in which α_1 and α_2 $GABA_A$ receptor subunits are downregulated, suggesting a mechanistic link between enhanced anxiety in 5-$HT_{1A}^{-/-}$ mice and GABAergic function *(15)*. However, the role of $GABA_A$ receptors in the development of anxiety symptoms is unclear. 5-$HT_{1A}^{-/-}$ mice expressed on a C57Bl6 background retain the anxiety phenotype but do not express resistance to benzodiazepines or changes in $GABA_A$ receptor subunit expression *(16)*.

In contrast to mice lacking the 5-HT_{1A} receptor, animals *overexpressing* the 5-HT_{1A} receptor might demonstrate an opposite and anxiolytic phenotype. These animals spent more time, made more entries, and had more head dips in the open arms of the elevated plus maze, although they were similar to wild-type animals in an open field test *(17)*. In these experiments, overexpression of the 5-HT_{1A} receptor was only observed during early development, providing further evidence that alterations in 5-HT_{1A} receptor expression during this critical period results in profound changes in anxiety behavior.

Taken together, these findings suggest that the predominant components of the anxiogenic phenotype observed in 5-$HT_{1A}^{-/-}$ mice are the result of unspecified long-term and permanent compensatory effects emerging from the absence of 5-HT_{1A} receptors during early development. The general conclusion that genetic determinates of 5-HT_{1A} receptor function are important in anxiety phenotypes is further validated by recent reports in humans in which polymorphisms in the 5-HT_{1A} receptor gene were associated with panic disorder *(18,19)*.

2.2. *5-HT_{1B} Receptors*

It has been suggested that 5-$HT_{1A}^{-/-}$ and 5-$HT_{1B}^{-/-}$ mice represent two opposing behavioral phenotypes (e.g., ref. *11*). Indeed, 5-$HT_{1A}^{-/-}$ mice demonstrate a clear anxiogenic phenotype across several laboratories and behavioral tests. Although 5-$HT_{1B}^{-/-}$ mice are reported to demonstrate an anxiolytic phenotype under some circumstances, overall findings regarding animals lacking 5-HT_{1B} receptors (5-$HT_{1B}^{-/-}$) are less clear. Careful inspection of the available published literature suggests that 5-$HT_{1B}^{-/-}$ mice express some behaviors that are indicative of decreased anxiety levels, but are generally similar to wild-type animals in the more common measures of anxiety. In particular, 5-$HT_{1B}^{-/-}$ mice were not different from wild-type mice in time spent in the center of an open

Table 1
Effects of 5-HT-Related Genes on Behavioral Tests of Anxiety

Genotype	Test	Outcome	Ref.
5-$HT_{1A}^{-/-}$	Open field	⇓ Center entries[a]	5
		⇓ Center time[a]	
		⇓ Center distance[a]	
		⇔ Activity	
	Open field	⇓ Center entries	6
		⇓ % Center entries[a]	
		⇓ Center time	
		⇔/⇑ Activity[b]	
	Open field	⇓ Center time[c]	7
		⇓ Center distance[c]	
		⇓ Activity	
	Open field	⇔ Center time	11
		⇓ Center/total distance	
		⇓ Rearings	
		⇓ Total distance	
	Open field	⇓ Center time response to diazepam	15
		⇓ Center distance response to diazepam	
		⇔ Activity response to diazepam	
	Open field	⇓ Center activity	10
		⇓ Activity	
	Open field	⇓ Center time[d]	16
		⇔ Activity[d]	
		⇓ Center time[e]	
		⇓ Activity[e]	
	Elevated zero maze	⇓ Open quadrant time[a]	5
		⇓ Open quadrant distance[a]	
		⇓ Head dips[a]	
		⇔ Activity	
	Elevated plus maze	⇓ Open arm time	7
		⇓ Open arm entries	
		⇓ Head dips	
		⇔ Activity	
	Elevated plus maze	⇓ Open arm time response to diazepam	15
		⇓ Open arm entries response to diazepam	
		⇔ Activity	
	Elevated plus maze	⇓ Open arm entries	10
	Elevated plus maze	⇓ Open arm time[d]	16
		⇓ Open arm entries[d]	
		⇓ Closed arm entries[d]	

Table 1 *(continued)*

Genotype	Test	Outcome	Ref.
		⇓ Open arm time response to diazepam[d]	
		⇓ Open arm entries response to diazepam[d]	
		⇓ Open arm time[e]	
		⇓ Open arm entries[e]	
		⇔ Closed arm entries[e]	
		⇔ Open arm time response to diazepam[e]	
		⇔ Open arm entries response to diazepam[e]	
	Novel object	⇑ Latency	*5*
		⇑ Time in nest area	
		⇓ Activity with object	
		⇔ Basal activity	
	Novelty-suppressed feeding	⇑ Latency to feed	*10*
	Novelty-suppressed feeding	⇑ Latency to feed	*12*
		⇓ Response to chronic fluoxetine	
		⇔ Response to chronic imipramine	
		⇔ Response to chronic desipramine	
		⇓ Response to 5-HT_{1A} agonist	
	Injection-stress	⇑ Body temperature response	*13*
		⇑ Heart rate response	
	Stress-induced hyperthermia	⇔ Body temperature response	*13*
		⇔ Heart rate response	
		⇓ Body temperature response to 5-HT_{1A} agonist	
		⇓ Heart rate response to 5-HT_{1A} agonist	
		⇓ Body temperature response to diazepam	
		⇓ Heart rate response to diazepam	
	Startle reactivity	⇔ Startle threshold	*65*
		⇔ Startle reactivity	
		⇔ Startle habituation	
		⇔ Activity	
	Foot-shock sensitization	⇔ Sensitization	*65*
5-$HT_{1A}^{+/+}$	Open field	⇔ All measures	*17*

(continued)

Table 1 *(continued)*

Genotype	Test	Outcome	Ref.
	Elevated plus maze	⇑ Open arm time ⇑ % Open arm entries ⇑ Head dips ⇑ Total entries	*17*
5-$HT_{1B}^{-/-}$	Open field	⇔ All measures ⇓ Response to 5-$HT_{1A/1B}$ agonist	*1*
	Open field	⇔ All measures	*20*
	Open field	⇔ Center time ⇑ Center/total distance ⇑ Rearings ⇑ Total distance	*11*
	Elevated plus maze	⇔ Open arm time ⇔ Open arm entries ⇑ Head dips ⇓ Defecation	*22*
	Elevated plus maze	⇔ All measures	*21*
	Elevated plus maze	⇑ Open arm time response to toluene ⇑ Open arm entry response to toluene	*23*
	Light/dark	⇔ All measures	*20*
	Probe burying	⇓ Basal burying ⇑ Time burying response to toluene ⇑ Latency to bury response to toluene	*23*
	Novel object	⇓ Thigmotaxis ⇑ Object exploration ⇔ Habituation ⇔ Activity	*21*
	Stress-induced hyperthermia	⇔ Body temperature response ⇔ Plasma corticosterone ⇔ Body temperature response to 5-HT_{1A} agonist	*66*
	Startle reactivity	⇓ Startle threshold ⇓ Startle reactivity ⇔ Startle habituation ⇑ Activity	*65*
	Foot-shock sensitization	⇓ Sensitization	*65*
5-$HTT^{-/-}$	Open field	⇓ % Center time ⇓ Activity	*14*
	Open field	⇔ All measure	*25*

Table 1 *(continued)*

Genotype	Test	Outcome	Ref.
	Elevated plus maze	⇔ Open arm entries[f] ⇔ % Open arm time[f] ⇔ Total arm entries[f] ⇓ Open arm entries[e] ⇓ % Open arm time[e] ⇓ Total arm entries[e]	24
	Elevated plus maze	⇔ % Open arm entries ⇔ Open arm time ⇓ Total arm entries ⇓ Head dips	25
	Elevated plus maze	⇓ % Open arm entries[e] ⇓ % Open arm time[e] ⇔ Closed arm entries[e] ⇔ Rears ⇑ % Open are time response to 5-HT_{1A} antagonist ⇑ % Open are entries response to 5-HT_{1A} antagonist	14
	Light/dark	⇓ Time in light[e] ⇓ Light/dark transitions[e] ⇔ Time in light[f] ⇔ Light/dark transitions[f]	24
	Light/dark	⇓ Time in dark[e,b] ⇓ Light/dark transitions[e]	14
	Emergence	⇓ Light/dark transitions[e] ⇓ Time out of shelter[e]	14
	Immobilization stress	⇑ Plasma epinepherine ⇔ Plasma norephinepherine ⇔ Plasma ACTH ⇔ Plasma corticosterone	67
	Novelty-suppressed feeding	⇑ Latency to feed	25
	Stress-induced analgesia	⇔ Pain sensitivity	25
	Shock avoidance	⇑ Escape latency ⇓ Escape ⇔ Activity	25
	Foot-shock sensitization	⇔Sensitization	25

(continued)

Table 1 *(continued)*

Genotype	Test	Outcome	Ref.
5-$HT_{2C}^{-/-}$	Emergence	⇓ Latency to enter light	30
5-$HT_{3A}^{-/-}$	Open field	⇔ All measures	34
	Open field	⇔ All measures	32
	Open field	⇔ Activity ⇔ Habituation	33
	Elevated plus maze	⇑ Open arm entries	33
	Elevated plus maze	⇑ Open arm time ⇑ % Open arm time ⇔ Open arm entries ⇔ % Open arm entries ⇓ Closed arm time ⇓ % Closed arm time ⇔ Total activity	32
	Light/dark	⇑ Time in the light ⇑ Latency to enter the dark	33
	Light/dark	⇔ All measures	32
	Novel object	⇑ Activity in object area ⇑ Activity with object	33
5-$HT_{4}^{-/-}$	Open field	⇓ Activity ⇓ Habituation	35
	Stress-suppressed feeding	⇔ Baseline food intake ⇓ Suppression of feeding	35
5-$HT_{7}^{-/-}$	Elevated plus maze	⇔ Open arm time ⇔ Open arm entries	44

Note: ⇔ equal to wild type. ⇑ increased compared to wild type. ⇓ decreased compared to wild type.

[a]More pronounced effect in males compared to females.

[b]Females only.

[c]Males only.

[d]Swiss–Webster background.

[e]C57BL/6J background.

[f]129S6 background.

field *(1,20)*, time spent in the open arms of an elevated plus maze *(21)*, or time in the light in the light/dark test *(20)*, although one report suggested an increase in the center-to-total distance ratio, rearings, and total distance traveled in the open field test *(11)*. Similarly, genotypic differences were not observed in the traditional measures of anxiety in the elevated plus maze (i.e., center time

and center entries) *(21,22)*. However, some suggestion of decreased anxiety was provided by increased head dips and decreased defecation *(22)*.

In contrast to the more commonly used behavioral tests of anxiety, other tests involving responses to environmental stimuli suggest that 5-$HT_{1B}^{-/-}$ mice might be hyperreactive to these stimuli and therefore demonstrate decreased anxiety. Specifically, 5-$HT_{1B}^{-/-}$ mice showed decreased thigmotaxis and greater exploration of an object in a novel object test, signs of reduced anxiety *(21)*. 5-$HT_{1B}^{-/-}$ mice also showed decreased burying of a shock probe in the defensive burying test *(23)*. These types of response further generalized to other behavioral tests, including impulsive aggression tests, where 5-$HT_{1B}^{-/-}$ mice attacked intruders more quickly and with greater intensity than wild-type mice in a resident intruder test *(1,20)*. In general, these findings are consistent with the suggestion that 5-$HT_{1A}^{-/-}$ and 5-$HT_{1B}^{-/-}$ mice display distinct but not necessarily opposing anxiety phenotypes.

2.3. 5-HT Transporters

Tests of the effects of 5-HTT gene deletion have yielded a somewhat mixed pattern of results. In general, mice lacking the 5-HTT gene (5-$HTT^{-/-}$) seem to express an anxiogenic phenotype that is only revealed under specific genetic backgrounds. For example, 5-$HTT^{-/-}$ mice on a 129 background appeared similar to wild-type animals in the elevated plus maze *(24,25)*, showing only hints of increased anxiety such as decreased head dips *(25)*, whereas 5-$HTT^{-/-}$ mice on a C57BL6 background were reported to show heightened anxiety in this test *(14,24)*. This finding is mirrored in another test of anxiety, the light/dark box. Specifically, 5-$HTT^{-/-}$ mice on a 129 background were similar to wild-type mice, whereas 5-$HTT^{-/-}$ mice on a C57BL6 background demonstrated elevated anxiety levels compared to their wild-type counterparts *(24)*. These investigators suggest that this pattern of findings is the result of a ceiling effect observed in the 129 strain. In ref. *24*, it seems likely that wild-type 129 mice were already expressing a high level of anxiety, preventing further elevation by the deletion of the 5-HTT gene.

More recent findings suggest that the role of 5-HT reuptake in anxiety might be particularly critical during early development *(26)*. The selective serotonin reuptake inhibitor (SSRI) fluoxetine given postnatally to wild-type mice for approximately 2 wk (between postnatal days 4 and 21) resulted in anxiety behavior when tested as adults, and this was similar to that of 5-$HTT^{-/-}$ mice. Perhaps because these experiments were conducted in 5-$HTT^{-/-}$ mice on a 129 background, neither 5-$HTT^{-/-}$ nor postnatally SSRI-treated mice exhibited anxiety in the most typical measures of anxiety (see supporting online material *[26]*). 5-$HTT^{-/-}$ and postnatal SSRI-treated mice did not demonstrate decreased center time in the open field or decreased open arm time or entries in the

elevated plus maze. However, 5-HTT$^{-/-}$ mice and those treated with an SSRI during early development displayed similar decreased exploratory behavior, as evidenced by lower activity levels compared to wild-type mice in these two tests. Further, early fluoxetine treatment increased the latency to feed in a novelty-suppressed feeding test to 5-HTT$^{-/-}$ levels in 5-HTT$^{+/-}$ mice *(26)*. These findings suggest that reuptake of 5-HT from the synapse during development might serve to decrease anxiety or, potentially, that the presence of 5-HT in the synapse during development is a signal for anxiety.

The precise developmental changes that are caused by decreased 5-HTT function that result in altered behaviors is relatively unknown. However, it has been reported that 5-HTT$^{-/-}$ mice exhibited decreased 5-HT$_{1A}$ receptor density in the dorsal raphe *(27)* and altered responding to the 5-HT$_{1A}$ receptor antagonist WAY 100635 *(14)*. It has been reported recently that human subjects expressing the short form of the 5-HTT gene-linked polymorphic region (5-HTTLPR) also demonstrate reduced 5-HT$_{1A}$ binding *(28)*. The suggestion that disruption of 5-HT uptake might result in an anxiogenic phenotype is in remarkable agreement with human studies demonstrating that expression of the short form of the 5-HTTLPR resulted in greater 5-HT transmission, via decreased reuptake, and is positively associated with anxiety-related personality traits *(29)*. In summary, decreases in the developmental function of the 5-HTT are correlated with increases in anxiety behavior in both humans and animals, and these behavioral alterations might result from changes to the 5-HT$_{1A}$ receptor system.

2.4. 5-HT$_{2C}$ Receptors

5-HT$_{2C}$$^{-/-}$ mice might demonstrate an anxiolytic phenotype. This conclusion is substantiated by a report that 5-HT$_{2C}$$^{-/-}$ mice exhibited decreased latency to enter the light in an emergence test *(30)*. Unfortunately, the goal of this series of experiments was to characterize the state of the dentate gyrus in 5-HT$_{2C}$$^{-/-}$ mice, so no further information was provided regarding other anxiety measures. However, 5-HT$_{2C}$$^{-/-}$ mice did demonstrate impaired dentate gyrus function as evidenced by deficits in hippocampal long-term potentiation and alterations in an array of behavioral tests associated with the dentate gyrus *(30)*. Because interest in the role of 5-HT$_{2C}$ receptors as a target for anxiolytic drugs has been generated from pharmacological studies with selective antagonists *(31)*, further studies to examine this phenotype more thoroughly are warranted.

2.5. 5-HT$_{3A}$ Receptors

Mice lacking 5-HT$_{3A}$ receptors (5-HT$_{3A}$$^{-/-}$) might demonstrate an anxiolytic phenotype. These mice spent more time in the open arms of the elevated plus maze *(32,33)*, were more active with a novel object *(33)* and, under certain conditions, spent more time in the illuminated area in the light/dark test *(33)*

than wild types, although no differences from wild types were reported for this strain when tested in the open field *(32–34)*. Bhatnagar et al. *(32)* suggested that the discordance in these findings might result from differential housing conditions between laboratories. Nonetheless, these authors did report decreased anxiety using the elevated plus maze and altered acute stress-induced hypothalamic–pituitary–adrenal activity in 5-$HT_{3A}^{-/-}$ mice *(32)*.

2.6. 5-HT_4 Receptors

5-HT_4 knockout mice (5-$HT_4^{-/-}$) were reported to demonstrate decreased activity in the open field, suggesting that the 5-HT_4 receptor might be important in the exploratory response to novelty *(35)*. These mice also showed decreased stress-induced suppression of feeding *(35)*, indicating that the 5-HT_4 receptor might also serve to initiate stress induced anorexia.

3. Tests Sensitive to the Effects of Antidepressant Drugs

The forced swimming and tail suspension tests are two behavioral tests that are commonly used to investigate the mechanisms underlying depression and its treatment. During both of these tests, animals are placed into inescapable situations during which the duration of passive behavior is measured. In general, animals initially attempt to escape and then adopt a passive immobile posture that is thought to reflect behavioral despair. Animals given antidepressants struggle more or persist in attempting to escape, resulting in decreased measures of immobility *(36,37)*. Some criticize these tests by suggesting that they possess only predictive validity for the efficacy of antidepressants and, therefore, provide little information about the mechanisms of depression. However, a growing body of literature demonstrates that these tests might be useful indicators of mechanisms underlying stress resilience or, in contrast, vulnerability that can lead to depression *(37)*. Results with genetically modified mice examined in these tests suggest physiological mechanisms underlying the expression of stress-induced behavioral depression. Furthermore, when used in combination with antidepressant treatment, these tests provide information on mechanisms underlying changes in stress-related behaviors that are relevant to clinical treatment. Findings regarding 5-HT related knockouts and tests of antidepressant efficacy are summarized in Table 2.

3.1. 5-HT_{1A} Receptors

5-$HT_{1A}^{-/-}$ mice express an antidepressant phenotype in tests of antidepressant efficacy. In particular, several laboratories have demonstrated that 5-$HT_{1A}^{-/-}$ mice demonstrated decreased immobility in the forced swimming *(6,7)* and tail suspension tests *(5,38)*. Because decreased immobility could not be reproduced in wild-type mice by the use of a 5-HT_{1A} receptor antagonist, the

Table 2
Effects of 5-HT-Related Genes on Behavioral Tests of Antidepressant Effects

Genotype	Test	Outcome	Ref.
5-HT$_{1A}^{-/-}$	Forced swimming	⇓ Immobility	6
	Forced swimming	⇓ Immobility[a]	7
	Tail suspension	⇓ Immobility[b]	5
	Tail suspension	⇓ Immobility	38
		⇓ Response to fluoxetine	
		⇓ Response to paroxetine	
		⇔ Immobility effect of desipramine	
		⇔ Immobility PCPA	
		⇑ Response to AMPT	
	Tail suspension	⇓ Immobility	41
		⇔ Response to PCPA	
5-HT$_{1B}^{-/-}$	Forced swimming	⇓ Immobility[c]	41
	Tail suspension	⇔ Immobility	38
		⇑ Response to fluoxetine	
		⇔ Response to desipramine	
	Tail suspension	⇓ Immobility[c]	41
		⇑ Immobility with PCPA[c]	
		⇔ Response to fluoxetine	
5-HTT$^{-/-}$	Forced swimming	⇑ Immobility[d]	42
		⇔ Immobility[e]	
	Forced swimming	⇑ Immobility	25
	Tail suspension	⇓ Immobility[d]	42
		⇔ Immobility[e]	
		⇓ Response to fluoxetine[e]	
		⇔ Response to desipramine[e]	
		⇔ Response to imipramine[e]	
	Tail suspension	⇓ Immobility	25
5-HT$_{2C}^{-/-}$	Tail suspension	⇔ Immobility	43
		⇑ Response to fluoxetine	
5-HT$_{3A}^{-/-}$	Forced swimming	⇑ Immobility[a,c]	34
		⇓ Swimming[a]	
		⇔ Climbing	
5-HT$_{7}^{-/-}$	Forced swimming	⇓ Immobility	44

Note: ⇔ equal to wild type. ⇑ increased compared to wild type. ⇓ decreased compared to wild type.

[a]Second test only.

[b]More pronounced effect in males compared to females.

[c]Females only.

[d]129S6 background.

[e]C57BL/6J background.

antidepressant phenotype at baseline was likely the result of developmental compensation *(38)*. Further, examination of 5-$HT_{1A}^{-/-}$ mice following 5-HT depletion, via *para*-chlorophenylalanine (PCPA), revealed that behavior did not return to wild-type levels, suggesting that increased 5-HT from the absence of 5-HT_{1A} receptors was not the cause of this phenotype. However, catecholamine depletion, via α-methyl-*para*-tyrosine (AMPT), was more effective at increasing immobility in 5-$HT_{1A}^{-/-}$ mice compared to wild-type mice, suggesting that reduced immobility in 5-$HT_{1A}^{-/-}$ mice might be mediated by altered norepinephrine or dopamine transmission *(38)*.

5-$HT_{1A}^{-/-}$ mice were also less sensitive to the SSRIs fluoxetine and paroxetine in the tail suspension test *(38)*. It is unlikely that this finding reflects a floor effect because the norepinpherine reuptake inhibitor (NRI) desipramine was able to decrease immobility. The pattern of these results suggests that 5-HT_{1A} receptors are important for expression of the behavioral effects of SSRIs and that separate mechanisms mediate the effects of different types of antidepressant drug, the SSRIs and NRIs. The pattern of acute antidepressant drug effects using the tail suspension test was similar to subsequent studies conducted with 5-$HT_{1A}^{-/-}$ mice that evaluated the response to chronic antidepressant drug treatments. In these studies, the effect of chronic administration of the SSRI fluoxetine to decrease novelty-induced suppression of feeding behavior and increase neurogenesis in the hippocampus were blocked in 5-$HT_{1A}^{-/-}$ mice *(12)*. In contrast, chronic treatment of 5-$HT_{1A}^{-/-}$ mice with the NRI desipramine was able to decreased novelty-induced suppression of feeding. These results further support the ideas that (1) distinct neural mechanisms mediate the effects of different types of antidepressant drug, the SSRIs and NRIs, following their chronic administration and that (2) 5-HT_{1A} receptors are important for the behavioral effects of SSRIs.

Although many studies support the hypothesis that the 5-HT_{1A} receptor is an important physiological substrate for the behavioral effects of SSRIs, many of these studies have been conducted in constitutive 5-$HT_{1A}^{-/-}$ mice. The antidepressantlike phenotype in constitutive 5-$HT_{1A}^{-/-}$ mice are likely reflective of predisposing behavioral disturbances from altered development, similar to those shown on anxiety tests *(10)*. The altered baseline, however, is problematic for unequivocally determining the effects of antidepressant drugs. The use of inducible deletions would help to distinguish the critical role of developmental dysfunction from the absence of 5-HT_{1A} receptors. Tissue-specific rescue strategies could help to identify the critical neural circuitry necessary for modifying the behavioral response to SSRIs. The important genetic role of the 5-HT_{1A} receptor in depression-related behaviors is further corroborated by recent findings in humans demonstrating that polymorphisms in the 5-$HT_{1A}^{-/-}$ receptor gene are associated with depression *(39)* and antidepressant efficacy *(40)*.

3.2. 5-HT$_{1B}$ Receptors

Initial findings regarding the deletion of the 5-HT$_{1B}$ receptor gene suggested a possible role for the receptor in antidepressant efficacy. This suggestion was derived from findings demonstrating 5-HT$_{1B}^{-/-}$ mice were more sensitive than wild types to the effects of fluoxetine in the tail suspension test, whereas differences in baseline immobility were not observed *(38)*. In contrast, the effects of desipramine in the tail suspension test were unaltered in 5-HT$_{1B}^{-/-}$ mice. The pattern of these effects again underscores how different mechanisms regulate the behavioral effects of different types of antidepressant drugs.

More recent findings demonstrate that the deletion of the 5-HT$_{1B}$ receptor has profound effects, particularly in female mice. Female, but not male, 5-HT$_{1B}^{-/-}$ mice demonstrated decreased immobility at baseline in both the forced swimming and tail suspension tests *(41)*. These effects seem to be mediated in part by higher 5-HT levels, as depletion of 5-HT with PCPA increased female 5-HT$_{1B}^{-/-}$ mouse immobility to wild-type levels *(41)*.

3.3. 5-HT Transporters

The impact of deletion of the 5-HT transporter gene has varying effects on depression-related behavior, which depend on the background strain. Mice lacking the 5-HT transporter on the 129 background showed increased immobility in the forced swimming test and decreased immobility in the tail suspension test *(25,42)*. It should be noted, however, that the increased immobility observed in the forced swimming test was likely the result of decreased muscular strength in these animals. On the other hand, the prominent phenotype in the tail suspension test was an antidepressant-like response. In contrast, 5-HTT$^{-/-}$ mice on the C57BL6 background were not different from wild-type animals in baseline performance on either of these two tests *(42)*. 5-HTT$^{-/-}$ mice on the C57BL6 background showed decreased sensitivity in the tail suspension test to the SSRI fluoxetine, whereas their responses to the NRI desipramine and the mixed 5-HT/norepinepherine reuptake inhibitor imipramine were unaffected *(42)*. These findings suggest that the deletion of the 5-HT transporter results in an antidepressant phenotype depending on testing conditions. Also, these animals are likely insensitive to the effects of SSRIs, although they respond to other antidepressents.

3.4. 5-HT$_{2C}$ Receptors

Under basal conditions, 5-HT$_{2C}^{-/-}$ mice demonstrated immobility levels in the tail suspension test that are comparable to wild-type mice. However, they exhibited enhanced responding to fluoxetine *(43)*. Additional experiments revealed that the SSRI citalopram and the 5-HT$_{2B/2C}$ receptor antagonist SB 206553 given in combination to wild-type mice decreased immobility in

the tail suspension test, whereas neither drug given alone altered their response *(40)*. These findings suggest that the blockade of the 5-HT_{2C} receptor might enhance antidepressant efficacy.

3.5. 5-HT_{3A} Receptors

Animals lacking the 5-HT_{3A} receptor were reported to display a depression-like phenotype and it is suggested that these effects might be specific to female 5-$HT_{3A}^{-/-}$ mice. In particular, female 5-$HT_{3A}^{-/-}$ mice were reported to demonstrate increased immobility compared to male 5-$HT_{3A}^{-/-}$ mice and wild-type mice of both sexes. Similarly, female 5-$HT_{3A}^{-/-}$ mice reportedly showed decreased swimming compared to male 5-$HT_{3A}^{-/-}$ and both sexes of wild-type mice *(34)*. These findings implicate blockade of the 5-HT_{3A} receptor as a potential target for antidepressant therapy, especially in females.

3.6. 5-HT_7 Receptors

5-$HT_7^{-/-}$ mice demonstrated an antidepressant phenotype, as evidenced by decreased immobility in the forced swimming test *(44)*. Wild-type mice tested in the forced swimming test during the light phase of their light cycle and treated with the 5-HT_7 receptor antagonist SB-258719 did not show the reduce immobility levels that were observed in 5-$HT_7^{-/-}$ mice. These findings might suggest that the antidepressant phenotype observed in 5-$HT_7^{-/-}$ mice results from developmental compensations in the absence of the 5-$HT_7^{-/-}$ receptor, rather than a consequence of the absence of functional receptors in the 5-$HT_7^{-/-}$ mice *(44)*. However, additional studies demonstrated that SB-258719 did have antidepressant effects in wild-type animals tested during the dark phase of their light cycle *(44)*. These findings are likely the result of the association between 5-HT_7 receptor and circadian rhythm regulation.

4. Neurotransmission as Measured by In Vivo Microdialysis

In vivo microdialysis is a technique that allows sampling of extracellular levels of neurotransmitters in discrete regions of the brain. The extracellular neurotransmitter levels provide an indication of the net activity of a particular set of neurons, including release, synthesis, and uptake, from conscious unanesthetized animals. For example, in vivo microdialysis studies have shown that extracellular 5-HT levels measured under appropriate conditions are dependent on the concentration of Ca^{2+} or K^+ in the perfusion fluid, is inhibited by tetrodotoxin, and is predominately neuronal in origin *(45)*. In addition, specific neural processes can be measured after the local application of agents through the microdialysis probe, such as release after application of hypertonic KCl, rate of synthesis after synthesis inhibitors, or the local effects of drugs *(46)*. This technique has made it possible to more accurately quantitate and characterize the

changes in extracellular neurotransmitter concentrations produced by psychoactive drugs. These measures are routinely collected in single awake and unrestrained animals and have recently been adapted to address specific questions concerning neurotransmission with genetically altered mice. Table 3 summarizes in vivo microdialysis findings obtained in 5-HT-related knockouts.

4.1. 5-HT$_{1A}$ Receptors

Because 5-HT$_{1A}$ autoreceptors are somatodendritic autoreceptors, their deletion might have been expected to increase basal levels of 5-HT. However, most in vivo microdialysis studies have not shown basal levels of 5-HT to differ between 5-HT$_{1A}^{-/-}$ and wild-type mice when measured in either the striatum, hippocampus, or raphe nucleus *(47–49)*. In contrast, one laboratory reported that basal 5-HT levels are elevated in both the ventral hippocampus and the frontal cortex of 5-HT$_{1A}^{-/-}$ mice *(50)*. It is difficult to resolve this discrepancy, although it is possible that the older age of the animals in this study might contribute to the difference. However, the findings reported earlier showing no change in baseline 5-HT levels in 5-HT$_{1A}^{-/-}$ mice are consistent with a larger body of literature suggesting that 5-HT$_{1A}$ receptors are not tonically active; that is, selective 5-HT$_{1A}$ receptor antagonists do not increase extracellular levels of 5-HT when given to animals with 5-HT$_{1A}$ receptors intact (for review, *see* ref. *51*). These data support the idea that somatodendritic 5-HT$_{1A}$ autoreceptors do not regulate basal levels of 5-HT, but might be more important in the regulation of 5-HT levels during periods of activation or challenge.

As expected for the somatodendritic autoreceptor, administration of 5-HT$_{1A}$ receptor agonists decrease extracellular 5-HT levels in terminal regions. However, 5-HT levels in the ventral hippocampus, striatum *(52)*, and dorsal raphe *(48)* remained unchanged following administration of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT to 5-HT$_{1A}^{-/-}$ mice. Thus, the deletion of 5-HT$_{1A}$ receptors prevents the regulation of extracellular 5-HT. However, it is unclear whether this regulation is exerted directly by somatodendritic 5-HT$_{1A}$ autoreceptors or involves reciprocal regulation of the dorsal raphe from postsynaptic 5-HT$_{1A}$ receptors in terminal regions (e.g., ref. *53*). Interestingly, 5-HT$_{1A}^{-/-}$ mice demonstrated enhanced responsiveness to the effects of 5-HT$_{1B}$ receptor agonists when measured in the striatum, but not when measured in the hippocampus *(49,52)*. These findings suggest the occurrence of developmentally induced compensatory changes in response to the deletion of the 5-HT$_{1A}$ receptor. Striatal terminal 5-HT$_{1B}$ autoreceptors might become sensitized after the deletion of 5-HT$_{1A}$ somatodendritic autoreceptors, perhaps to preserve regulation of 5-HT transmission in this region.

Deletion of the 5-HT$_{1A}$ receptor leads to enhanced extracellular 5-HT levels in response to systemic administration of selective serotonin reuptake inhibitors

(SSRIs). Although basal levels remained unchanged, fluoxetine induced greater extracellular 5-HT levels in the striatum *(47,49,53)*, frontal cortex *(50)*, and dorsal raphe *(48)* of 5-$HT_{1A}^{-/-}$ mice. Some findings suggest that the regulation of 5-HT neurotransmission by the 5-HT_{1A} receptor might be more pronounced in specific brain regions than in others. In particular, it has been reported that 5-$HT_{1A}^{-/-}$ mice show greater augmentation of serotonin in response to fluoxetine in the striatum *(49)* and frontal cortex *(50)* than in the hippocampus. In addition, increases in 5-HT levels produced by local infusion of citalopram into the dorsal raphe were not augmented in 5-$HT_{1A}^{-/-}$ mice, suggesting that the absence of 5-HT_{1A} receptors in terminal regions might be responsible for augmented fluoxetine responses observed in 5-$HT_{1A}^{-/-}$ mice. This suggestion would be further supported by future experiments examining the effects of fluoxetine on 5-HT levels in mice where forebrain 5-$HT_{1A}^{-/-}$ receptors have been rescued (i.e., ref. *10*).

4.2. 5-HT_{1B} Receptors

In vivo microdialysis findings have demonstrated that deletion of the 5-HT_{1B} receptor modified transmission of 5-HT in several brain areas. Male mice lacking the 5-HT_{1B} receptor have normal basal extracelluar 5-HT levels in the hippocampus, striatum, and cortex *(41,49,54–59)*. However, an exception was recently reported demonstrating that *female* 5-$HT_{1B}^{-/-}$ mice have greater basal extracellular 5-HT levels in the ventral hippocampus *(41)*. This suggests that the effects of genetic deletion of 5-HT terminal autoreceptors are gender-specific. It is not known, however, whether gender-related effects are the result of immutable developmental programming or a different hormonal environment.

As expected for the terminal 5-HT_{1B} autoreceptor, 5-HT_{1B} receptor agonists decreased extracellular 5-HT levels following either local or systemic administration. Unlike their wild-type counterparts, 5-$HT_{1B}^{-/-}$ mice did not demonstrate decreased 5-HT levels in any brain area following treatment with the 5-HT_{1B} receptor agonists CP-93,129 and CP-94,253 *(52,56–58)*. However, 5-$HT_{1B}^{-/-}$ mice were less responsive to the effects of the systemic 5-HT_{1A} receptor agonist 8-OH-DPAT when they were measured in the ventral hippocampus, but not the striatum *(52)*. This is in agreement with the hypothesis that terminal 5-HT_{1B} autoreceptors might be more important than somatodendritic 5-HT_{1A} autoreceptors for the regulation of 5-HT neurotransmission in the hippocampus.

The 5-$HT_{1B}^{-/-}$ mice demonstrated augmented increases of 5-HT levels in response to systemic administration of SSRIs, compared to wild-type mice when measured in the ventral hippocampus *(41,49,54–56,60)*. However, when the effects of SSRIs were measured in other regions of 5-$HT_{1B}^{-/-}$ mice, their effects were not different from wild-type mice. For example, the effects of systemic paroxetine in the prefrontal cortex *(54)*, or systemic fluoxetine in

Table 3
Effects of 5-HT-Related Genes on In Vitro Microdialysis

Genotype	Brain area	Transmitter	Outcome	Ref.
5-$HT_{1A}^{-/-}$	Ventral hippocampus	5-HT	⇓ Response to 5-HT_{1A} agonist ⇔ Response to 5-HT_{1B} agonist ⇔ Response to fluoxetine with 5-$HT_{1B/1D}$ antagonist	*52*
		5-HT	⇔ Basal ⇔ Response to fluoxetine ⇓ Response to fluoxetine with 5-HT_{1A} antagonist[b]	*49*
		5-HT	⇑ Basal ⇔ Response to fluoxetine ⇓ Response to fluoxetine with 5-HT_{1A} antagonist ⇔ No response to open field	*50*
	Striatum	5-HT	⇔ Basal ⇔ Response to K^+ ⇑ Response to fluoxetine	*47*
		5-HT	⇓ Response to 5-HT_{1A} agonist ⇑ Response to 5-HT_{1B} agonist ⇑ Response to fluoxetine with 5-$HT_{1B/1D}$ antagonist; no effect in wild type	*52*
		5-HT	⇔ Basal ⇑ Response to fluoxetine ⇓ Response to fluoxetine with 5-HT_{1A} antagonist	*49*
		Dopamine	⇔ Basal ⇔ Response to K^+ ⇔ No response to fluoxetine	*47*
	Frontal cortex	5-HT	⇑ Basal ⇑ Response to fluoxetine ⇓ Response to fluoxetine with 5-HT_{1A} antagonist ⇑ Response to open field; no effect in wild type	*50*
	Dorsal raphe	5-HT	⇔ Basal ⇔ Response to local citalopram ⇓ Response to 5-HT_{1A} agonist with citalopram	*48*

Table 3 *(continued)*

Genotype	Brain area	Transmitter	Outcome	Ref.
			⇔ Response to 5-HT_{1B} agonist with citalopram ⇑ Response to fluoxetine ⇑ Response to handling with citalopram; no effect in wild type	
5-$HT_{1B}^{-/-}$	Ventral hippocampus	5-HT	⇔ Basal ⇔ Response to K^+ ⇓ Response to K^+ with local 5-HT_{1B} agonist	*59*
		5-HT	⇓ Response to 5-HT_{1B} agonist ⇓ Response to 5-HT_{1A} agonist ⇓ Response to fluoxetine with 5-HT_{1A} antagonist	*52*
		5-HT	⇔ Basal ⇑ Response to fluoxetine ⇓ Response to fluoxetine with 5-$HT_{1B/1D}$ antagonist	*49*
		5-HT	⇔ Basal ⇑ Response to paroxetine ⇓ Response to paroxetine with 5-$HT_{1B/1D}$ antagonist	*55*
		5-HT	⇔ Basal ⇓ Response to local 5-HT_{1B} agonist ⇑ Response to paroxetine ⇑ Response to local fluvoxamine	*56*
		5-HT	⇔ Basal ⇑ Response to fluoxetine	*60*
		5-HT	⇔ Basal ⇑ Response to paroxetine ⇔ No response to chronic paroxetine	*54*
		5-HT	⇑ Basal[a] ⇑ Response to fluoxetine	*41*
	Striatum	5-HT	⇓ Response to 5-HT_{1B} agonist ⇔ Response to 5-HT_{1A} agonist ⇔ Response to fluoxetine with 5-HT_{1A} antagonist	*52*

(continued)

Table 3 *(continued)*

Genotype	Brain area	Transmitter	Outcome	Ref.
		5-HT	⇔ Basal ⇔ Response to fluoxetine ⇔ No response to fluoxetine with 5-$HT_{1B/1D}$ antagonist	*49*
		5-HT	⇔ Basal ⇓ Response to local 5-HT_{1B} agonist ⇔ Response to local fluvoxamine ⇔ Response to local fenfluramine	*58*
		Dopamine	⇔ Basal ⇔ Response to local 5-HT_{1B} agonist ⇔ Response to local fluvoxamine ⇔ Response to local fenfluramine ⇔ Response to local tetrodotoxin with 5-HT_{1B} agonist	*58*
	Frontal cortex	5-HT	⇔ Basal ⇔ Response to K^+ ⇓ Response to on K^+ with 5-HT_{1B} agonist ⇓ Response to K^+ with local 5-$HT_{1B/1D}$ agonist	*59*
		5-HT	⇔ Basal ⇔ Response to paroxetine ⇔ No response to paroxetine with 5-$HT_{1B/1D}$ antagonist	*55*
		5-HT	⇔ Basal ⇔ Response to fluoxetine	*60*
		5-HT	⇔ Basal ⇓ Response to local 5-HT_{1B} agonist ⇔ No response to 5-HT_{1B} antagonist ⇑ Response to local fluvoxamine	*57*

Table 3 *(continued)*

Genotype	Brain area	Transmitter	Outcome	Ref.
			⇓ Response to local fluvoamine with 5-HT_{1B} antagonist	
		5-HT	⇔ Basal ⇔ Response to paroxetine ⇔ No response to chronic paroxetine	*54*
5-$HTT^{-/-}$	Frontal cortex	5-HT	⇑ Basal	*62*
		Dopamine	⇔ Basal ⇔ Response to cocaine	*62*
	Caudate-putamen	5-HT	⇑ Basal ⇑ Response to DAT blocker; no response in wild type ⇔ Response to local cocaine	*62*
		Dopamine	⇔ Basal ⇔ Response to cocaine ⇔ No response to fluoxetine ⇔ Response to local cocaine	*62*
	Nucleus accumbens	5-HT	⇑ Basal	*62*
		Dopamine	⇔ Basal ⇔ No response to fluoxetine ⇔ Response to cocaine	*62*
5-$HT_{2C}^{-/-}$	Frontal cortex	5-HT	⇔ Basal ⇑ Response to fluoxetine	*43*

Note: ⇔ equal to wild type. ⇑ increased compared to wild type. ⇓ decreased compared to wild type.

[a] Only in females.

[b] Depending on dose.

the striatum *(49)*, were not augmented in 5-$HT_{1B}^{-/-}$ mice. Local administration of fluvoxamine into the frontal cortex produced an augmented increase of 5-HT in 5-$HT_{1B}^{-/-}$ mice *(54,57)*, but the effects of fenfluramine or fluvoxamine administered locally into the striatum did not differ from wild-type mice *(58)*.

These patterns in the regulation of extracellular 5-HT levels by SSRIs in 5-$HT_{1B}^{-/-}$ mice, in combination with those from 5-$HT_{1A}^{-/-}$ mice reviewed previously, suggest that there are important topographical differences in regulation of extracellular 5-HT levels by 5-HT_{1A} and 5-HT_{1B} autoreceptors. The existence of such differences is supported by corresponding studies using pharmacological antagonists of 5-HT_{1A} and 5-HT_{1B} receptors, respectively, conducted in wild-type mice (*see* ref. *52*). Regional differences might be related to the origin of the

5-HT afferents projecting to individual terminal areas. Thus, the effects of SSRIs in areas with predominate innervation from the dorsal raphe are augmented by 5-HT_{1A} receptor antagonists or in 5-$HT_{1A}^{-/-}$ mice, such as the frontal cortex and striatum, but are less effective (or not at all) in the median raphe-innervated dorsal hippocampus.

Different regional patterns of 5-HT efflux associated with 5-HT receptor mutants could contribute to varying expression of behavioral phenotypes *(11)* or, in the case of humans with relevant polymorphisms, to distinct behavioral traits, vulnerabilities, or drug interactions *(3)*. For example, enhanced sensitivity of 5-HT_{1B} receptor mutants to the antidepressant-like response of fluoxetine in the tail suspension test could be mediated by increased release of 5-HT in the hippocampus *(38)*. 5-HT_{1A} receptor knockout mice demonstrated increased release of 5-HT in the frontal cortex but not the hippocampus after exposure to an open field *(50)*, and this topographical pattern could be related to their anxiogenic phenotype. Finally, because the 5-HT_{1A} and 5-HT_{1B} receptor antagonist pindolol is used to augment the clinical effects of fluoxetine, regional variations in the regulation of extracellular 5-HT might be pertinent to understanding its mechanism of action *(61)*.

4.3. 5-HT Transporters

Mice lacking the 5-HT transporter demonstrated higher extracellular levels of 5-HT. In particular, 5-$HTT^{-/-}$ mice showed higher basal levels of 5-HT in the caudate-putamen, nucleus accumbens, and prefrontal cortex, whereas dopamine levels in these areas were similar to that of wild-type mice *(62)*. Further, the blockade of dopamine transporters in the caudate-putamen with GBR12909 led to higher extracellular levels of 5-HT in the absence of the 5-HTT *(62)*, but not in wild-type mice. These findings suggest, then, that elimination of the 5-HT transporter might lead to reuptake of 5-HT by other monoamine transporters and potentially facilitate its release as a "false transmitter" by other monoamine neurons *(62)*.

4.4. 5-$HT_{2C}^{-/-}$ Receptors

Recent findings regarding the effects of 5-HT_{2C} receptor deletion in combination with pharmacological experiments suggest that this receptor system might be an important target for antidepressant efficacy *(43)*. Cremers et al. *(43)* reported that the increase of extracellular levels of 5-HT in the frontal cortex produced by systemic fluoxetine was enhanced in 5-$HT_{2C}^{-/-}$ mice. This effect was reflected in experiments with rats where ketanserin augmented the response of SSRIs on extracellular 5-HT levels when given in combination with citalopram in the frontal cortex, and with citalopram, fluoxetine, or sertraline in the hippocampus, *(43)*. These authors reported that basal frontal cortex levels of 5-HT were not

altered in 5-$HT_{2C}^{-/-}$ mice. Concordant results were obtained in rats with the nonselective 5-$HT_{2A/2C}$ receptor antagonist ketanserin, which did not alter extracellular 5-HT levels in either the frontal cortex or hippocampus *(43)*. Taken together, these findings suggest that the augmented response to SSRIs in 5-$HT_{2C}^{-/-}$ mice is not the result of developmental compensation.

The significance of the interaction between SSRIs and 5-$HT_{2C}^{-/-}$ mice might be important for understanding the enhanced clinical antidepressant effects of SSRIs reported when combined with many atypical antipsychotic drugs *(63)*. Because many atypical antipsychotic drugs have high affinity for 5-HT_{2C} receptors *(64)*, the utility of the atypical antipsychotic drugs to augment the effects of SSRIs in treatment-resistant depressions might involve enhanced effects on monoamine levels in different brain regions when these drugs are given in combination *(65)* as a result, in part, of the blockade of 5-HT_{2C} receptors.

5. Concluding Remarks

Since the development of the first 5-HT receptor genetic mutant by Saudou et al. *(1)*, additional 5-HT receptor-related mutants have been generated. Although initially conceived as sophisticated versions of pharmacological antagonists, further investigation of new 5-HT-related mutant models have revealed developmental components contributing to many of the observed phenotypes. In some cases, the absence of the receptor during a critical developmental period appears to contribute more to the phenotype than the absence of the gene target itself at the time of testing. This concern has often made it difficult to distinguish phenotypes resulting from the actual deletion of a gene target from those resulting from developmental compensation.

It has been subsequently suggested that 5-HT receptor genetic mutant mice are better suited as models of the effects of long-term gene dysfunction in behavioral tests of mood disorders. In some instances, such as the 5-$HTT^{-/-}$ and 5-$HT_{1A}^{-/-}$ mice, 5-HT receptor genetic deletion models yield phenotypic effects that are similar to that of known human polymorphisms. The main problem in comparing genetic deletion models to human polymorphisms is that polymorphisms typically result in a partial loss of function, rather than complete loss of function. Therefore, the use of heterozygous animals, where one allele is spared, might more closely model the effects of human polymorphisms if they retain some functional activity.

Future experiments should take advantage of new technologies that are increasingly becoming available to enhance our understanding of the role of 5-HT-related genes in mood disorders. In particular, the use of inducible gene deletion helps to distinguish between the effects of receptor deletion and the developmental compensation, resulting from receptor deletion. Confining

genetic deletions to particular brain regions might be invaluable for determining the role of specific synaptic targets, such as the role of presynaptic vs postsynaptic 5-HT_{1A} and 5-HT_{1B} receptors. Similarly, the rescue of 5-HT receptor genetic deletions on particular neuronal cell types is now possible and can provide more precise information on the physiological role of 5-HT receptors in neural assemblies. Finally, because the pharmacological tools to investigate the effects of some 5-HT targets are still lacking, the contribution of some 5-HT receptors to mood disorders has yet to be evaluated.

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