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Cloning and Functional Expression of a Human 5-Hydroxytryptamine Type $3A_S$ Receptor Subunit

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SUMMARY

A human 5-hydroxytryptamine receptor type $3A_S$ (5-HT_3R-A_S) subunit has been cloned from an amygdala cDNA library. We report the nucleotide and predicted amino acid sequence of the human subunit, which possesses 85% and 84% amino acid sequence identity with mouse and rat 5-HT_3R-A_S subunits, respectively. Acting on *Xenopus laevis* oocytes injected with RNA transcripts of the clone, 5-HT and selective 5-HT_3 receptor agonists elicited inwardly directed current responses that displayed desensitization. Such currents were blocked in a concentration-dependent manner by selective and nonselective 5-HT_3 receptor antagonists but were unaffected by compounds acting at G protein-linked 5-HT receptors. A quantitative comparison of the pharmacological profiles of human and mouse recombinant 5-HT_3R-A_S receptor complexes revealed differences in the potencies of some antagonist or agonist compounds tested, the most dramatic example being (+)-tubocurarine, which demonstrated an ~1800-fold discrepancy in antagonist potency. In view of the small number of sequence substitutions that occur between the human and mouse homologues of the 5-HT_3R-A_S in the extracellularly located amino-terminal domain, compounds such as (+)-tubocurarine, in conjunction with site-directed mutagenesis, may prove to be valuable in locating amino acid residues that contribute to the ligand binding site(s) of the 5-HT_3 receptor. Also, when methodological differences are taken into account, the present study suggests that a homo-oligomeric assembly of human 5-HT_3R-A_S subunits can account for the distinctive ligand binding properties of human 5-HT_3 receptors established in postmortem brain tissue.

5-HT_3 receptors are ligand-gated, cation-selective ion channels that mediate neuronal excitation and the exocytotic release of neurotransmitters within the peripheral and central nervous systems (1, 2; reviewed in Ref. 3). The receptor is also expressed at high density in several neuronal clonal cell lines that have proved valuable in assessing the structural, biochemical, electrophysiological, and pharmacological characteristics of the 5-HT_3 receptor (4–6). Operationally, 5-HT_3 receptors are distinguished from multiple subclasses of G protein-linked 5-HT receptor by their selective activation by 2-Me-5-HT and biguanide derivatives and by their highly selective blockade by compounds that include bemesetron, tropisetron, ondansetron, and granisetron (7, 8). Functional studies conducted on isolated tissues (9, 10) or single cells (11), using these and other compounds, have revealed substantial differences in agonist potencies and efficacies and antagonist affinities across 5-HT_3 receptor-containing preparations. Such variation has also been detected by the direct labeling of 5-HT_3 receptor recognition sites present within membrane homogenates derived from central and peripheral nervous tissues and neuronal cell lines (12–14). Interspecies variation in 5-HT_3 receptor pharmacology is responsible for much of this heterogeneity (9–13), with intraspecies variability perhaps being a contributory factor (14). A striking example is presented by 5-HT_3 receptors expressed by guinea pig tissues, in which antagonists display a different rank order of effectiveness and relatively low affinity in comparison to their actions on receptors in rat and rabbit preparations (9–11, 13). Also, although PBG acts as an agonist at 5-HT_3 receptors expressed by peripheral neurons of the rat (10), rabbit (15), and mouse (10), it is reported to be devoid of efficacy in 5-HT_3 receptor-containing preparations derived from the guinea pig (9, 10). Together with the non-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-HT_3R-A_S, 5-hydroxytryptamine receptor type $3A_S$; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; PBG, 1-phenylbiguanide; HEK, human embryonic kidney; RIN, rat insulin secreting; SSPE, sodium chloride/sodium phosphate/EDTA buffer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *m*-CPBG, *meta*-chlorophenylbiguanide; BRL 46470, endo-*N*-(8 methyl-8-azabicyclo-[3,2,1]oct-3-yl)-2,3-dihydro-3,3-dimethyl-indol-1-carboxamide.

selective antagonists (+)-tubocurarine and cocaine (10, 11, 15–17), the biguanides also discriminate, albeit less dramatically, among 5-HT$_3$ receptors present in mouse, rat, and rabbit (12, 13).

Heterogeneity in the properties of 5-HT$_3$ receptors extends to their biophysical characteristics. In the murine neuronal cell lines N18 and N1E-115, the conductance of the channel integral to the receptor complex, inferred by fluctuation analysis of whole-cell currents recorded under voltage-clamp, is <1 pS (2, 17, 18). In contrast, 5-HT$_3$ receptor-mediated single-channel currents yielding conductances within the range of 9–19 pS have been directly resolved in membrane patches of neurons of the guinea pig submucous plexus (1), rat superior cervical ganglion (19), and rabbit nodose ganglion (15). Interestingly, such differences are apparent within a species; in contrast to the aforementioned mouse cell lines, superior cervical ganglion neurons of the mouse possess a 5-HT$_3$ receptor channel with a substantial (~10 pS) conductance (18). Also, for both rat and mouse superior cervical ganglion neurons (18, 19), marked discrepancies between direct determinations of channel conductance and values inferred by fluctuation analysis have led to the suggestion that 5-HT$_3$ receptors with differing subunit composition may exist within one cell (18).

The structural basis of 5-HT$_3$ receptor heterogeneity is unknown. For other ligand-gated ion channels, subunit composition, the expression of alternatively spliced subunits, and RNA editing have been shown to be important determinants of receptor function. Only one 5-HT$_3$ receptor subunit, called 5-HT$_3$R-A, which was originally cloned from an NCB-20 hybridoma cell expression library, has been identified (4). Diversity within this receptor family is limited to mouse and rat homologues of the 5-HT$_3$R-A gene (20, 21) and the existence of mRNA splice variants (22, 23) encoding subunits that vary by their possession (5-HT$_3$R-A) or lack (5-HT$_3$R-A$_S$) of six consecutive amino acids in the putative second intracellular loop. Both splice variants of the mouse 5-HT$_3$R-A express efficiently as homo-oligomeric complexes in *Xenopus laevis* oocytes (4, 22, 24, 25), HEK 293 (26), or RIN cells (18, 23) and retain properties characteristic of 5-HT$_3$ receptors endogenous to murine cell lines, i.e., an exceptionally low single-channel conductance (~400 fS [18, 26]) and high affinity toward (+)-tubocurarine (IC_{50} ~1–2 nM [4, 18, 22, 25, 26]). Functional characterization of the rat homologue of the 5-HT$_3$R-A$_S$, which possesses 95% sequence identity to the mouse, has not been reported in depth. However, one intriguing observation is the relatively low potency of (+)-tubocurarine at homo-oligomeric receptors formed from that subunit (20), a feature that is shared with 5-HT$_3$ receptors endogenous to neurons of the rat (10, 19).

Due to the lack of a suitable experimental preparation, human 5-HT$_3$ receptors remain to be characterized functionally *in vitro*. However, it is evident from the results of radioligand binding studies using membrane homogenates prepared from postmortem human brain that the pharmacological profile of the human receptor is somewhat different to that encountered in other species (27, 28), highlighting the need for a robust functional assay. The human amygdala, in comparison with other limbic structures, expresses a relatively high density of 5-HT$_3$ binding sites (27), and in at least the rat, such receptors mediate fast excitatory postsynaptic potentials in response to neuronally released 5-HT (29), establishing a fast neurotransmitter role for this indoleamine in the mammalian central nervous system. In the present study, we used homology screening to isolate, from a human amygdala cDNA library, a clone encoding the human homologue of the 5-HT$_3$R-A$_S$. We report the nucleotide and deduced primary amino acid sequences of the human 5-HT$_3$R-A$_S$ subunit and demonstrate that cRNA transcripts derived from this clone direct the formation of homo-oligomeric 5-HT$_3$ receptor complexes in *X. laevis* oocytes. In electrophysiological studies, the pharmacological profile of the human recombinant receptor was found to differ from that established for the mouse homologue of the 5-HT$_3$R-A$_S$ or 5-HT$_3$ receptors native to rat, rabbit, or guinea pig neurons.

Materials and Methods

Isolation and sequencing of the human 5-HT$_3$R-A$_S$ cDNA. A commercially available λgt10 cDNA library constructed from human amygdala RNA (Clontech) was screened under conditions of reduced stringency using the coding region of the published mouse 5-HT$_3$R-A (4). The library was plated, and plaque lifts were performed as described by Sambrook *et al.* (30). Filters were hydridized in 50% formamide, 5× SSPE, 1× Denhardt's, 250 μg/ml salmon sperm DNA, and 0.2% sodium dodecyl sulfate at 42° for 16 hr and subsequently washed in 2× SSPE, 0.2% sodium dodecyl sulfate at 42° for 30 min. After autoradiography (−80° for 24 hr), positively hybridizing plaques were picked and subjected to several rounds of screening until plaque pure. Recombinant phage clones were analyzed by restriction analysis and partial sequence analysis. A 1.9-kb *Eco*R1 fragment corresponding to the longest clone was subcloned to pUC18 and subjected to complete sequence analysis using an automated sequencer (Applied Biosystem Model 373A). For expression studies, the human putative 5-HT$_3$ receptor cDNA was subcloned into the unique *Eco*R1 site in pBluescript SK.

Preparation of *in vitro* transcripts. The human 5-HT$_3$R-A$_S$ cDNA was linearized using the restriction enzyme *Hin*dIII. A Riboprobe System II transcription kit (Promega Limited) was used to synthesize capped *in vitro* transcripts from template cDNA using T_3 RNA polymerase. The integrity of the RNA transcripts was determined by electrophoresis through a denaturing 1% agarose formaldehyde gel (30) with standard RNA size markers (GIBCO-BRL).

Expression and electrical recordings. *X. laevis* oocytes were isolated as previously described (22, 25) and incubated in Barth's saline composed of (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgSO_4$ 1, $CaCl_2$ 0.5, $Ca(NO_3)_2$ 0.5, and HEPES 15, pH 7.5. Stage VI oocytes were identified and injected with 30–50 nl of nuclease free water containing 20–50 ng of cRNA (Drummond Digital Microdispenser 510, Drummond Scientific Co.). Injected oocytes were individually stored in wells of 96-well microtiter plates containing 200 μl of Barth's saline supplemented with gentamycin (100 μg/ml). Oocytes were maintained at 19–20° for 2–10 days before use.

Electrical recordings were routinely made in modified Barth's solution containing (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgCl_2$ 1, $CaCl_2$ 1, and HEPES 15, pH 7.5 (adjusted with NaOH). A small number of experiments used a solution in which the sodium salt of the poorly permeant anion glucuronate partially replaced NaCl to yield an extracellular chloride ion concentration of 10 mM (i.e., an approximate decade change in extracellular chloride ion concentration). In such experiments, the standard Ag/AgCl reference electrode was replaced with a low-resistance electrode filled with 3 M KCl, and the membrane potential was recorded differentially. Unless stated otherwise, agonist-induced currents were recorded at a holding potential of −60 mV with an Axoclamp 2A (Axon Instruments) voltage-clamp amplifier in the twin-electrode voltage-clamp mode. The voltage-sensing and current-passing microelectrodes were filled with 3 M

KCl and 3 M CsCl, respectively, and had resistances of 0.6–1.5 MΩ when determined in the standard extracellular saline. Oocytes were continuously superfused at a rate of 7–10 ml/min. Agonist and antagonist compounds were applied via the superfusion system. Antagonist compounds were preapplied for 1 min before their simultaneous application with the appropriate agonist for an additional 20–60 sec. Membrane currents were low-pass filtered at 250 Hz (two-pole Bessel filter), recorded onto magnetic tape via a Racal Store 4DS F/M tape recorder, and simultaneously displayed on a chart recorder (Multitrace 2, Lectromed). All recordings were performed at ambient temperature (18–23°).

Data analysis. The peak amplitudes of agonist-evoked currents were measured manually from chart recorder traces. EC_{50} values of agonists, together with the Hill coefficient or slope factor, were derived from full log concentration-effect relationships. Concentration-response data were fitted, by use of Fig P version 6c (Biosoft), with the sigmoidal function:

$$\frac{I}{I_{max}} = \frac{[A]^{n_H}}{[A]^{n_H} + [EC_{50}]^{n_H}}$$

where A is agonist concentration, I_{max} is the maximum inward current evoked by a saturating concentration of agonist, I is inward current, EC_{50} is the concentration of agonist evoking a half-maximal response, and n_H is the Hill coefficient. A similar equation, with antagonist concentration replacing A, inward current amplitude in the absence of antagonist replacing I_{max}, and the concentration of antagonist (IC_{50}) producing a 50% block of the response replacing EC_{50}, was used to analyze concentration-inhibition data obtained with antagonist compounds.

EC_{50} and IC_{50} values are reported as the geometric mean ± standard error. All other data are presented as the arithmetic mean ± standard error.

Drugs. Reagents were obtained from the following sources: 5-HT creatinine sulfate, 5-methoxytryptamine, metoclopramide hydrochloride, cocaine hydrochloride, and (+)-tubocurarine chloride (Sigma Chemical Co.), 2-Me-5-HT maleate (Research Biochemicals), PBG (Aldrich Chemical Co.), *m*-CPBG dihydrochloride and ondansetron hydrochloride (Glaxo), granisetron hydrochloride and BRL 46470 hydrochloride (SmithKline Beecham), methysergide hydrogen maleate (Sandoz), and ketanserin tartrate (Janssen). All drugs were freshly prepared and dissolved as concentrated stock solutions in either twice-distilled water or *Xenopus* Ringers' solution.

Results

Nucleotide and Predicted Primary Amino Acid Sequence of the Human 5-HT_3R-A_S

The human 5-HT_3R-A_S cDNA contains an open reading frame of 1434 basepairs preceded by 42 basepairs of 5′ noncoding sequence. The nucleotide sequence presented in Fig. 1 predicts a putative 23 residue signal peptide and a mature receptor subunit of 455 amino acids with a calculated molecular mass of 52,786 Da. The human subunit exhibits 85% and 84% amino acid sequence identity to the mouse and rat 5-HT_3R-A_S subunits, respectively (Fig. 2). Features common to human, mouse, and rat homologues include the presence of putative *N*-glycosylation sites (i.e.,. N-x-S/T) at N81, N147,

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AATTCCGGGTCACTCCCCCTCTCTGAGCTTGGAAAGCTCGCTATGCTGCTGTGGGTCCAGCAGGCGCTGCTCGCCTTGCTCCTCCCCACACTCCTGGCACAGGGAGAAGCCAGGAGG      6
                                          M  L  L  W  V  Q  Q  A  L  L  A  L  L  L  P  T  L  L  A  Q  G  E  A  R  R      2
                                                                Signal Sequence
AGCCGAAACACCACCAGGCCCGCTCTGCTGAGGCTGTCGGATTACCTTTTGACCAACTACACGAAGGGTGTGCGCCCCGTGAGGGACTGGAGGAAGCCAACCACCGTATCCATTGAC     123
 S  R  N  T  T  R  P  A  L  L  R  L  S  D  Y  L  L  T  N  Y  T  K  G  V  R  P  V  R  D  W  R  K  P  T  T  V  S  I  D      41
    ◆                                                   ◆
GTCATTGTCTATGCCATCCTCAACGTGGATGAGAAGAATCAGGTGCTGACCACCTACATCTGGTACCGGCAGTACTGGACTGATGAGTTTCTCCAGTGGAACCCTGAGGACTTTGAC     240
 V  I  V  Y  A  I  L  N  V  D  E  K  N  Q  V  L  T  T  Y  I  W  Y  R  Q  Y  W  T  D  E  F  L  Q  W  N  P  E  D  F  D      80

AACATCACCAAGTTGTCCATCCCCACGGACAGCATCTGGGTCCCGGACATTCTCATCAATGAGTTGGTGGATGTGGGGAAGTCTCCAAATATCCCGTACGTGTATATTCGGCATCAA     357
 N  I  T  K  L  S  I  P  T  D  S  I  W  V  P  D  I  L  I  N  E  F  V  D  V  G  K  S  P  N  I  P  Y  V  Y  I  R  H  Q     119
 ◆
GGCGAAGTTCAGAACTACAAGCCCCTTCAGGTGGTGACTGCCTGTAGCCTCGACATCTACAACTTCCCCTTCGATGTCCAGAACTGCTCGCTGACCTTCACCAGTTGGCTGCACACC     474
 G  E  V  Q  N  Y  K  P  L  Q  V  V  T  A  C  S  L  D  I  Y  N  F  P  F  D  V  Q  N  C  S  L  T  F  T  S  W  L  H  T     158
                                                                                    ◆
ATCCAGGACATCAACATCTCTTTGTGGCGCTTGCCAGAAAAGGTGAAATCCGACAGGAGTGTCTTCATGAACCAGGGAGAGTGGGAGTTGCTGGGGGTGCTGCCCTACTTTCGGGAG     591
 I  Q  D  I  N  I  S  L  W  R  L  P  E  K  V  K  S  D  R  S  V  F  M  N  Q  G  E  W  E  L  L  G  V  L  P  Y  F  R  E     197
    ◆
TTCAGCATGGAAAGCAGTAACTACTATGCAGAAATGAAGTTCTATGTGGTCATCCGCCGGCGGCCCCCTCTTCTATGTGGTCAGCCTGCTACTGCCCAGCATCTTCCTCATGGTCATG     708
 F  S  M  E  S  S  N  Y  Y  A  E  M  K  F  Y  V  V  I  R  R  R  P  L  F  Y  V  V  S  L  L  L  P  S  I  F  L  M  V  M     236
                                                                                    M1
GACATCGTGGGCTTCTACCTGCCCCCCAACAGTGGCGAGAGGGTCTCTTTCAAGATTACACTCCTCCTGGGCTACTCGGTCTTCCTGATCATCGTTTCTGACACGCTGCCGGCCACT     825
 D  I  V  G  F  Y  L  P  P  N  S  G  E  R  V  S  F  K  I  T  L  L  L  G  Y  S  V  F  L  I  I  V  S  D  T  L  P  A  T     275
                                  ■                                 M2
GCCATCGGCACTCCTCTCATTGGTGTCTACTTTGTGGTGTGCATGGCTCTGCTGGTGATAAGTTTGACCGAGACCATCTTCATTGTGCGGCTGGTGCACAAGCAAGACCTGCAGCAG     942
 A  I  G  T  P  L  I  G  V  Y  F  V  V  C  M  A  L  L  V  I  S  L  T  E  T  I  F  I  V  R  L  V  H  K  Q  D  L  Q  Q     314
                         M3
CCCGTGCCTGCTTGGCTGCGTCACCTGGTTCTGGAGAGAATCGCCTGGCTACTTTGCCTGAGGGAGCAGTCAACTTCCCAGAGGCCCCCAGCCACCTCCCAAGCCACCAAGACTGAT     1059
 P  V  P  A  W  L  R  H  L  V  L  E  R  I  A  W  L  L  C  L  R  E  Q  S  T  S  Q  R  P  P  A  T  S  Q  A  T  K  T  D     353
                                                                                                 ●
GACTGCTCAGCCATGGGAAACCACTGCACCCACATGGGAGGACCCCAGGACTTCGAGAAGAGCCCGAGGGACAGATGTAGCCCTCCCCCACCACCTCGGGAGGCCTCGCTGGCGGTG     1176
 D  C  S  A  M  G  N  H  C  T  H  M  G  G  P  Q  D  F  E  K  S  P  R  D  R  C  S  P  P  P  P  P  R  E  A  S  L  A  V     392

TGTGGGCTGCTGCAGGAGCTGTCCTCCATCCGGCAATTCCTGGAAAAGCGGGATGAGATCCGAGAGGTGGCCCGAGACTGGCTGCGCGTGGGCTCCGTGCTGGACAAGCTGCTATTC     1293
 C  G  L  L  Q  E  L  S  S  I  R  Q  F  L  E  K  R  D  E  I  R  E  V  A  R  D  W  L  R  V  G  S  V  L  D  K  L  L  F     431

CACATTTACCTGCTAGCGGTGCTGGCCTACAGCATCACCCTGGTTATGCTCTGGTCCATCTGGCAGTACGCTTGAGTGGGTACAGCCCAGTGGAGGAGGGGGTACAGTCCTGGTTAG     1410
 H  I  Y  L  L  A  V  L  A  Y  S  I  T  L  V  M  L  W  S  I  W  Q  Y  A                                                 455
                   M4
GTGGGGACAGAGGATTTCTGCTTAGGCCCCTCAGGACCCAGGGAATGCCAGGGACATTTTCAAGACACAGACAAAGTCCCGTGCCCTGTTTCCAATGCCAATTCATCTCAGCAATCA     1527

CAAGCCAAGGTCTGAACCCTTCCACCAAAAACTGGGTGTTCAAGGCCCTTACACCCTTGTCCCACCCCCAGCAGCTCACCATGGCTTTAAAACATGCTCTCTTAGATCAGGAGAAAC     1644

TCGGGCACTCCCTAAGTCCACTCTAGTTGTGGACTTTTCCCCATTGACCCTCACCTGAATAAGGGACTTGGAATTTC                                              1721
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Fig. 1. The nucleotide and deduced amino acid sequence of the human 5-HT_3R-A_S subunit. Right, numbering of the nucleotides. Putative sites of *N*-linked glycosylation (◆) and phosphorylation by mammary gland casein kinase (■) and casein kinase II (●) are indicated. *Underline*, the four predicted transmembrane sequences (M1–M4).

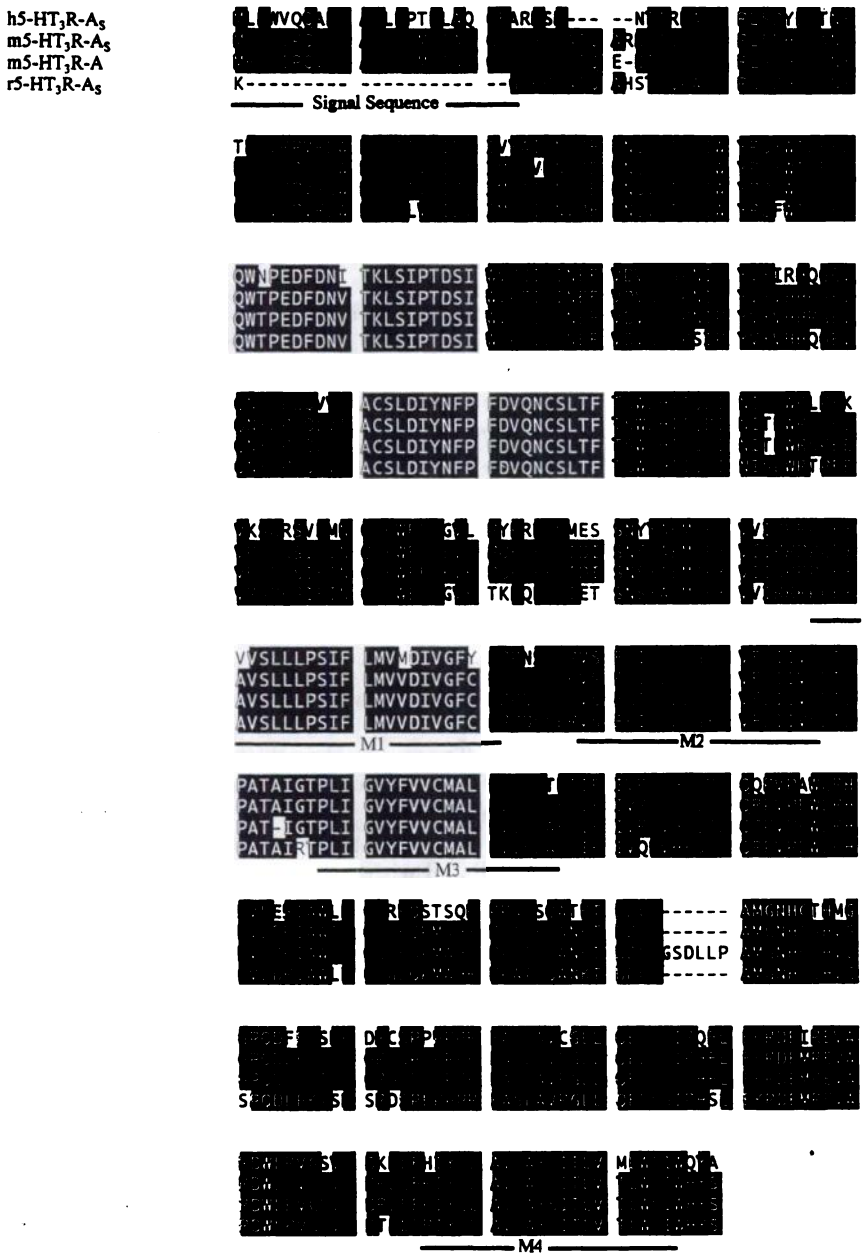

Fig. 2. A comparison of the predicted amino acid sequence of the mouse, rat, and human 5-HT$_3$R-A$_S$ subunit homologues and the mouse 5-HT$_3$R-A subunit. The alignment was performed with the DNAsis program. *Shading*, amino acid identities.

and N163 within the presumably extracellularly located amino-terminal domain and a consensus sequence for phosphorylation by mammary gland casein kinase (i.e.,. S-x-E) at S247 located between the presumptive transmembrane 1 and transmembrane 2 domains. In the human subunit, additional putative sites of *N*-glycosylation occur at residues N5 and N21, and an additional consensus sequence for phosphorylation by casein kinase II (i.e., S/T-x-x-D/E) is present at T350. A consensus tyrosine kinase site identified in both mouse (4) and rat homologues (21) is lost from the human subunit due to the replacement of tyrosine by S424.

Functional Expression in *X. laevis* Oocytes

Agonist pharmacology. Injection of the cRNA encoding the human 5-HT$_3$R-A$_S$ subunit conferred sensitivity to 5-HT on all *X. laevis* oocytes studied. Bath-applied 5-HT (100 μM) acting on such cells elicited a transient inward current response with an average peak amplitude of 204 ± 29 nA (44 experiments) when studied over 2–10 days after injection. The peak current response to 5-HT was concentration dependent, with threshold and maximal effects of the agonist occurring to 0.6 μM and ≥100 μM 5-HT, respectively (Fig. 3). Analysis of the 5-HT concentration-response relationship yielded an EC_{50} value of 3.1 ± 0.1 μM (four experiments) and a Hill coefficient of 1.94 ± 0.14 (four experiments), suggesting that a minimum of two molecules of 5-HT are required for receptor activation. 5-HT$_3$ receptors native to peripheral neurons and neuronal cell lines are permeable to Ca^{2+} (17, 19). The potential contribution of the endogenous Ca^{2+}-dependent Cl^- conductance (31) of the oocyte to the current response to 5-HT was examined in ion-substitution experiments using a chloride-deficient extracellular medium. At a holding potential of −60 mV, the response to 5-HT (1.5 μM) was little affected when the latter replaced the standard solution, the peak current amounting to 103.5 ± 2.0% (four experiments) of control. Moreover, current responses to 5-HT

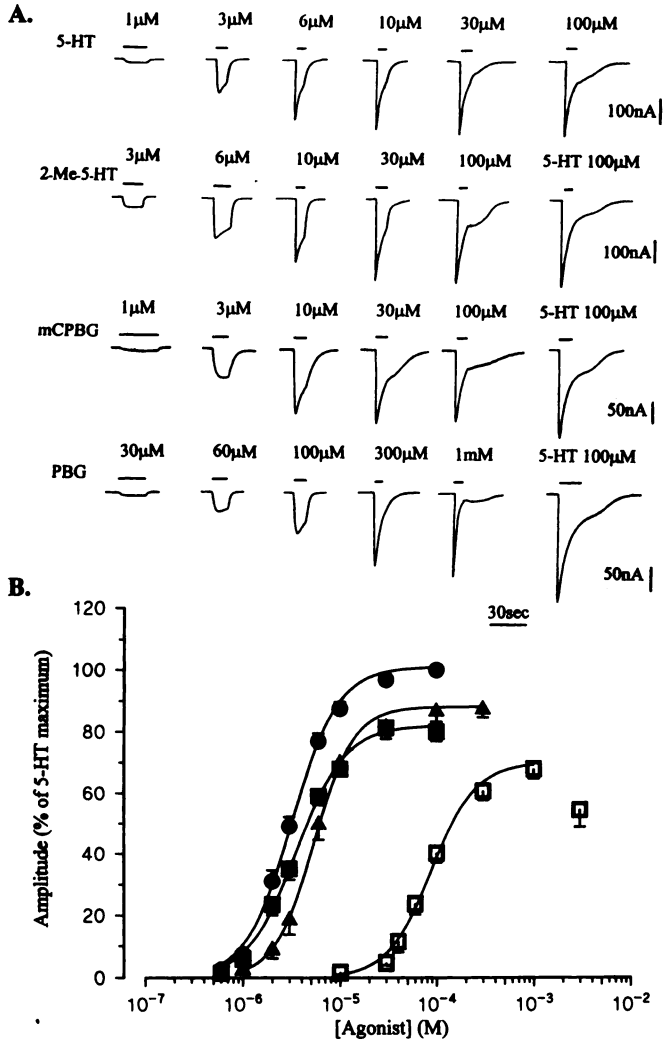

Fig. 3. Agonist pharmacology of the human 5-HT_3R-A_S homo-oligomeric receptor expressed in oocytes. A, Traces depicting concentration-dependent inward current responses to 5-HT and the selective 5-HT_3 receptor agonists 2-Me-5-HT *m*-CPBG and PBG. In each experiment, a complete concentration-response curve for the test agonist was constructed, and the maximal response to a saturating concentration of 5-HT (100 μM) was determined in the same oocyte. *Bars above each trace*, period of agonist application. Currents were recorded at a holding potential of −60 mV and low-pass filtered at 250 Hz. B, Concentration-effect relationships for 5-HT (●), 2-Me-5-HT (▲), m-CPBG (■), and PBG (□). To combine the data from different oocytes, the peak amplitude of the agonist-evoked current is expressed as a percentage of the maximal response to 5-HT (ordinate) and plotted against the concentration (log scale; abscissa) of bath-applied agonist. A logistic equation was fit to each set of data points as described in Materials and Methods to determine EC_{50} values, pseudo-Hill coefficients, and the current amplitude to saturating concentrations of agonist. Note that in the case of PBG, the data point obtained with the highest concentration of agonist tested (i.e., 3×10^{-3} M) was excluded from the analysis. Data points represent mean ± standard error of four observations obtained from separate oocytes.

reversed in sign, in a monophasic fashion, at potentials that were not significantly different in standard (−5.2 ± 1.3 mV; four experiments) and chloride ion-deficient (−7.0 ± 1.6 mV; four experiments) solutions. Collectively, these data suggest that any influx of Ca^{2+} that may occur during the response to 5-HT is insufficient to trigger the activity of Ca^{2+}-dependent chloride channels.

Inward current responses elicited by concentrations of 5-HT ≥2 μM declined from a peak value in the continued presence of 5-HT. The rate of fade of the current response, which presumably reflects receptor desensitization, increased with agonist concentration (Fig. 3). Qualitatively similar effects were observed with all other agonists tested (Fig. 3). The selective 5-HT_3 receptor agonist 2-Me-5-HT was ~2-fold less potent than 5-HT as an agonist of the human 5-HT_3R-A_S (Fig. 3). The EC_{50} for 2-Me-5-HT was estimated to be 5.3 ± 0.5 μM (four experiments), and the concentration-effect curve was associated with a Hill coefficient of 2.1 ± 0.04 (four experiments). The maximum current response to 2-Me-5-HT was 87.0 ± 2.7% (four experiments) of that evoked by a saturating concentration (100 μM) of 5-HT applied to the same population of oocytes. In contrast to 2-Me-5-HT, PBG acted as a weak agonist at the human 5-HT_3R-A, being ~27-fold less potent (EC_{50} = 84.3 ± 9.2 μM) than 5-HT (Fig. 3). The maximal current response to PBG (1 mM) was considerably less than that elicited by a saturating concentration of 5-HT (i.e., 67.9 ± 3.1% of the latter; four experiments). It has previously been noted that the introduction of a *meta*-chloro substituent into PBG greatly increases the potency of PBG as a 5-HT_3 receptor agonist (8). In the present study, the EC_{50} value for *m*-CPBG was found to be 3.6 ± 0.4 μM (four experiments), suggesting that the compound is equipotent with 5-HT as an agonist of the human 5-HT_3R-A (Fig. 3). The maximal current response to *m*-CPBG amounted to 81.3 ± 2% (four experiments) of that evoked by a saturating concentration of 5-HT. In common with 5-HT and 2-Me-5-HT, the agonist action of the biguanide compounds was associated with Hill coefficients of ~2 (Table 1). In contrast to 2-Me-5-HT, PBG, and *m*-CPBG, 5-methoxytryptamine (100 μM), which activates G protein-coupled 5-HT receptors but lacks efficacy at native 5-HT_3 receptors (4), failed to elicit a current response from oocytes demonstrably responsive to 5-HT (not illustrated).

Antagonist pharmacology. The interaction of selective and nonselective 5-HT_3 antagonists with the human 5-HT_3R-A_S was evaluated by quantifying their ability to block inward current responses elicited by 3 μM 5-HT, a concentration that approximates the EC_{50} of this agonist. The selective agents BRL 46470, granisetron, and ondansetron, at picomolar concentrations, exerted a concentration-dependent and slowly reversible blockade of the response (Fig. 4 and Table 2). The slow reversal of the blockade made it impractical to reestablish the control response to 5-HT between each concentration of antagonist examined. For this reason, IC_{50} values for BRL 46470 (770 ± 160 pM; four experiments), granisetron (314 ± 36 pM; four experiments),

TABLE 1

Summary of the agonist pharmacology of human (h) and mouse (m) 5-HT_3R-A_s homo-oligomeric receptors expressed in *Xenopus laevis* oocytes

pEC_{50}, n_H, and α values for the h5-HT_3R-A_s are derived from the present study. Those relating to the m5-HT_3R-A_s are reproduced from Ref. 25. α = fraction of maximal response obtainable with 5-HT.

Agonist	h5-HT_3R-A_s			m5-HT_3R-A_s			EC_{50} ratio
	pEC_{50}	n_H	α	pEC_{50}	n_H	α	h/m
5-HT	5.51	1.9	1.00	5.63	2.2	1.00	1.34
m-CPBG	5.44	1.8	0.81	6.08	1.8	0.88	4.34
2-Me-5-HT	5.27	2.1	0.87	4.82	2.2	0.09	0.35
PBG	4.07	2.1	0.68	4.57	2.0	0.73	3.12

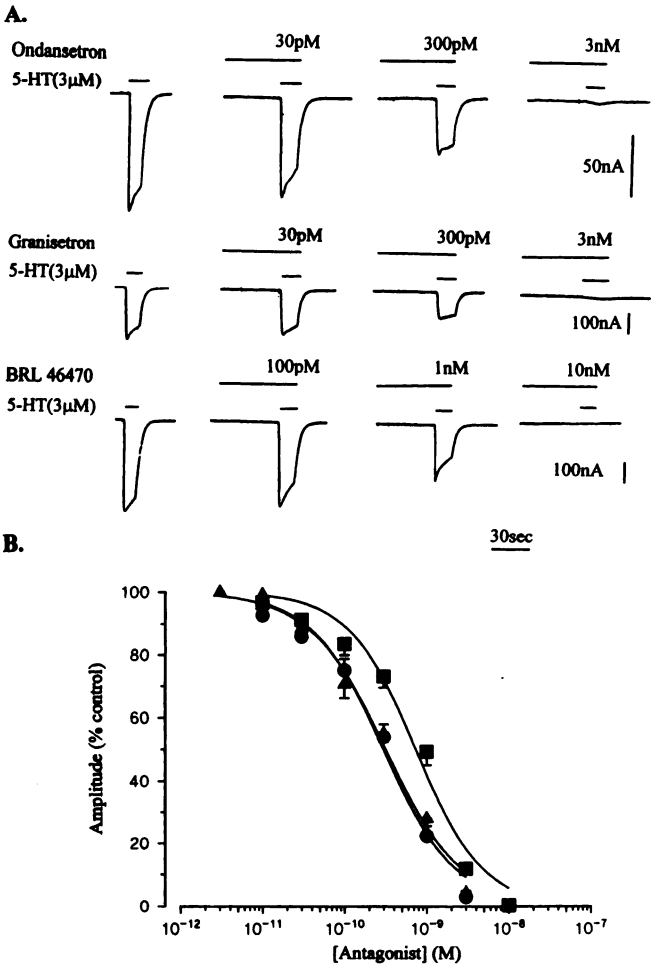

Fig. 4. Blockade of the human 5-HT$_3$R-A$_S$ homo-oligomeric receptor by selective 5-HT$_3$ receptor antagonists. A, Inward currents elicited by bath-applied 5-HT (3 μM) are inhibited in a concentration-dependent manner by ondansetron, granisetron, and BRL 46470. Note that oocytes were preincubated with bath-applied antagonist for 1 min (*top bar*) before the simultaneous delivery of agonist and antagonist (*bottom bar*). Although not illustrated, antagonism could be largely reversed on prolonged (>20 min) washout. Currents were recorded at a holding potential of −60 mV and low-pass filtered at 250 Hz. Concentration-inhibition curves for ondansetron (●), granisetron (▲), and BRL 46470 (■). The peak amplitude of the 5-HT-evoked current, expressed as a percentage of control (ordinate), is plotted against the concentration of bath-applied antagonist (log scale; abscissa). A logistic equation was fit to each set of data points as described in Materials and Methods to determine IC$_{50}$ values and slope factors for inhibition. Data points represent mean ± standard error of four observations obtained from separate oocytes.

and ondansetron (303 ± 98 pM; four experiments) were determined from repeated additions of antagonists in ascending concentrations. The nonselective antagonists metoclopramide, cocaine, and (+)-tubocurarine also produced a reversible suppression of the 5-HT-induced response, with IC$_{50}$ values of 177 ± 13 nM (four experiments), 756 ± 132 nM (four experiments), and 2.55 ± 0.15 μM (four experiments), respectively (Fig. 5 and Table 2). Responses to 5-HT (3 μM) were unaffected by the bath application of either methysergide or ketanserin at a concentration (1 μM) that would be expected to either mimic or abolish effects mediated by a variety of G protein-coupled 5-HT receptor subtypes (7) (not illustrated).

TABLE 2

Summary of the antagonist pharmacology of human (h) and mouse (m) 5-HT$_3$R-A$_S$ homo-oligomeric receptors expressed in *Xenopus laevis* oocytes

pIC$_{50}$ and n_H values for the h5-HT$_3$R-A$_S$ are derived from the present study. Those relating to the m5-HT$_3$R-A$_S$ are reproduced from Ref. 25.

Antagonist	h5-HT$_3$R-A$_S$		m5-HT$_3$R-A$_S$		IC$_{50}$ ratio
	pIC$_{50}$	*n_H*	*pIC$_{50}$*	*n_H*	*h/m*
Ondansetron	9.52	−1.0	8.92	−1.0	0.27
Granisetron	9.50	−0.9	9.85	−1.1	2.27
BRL 46470	9.11	−1.1	9.85	−1.4	5.50
Metoclopramide	6.75	−1.1	7.14	−1.3	2.46
Cocaine	6.12	−1.0	5.64	−1.1	0.34
(+)-Tubocurarine	5.59	−1.0	8.85	−0.9	1785.70

Discussion

The present study demonstrates that a cDNA isolated from a human amygdala cDNA library encodes functional, presumably homo-oligomeric receptors that possess the pharmacological attributes of the 5-HT$_3$ receptor class. Electron microscopic images of the receptor purified from insect cells infected with a recombinant baculovirus containing the mouse 5-HT$_3$R-A cDNA suggest that the expressed receptor exists as a pentameric complex (32). It is assumed that the individual subunits possess topographical features common to those proposed for nicotinic receptor subunits (4), but direct supporting evidence is sparse. Some information on the relative locations of the ligand binding domain and channel forming regions has been obtained with a chimeric construct comprising the amino-terminal domain of the chick nicotinic α7 subunit and the putative transmembrane and carboxyl-terminal domains of the 5-HT$_3$R-A (33). The chimera demonstrated nicotinic pharmacology and insensitivity to 5-HT along with ion channel properties that mimicked those associated with the 5-HT$_3$R-A (33).

The primary amino acid sequence of the human 5-HT$_3$R-A$_S$ reveals five putative sites of *N*-linked glycosylation within the presumptive extracellular domain, but the extent to which these sites are used is unknown. However, 5-HT$_3$ receptors solubilized from NCB 20 hybridoma cells and rat brain have been partially purified using lectin chromatography (5), and the glycoprotein nature of the mouse 5-HT$_3$R-A$_S$ is established (32). Recent studies suggest a role for glycosylation in the folding or oligomerization of 5-HT$_3$R-A$_S$ subunits (32). Several phosphorylation consensus sequences are present in mouse (4), rat (21), and human 5-HT$_3$ receptor subunits, and some electrophysiological studies have yielded data consistent with a modification of function by select protein kinases and phosphatases (34, 35). However, phosphorylation of the 5-HT$_3$ receptor has not been examined directly.

5-HT$_3$ receptors endogenous to central and peripheral neurons exhibit clear species differences in their pharmacological profile (see introductory paragraphs). A comparison of the potencies of antagonist compounds estimated in the present study with those obtained in voltage-clamp studies of either splice variant of the mouse 5-HT$_3$R-A expressed in *X. laevis* oocytes (4, 22, 25), RIN cells (18), or HEK 293 cells (26) identifies (+)-tubocurarine as a discriminatory ligand (Table 2). The potency of the latter in blocking current responses mediated by the mouse homologue (22) is almost 1800-fold higher than that found for the human receptor subunit under

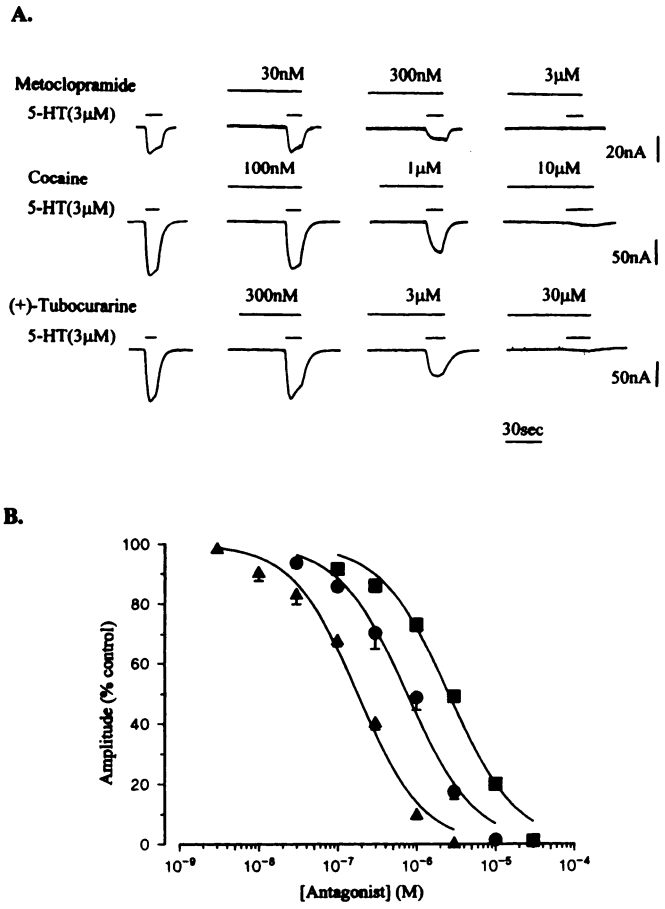

Fig. 5. Blockade of the human 5-HT_3R-A_S homo-oligomeric receptor by nonselective antagonists. A, Inward currents elicited by bath-applied 5-HT (3 μM) are inhibited by metoclopramide, cocaine, and (+)-tubocurarine in concentration-dependent manner. In all cases, oocytes were preincubated with bath-applied antagonist for 1 min (*top bar*) before the simultaneous delivery of agonist and antagonist (*bottom bar*). Although not illustrated, antagonism could be completely reversed on washout. Currents were recorded at a holding potential of −60 mV and low-pass filtered at 250 Hz. Concentration-inhibition curves for metoclopramide (▲), cocaine (●), and (+)-tubocurarine (■). The peak amplitude of the current elicited by 5-HT, expressed as a percentage of control (ordinate), is plotted against the concentration of bath-applied antagonist (log scale; abscissa). Data points were fitted with logistic equation as described in Materials and Methods to determine IC_{50} values and slope factors for inhibition. Each data point represents mean ± standard error of four observations obtained from separate oocytes.

similar experimental conditions. This observation reinforces the results obtained with native receptors, where (+)-tubocurarine exerts a potent antagonism of 5-HT_3 receptor-mediated depolarizing or inward current responses recorded from neurons (10, 11) or cell lines (16, 17) originating from the mouse. This antagonist is, however, much less potent when assayed on neurons derived from the rabbit (15) or rat (10) and is least effective as a blocker of 5-HT_3 receptors of the guinea pig (10). Moreover, the present data agree qualitatively with the results of a recent radioligand binding study, which demonstrated a pronounced difference in the affinity of (+)-tubocurarine for [^{3}H]granisetron-labeled 5-HT_3 receptor sites in membrane homogenates of human putamen and murine NG108–15 hybridoma cells (28). Interestingly, that investigation did not detect any compelling differences between the two preparations in the affinities of an additional eight antagonists. A similar finding is reported in Table 2, which, except for (+)-tubocurarine, documents only modest differences in the potencies of all other antagonists examined at human and mouse recombinant 5-HT_3 receptors. Nevertheless, such differences that exist are sufficient to convert the rank order of antagonist potencies from BRL 46470 = granisetron > ondansetron ≥ (+)-tubocurarine ≫ metoclopramide ≫ cocaine at the mouse homo-oligomer to ondansetron = granisetron > BRL 46470 ≫ metoclopramide > cocaine > (+)-tubocurarine at the human recombinant receptor. Interestingly, cocaine, which discriminates between 5-HT_3 receptor populations expressed by rabbit and mouse neurons (3), does not convincingly distinguish between recombinantly expressed human and mouse 5-HT_3 receptors (Table 2).

The structural basis of the discriminatory action of (+)-tubocurarine remains to be addressed. The voltage- and use-independent nature of the block in neuronal cell lines (16), apparently competitive kinetics in some functional studies (10), and, most dramatic, reduced potency against a chimeric construct bearing the amino-terminal domain of the nicotinic α7 subunit and transmembrane elements of the mouse 5-HT_3R-A (33) point to the extracellularly located ligand binding domain rather than the cation selective ion channel as the locus of tubocurarine action. Sequence comparisons among the amino-terminal domains of mouse, rat, and human homologues of the 5-HT_3R-A reveal only a limited number of amino acid substitutions that might contribute to their differing affinities toward (+)-tubocurarine. None of these occur at residues homologous to the aromatic amino acids implicated in the binding of curariform antagonists to nicotinic receptor α-subunits (reviewed in Ref. 36); such residues are conserved in all three species homologues of the 5-HT_3R-A_S (Fig. 2). However, several nonconservative substitutions cluster to a stretch of residues that define a neurotransmitter binding loop (loop C) in nicotinic, γ-aminobutyric $acid_A$, and glycine receptor subunits (36). The influence of these residues on the pharmacology of homo-oligomeric receptors formed from human and mouse 5-HT_3R-A_S subunits is under investigation.

Species differences in 5-HT_3 receptor pharmacology have also been detected with agonist compounds (9, 10, 12, 13). In the present study, the EC_{50} and Hill coefficient values for the natural agonist 5-HT at the human 5-HT_3R-A_S did not differ from values documented for the mouse splice variant (Ref. 25; Table 1). A similar comparison with the selective agents PBG and *m*-CPBG suggests that they discriminate weakly between the species homologues, with both compounds being slightly more potent as activators of the mouse 5-HT_3R-A_S (Table 1). However, the Hill coefficients associated with the biguanide compounds and the maximal current responses evoked by them, in relation to those evoked by a saturating concentration of 5-HT, do not vary between the two species homologues (Table 1). The difference, albeit small, in the potencies of PBG and *m*-CPBG at the two homo-oligomers may be significant in view of the fact that arylbiguanides have previously been suggested to discriminate among 5-HT_3 receptors expressed in mouse, rat, guinea pig, and rabbit tissues (9, 10, 12, 13) and also between receptors synthesized by different murine neuronal cell lines (37) or different tissues within a species (14). Studies with a range of recently described dichloro and trichloro derivatives of PBG with improved affinity for murine 5-HT_3 receptors (8) may prove

useful in further dissecting species differences in the properties of both recombinant and native receptors.

A further pharmacological difference between the human and mouse homologues of the 5-HT$_3$R-A$_S$ is suggested by a marked discrepancy in the actions of 2-Me-5-HT. In particular, the maximal current response elicited by 2-Me-5-HT at the human 5-HT$_3$R-A$_S$ is strikingly higher than that previously found for the mouse splice variant (22, 25) and is accompanied by a slightly increased potency at the former (Table 1). Unfortunately, the significance of this difference is blurred by the variable results that have been obtained with 2-Me-5-HT acting on recombinant 5-HT$_3$ receptors (18, 23, 25, 38). In a previous comparative study of the splice variants of the 5-HT$_3$R-A expressed in *Xenopus* oocytes, the normalized (with respect to 5-HT) maximal current response to 2-Me-5-HT acting at homo-oligomers assembled from 5-HT$_3$R-A subunits (i.e., 63%) greatly exceeded that elicited at homo-oligomers composed of 5-HT$_3$R-A$_S$ subunits (i.e., 9%). This difference was tentatively ascribed to the six-amino acid deletion in the putative intracellular loop of the 5-HT$_3$R-A$_S$ (25), a suggestion that is incompatible with the results of the present study. In qualitative agreement, 2-Me-5-HT acts essentially as a full agonist at homo-oligomers assembled from the 5-HT$_3$R-A subunit in HEK 293 cells but elicits much reduced maximal responses at the 5-HT$_3$R-A$_S$ complex expressed in the same cell line (38). In contrast, 2-Me-5-HT fails to discriminate between the two homo-oligomeric receptors expressed in RIN cells (23). At present, there is no satisfactory explanation of these disparate findings, although nonequivalent post-translational modifications in the different expression systems could be a contributory factor.

Subunits that may coassemble with the 5-HT$_3$R-A to form native 5-HT$_3$ receptors remain to be identified, although their existence has been predicted from electrophysiological and biochemical studies (5, 18). In this respect, it is important to consider how closely a species variant of a homo-oligomeric 5-HT$_3$R-A receptor mimics its endogenous counterpart. Unfortunately, a direct comparison of the pharmacological properties of the human 5-HT$_3$R-A subunit determined electrophysiologically with those of receptors endogenous to central and peripheral nervous tissues of human origin is complicated by the fact that the characterization of endogenous receptors has relied largely on radioligand binding assays using postmortem material (27, 28). A major confounding factor is the development of receptor desensitization, which in electrophysiological assays curtails the measured response before equilibrium between agonist and antagonist binding can be attained (reviewed in Ref. 3). This could explain why the antagonist pIC$_{50}$ values found in the present study, although in good correlation with pK_i values reported from binding studies using membrane homogenates of human putamen (Fig. 6), consistently suggest affinities that are considerably higher than those determined in binding assays. The opposite situation is obtained with 5-HT$_3$ receptor agonists. The apparent equilibrium dissociation constants (K_a or EC$_{50}$) determined functionally suggest affinities substantially lower than those determined in competition studies on human putamen homogenates (28). This might be attributed to the binding assay detecting a desensitized state of the receptor that demonstrates higher affinity toward agonists than the conducting state of the receptor assayed electrophysiologically (37, 39). In any event, the compounds examined here compete for [^{3}H]granisetron binding to membranes prepared from HEK 293 cells stably transfected with the human 5-HT$_3$R-A$_S$ subunit with affinity constants similar to those reported in human postmortem brain tissue (28, 40).

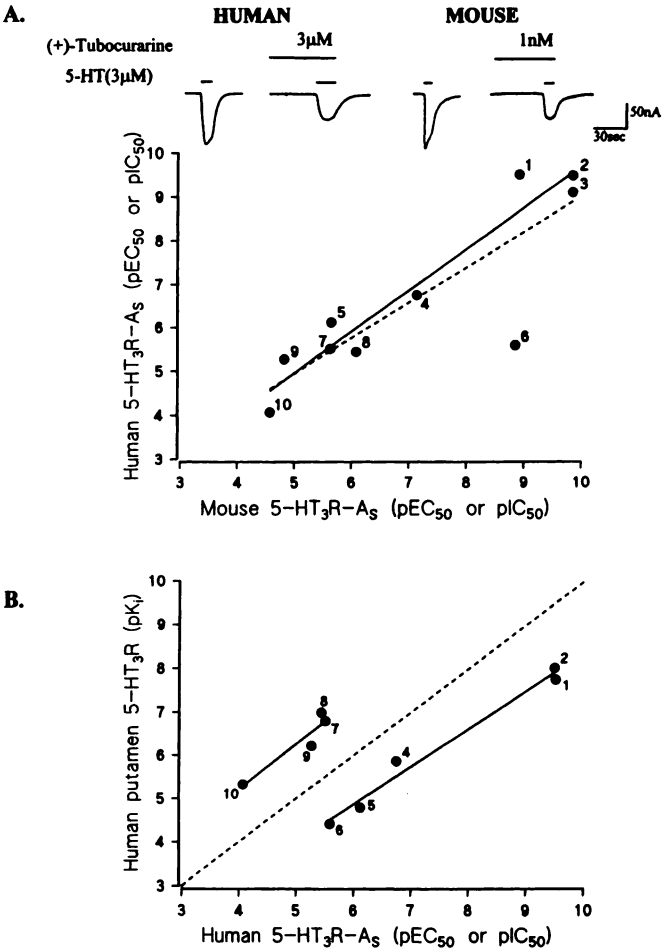

Fig. 6. Correlation between the pharmacological profile of the human 5-HT$_3$R-A$_S$ and mouse 5-HT$_3$R-A$_S$ homo-oligomeric receptors and human 5-HT$_3$ recognition sites in brain membranes. A, A comparison of pEC$_{50}$ and pIC$_{50}$ values of agonists and antagonists observed for the human (ordinate) and mouse (abscissa) 5-HT$_3$R-A$_S$ subunits expressed in oocytes. *Dashed line*, line fit by least-squares regression analysis to all data points yields a correlation coefficient (r) of 0.85 and slope of 0.82. Excluding (+)-tubocurarine from the analysis (uninterrupted line) produces an improved fit (r = 0.97) and a slope of 0.95, close to unity. *Inset*, an example of the large difference in the antagonist potency of (+)-tubocurarine at the human and mouse recombinant receptors. Recording conditions are as described in the legend to Fig. 5 and Ref. 25. B, A comparison of pEC$_{50}$ and pIC$_{50}$ values of agonists and antagonists observed for the human 5-HT$_3$R-A$_S$ subunit expressed in oocytes with pK_i values determined in ligand binding studies on postmortem human brain tissue. Ligand binding data were obtained with the radioligand [^{3}H] granisetron labeling 5-HT$_3$ recognition sites in membrane homogenates of human putamen. *Dashed line*, identity. Note that the data points for all agonists, i.e., compounds 7–10, lie above this line, whereas the opposite is apparent for all antagonists (see text for a possible explanation of these observations). *Two solid lines*, separate fits to the agonist (r = 0.94, slope = 1.04) and antagonist (r = 0.99, slope = 0.88) data performed by least-squares regression analysis. All binding data are abstracted from Bufton *et al.* (28). Compounds are denoted: *1*, ondansetron; *2*, granisetron; *3*, BRL 46470; *4*, metoclopramide; *5*, cocaine; *6*, (+)-tubocurarine; *7*, 5-HT; *8*, *m*-CPBG; *9*, 2-Me-5-HT; and *10*, PBG.

In conclusion, we describe the cloning and functional ex-

pression of a human 5-HT_3R-A_S subunit. Homo-oligomeric receptors assembled from the human 5-HT_3R-A_S display a pharmacology distinct from that of other species homologues currently isolated. The high degree of sequence conservation that is apparent within the amino-terminal domain of species homologues of the 5-HT_3R-A_S immediately suggests residues that may contribute to such differences. Future studies using site-directed mutagenesis to test such predictions may contribute to the definition of the ligand binding site(s) on the receptor. The current availability of a human receptor subunit will allow the direct assessment in functional tests of ligands targeting the 5-HT_3 receptor in humans.

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