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Pharmacology of polymorphic variants of the human 5-HT$_{1A}$ receptor

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Abstract

The 5-HT$_{1A}$ receptor is a critical mediator of serotonergic (5-HT) function. We have identified 13 potential single nucleotide polymorphisms resulting in amino acid changes throughout the human 5-HT$_{1A}$ receptor. The pharmacological profiles of these 13 polymorphic variants were then characterized using a high-throughput assay based on ligand-dependent transformation of NIH/3T3 cells. The majority of the polymorphic variants displayed wild-type pharmacological profiles in response to a panel of well-established agonists at the 5-HT$_{1A}$ receptor. However, the A50V polymorphic variant, which had an alanine to valine substitution in transmembrane 1, exhibited a loss of detectable response to 5-HT. Interestingly, all other agonists tested, including buspirone, lisuride, and (+)8-OH-DPAT, exhibited efficacies similar to that of the wild-type receptor. The competitive antagonist, methiothepin, also displayed a 19-fold decrease in potency at the A50V variant receptor. However, both 5-HT and methiothepin were able to compete for [^{3}H]WAY-100635 binding to the A50V variant with affinities similar to the wild-type receptor. Moreover, the B_{max} of [^{3}H]WAY-100635 binding was 14-fold lower for the A50V variant than for the wild-type receptor. Thus, the A50V receptor variant exhibited ligand-specific functional alterations in addition to lower expression levels. These data suggest a previously unappreciated role for transmembrane 1 in mediating 5-HT response at the 5-HT$_{1A}$ receptor. Furthermore, individuals that potentially harbor the A50V polymorphism might display aberrant affective behaviors and altered responses to drugs targeting the 5-HT$_{1A}$ receptor.

Keywords: 5-HT$_{1A}$ receptor; Serotonin; 5-HT; cSNP; Polymorphism; Site-directed mutagenesis

1. Introduction

The 5-HT$_{1A}$ receptor is a critical mediator of serotonergic (5-HT) function, and thus, has been implicated in a variety of neurophysiological behaviors, including mood, cognition, sexual behavior, and feeding. The 5-HT$_{1A}$ receptor is a member of the G-protein coupled receptor (GPCR) family and signals through $G_{i/o}$ proteins, leading to inhibition of the enzyme adenylyl cyclase [1,2]. 5-HT$_{1A}$ receptors are expressed by the 5-HT producing neurons in the raphe nuclei, and function as autoreceptors that negatively regulate 5-HT tone [3,4]. Postsynaptically, 5-HT$_{1A}$ receptors are located in structures that are implicated in mediating cognition, emotion, and affect: the cerebral cortex, hippocampus, and amygdala [3].

Multiple studies have indicated a role of the 5-HT$_{1A}$ receptor in mediating affective states, including anxiety and depression. Targeted deletion of the 5-HT$_{1A}$ receptor gene in mice creates a phenotype of increased anxiety behaviors, learning and memory deficits, as well as altered responses in behavioral paradigms for depression (reviewed in [5,6]). Most compelling are the clinical observations of the efficacy of 5-HT$_{1A}$ receptor drugs in the treatment of human mood disorders. Partial agonists of the 5-HT$_{1A}$ receptor, such as buspirone, are in clinical use as anxiolytics and may also have anti-depressant activity [7–9]. In addition, pindolol, a β-adrenergic receptor antagonist that also exhibits 5-HT$_{1A}$ receptor antagonism, has been reported to

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Abbreviations: 5-HT$_{1A}$ receptor, serotonin 1A receptor; PCR, polymerase chain reaction; R-SAT, receptor selection and amplification technology; 5-HT, 5-hydroxytryptamine; 5-CT, 5-carboxytryptamine; (+)8-OH-DPAT, (+)-*R*-8-hydroxy-2-(dipropylamino)tetralin; WAY-100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexanecarboxamide; TM, transmembrane domain.

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augment the therapeutic effects of anti-depressant medications in some studies [10] but not others [11].

While the 5-HT_{1A} receptor was one of the first GPCRs to be cloned [1], in comparison to other GPCRs, relatively few structure–function studies have been performed. In the majority of these studies, residues which align with those critical for function in other GPCRs were mutagenized. However, for the most part this approach has confirmed that these residues are also important for function of the 5-HT_{1A} receptor. For example, residues in TM2, 3, 5, and 7 have been shown to be important for ligand binding [12–14], residues in intracellular loop 3 have roles in G-protein coupling [15], and the intracellular loop 2 has been shown to be important downstream of G-protein coupling, such as calcium mobilization or inhibition of cAMP accumulation [16].

Recent advances in the sequencing of the human genome, and parallel studies specifically exploring the frequency and nature of SNPs have highlighted the greater than expected degree of polymorphism in the human genome [17,18]. A number of studies have documented the presence of single nucleotide polymorphisms (SNPs) within the human 5-HT_{1A} receptor gene, which include polymorphisms that change the coding sequence of the gene (cSNPs) [19–22]. Here we have functionally analyzed these and additional potential cSNPs that were identified by surveying the patent literature and searching for nonredundant 5-HT_{1A} coding sequences in the GenBank database (Fig. 1).

Most of the receptor variants exhibited a pharmacological profile similar to that of the wild-type receptor, suggesting that they are unlikely to have functional consequences *in vivo*. However, one variant, a C to T polymorphism that results in an alanine to valine substitution at amino acid 50 (A50V) in the receptor protein, displayed altered functional responses to serotonin. Additionally, the A50V receptor variant was expressed at more than 10-fold lower levels than the wild-type receptor. Surprisingly the A50V polymorphism is located in TM1. Thus, the A50V receptor variant exhibited *in vitro* alterations in function that, if present in humans, might lead to altered drug responses or abnormal central serotonergic physiology.

2. Materials and methods

2.1. Cloning of the human 5-HT_{1A} receptor cDNA

Oligonucleotide probes corresponding to the untranslated regions of the human 5-HT_{1A} receptor gene were used to amplify the entire coding exon by PCR. The sequences of the probes were: GGGTCTCTGAATTCCCTTCCTCCGAAACTT (sense), CAATGTCGACTCTCACAAACTCTCTGAATT (antisense). The 1642 base pair amplified product was cloned into the Topo 2.1 vector as per manufacturer's protocols (Invitrogen), subsequently subcloned into the mammalian expression vector pSI (Promega) and sequence verified. This designated wild-type 5-HT_{1A} receptor sequence corresponds to GenBank accession number M83181.

To construct plasmids encoding 5-HT_{1A} receptor variants, PCR mutagenesis was performed by standard protocols.

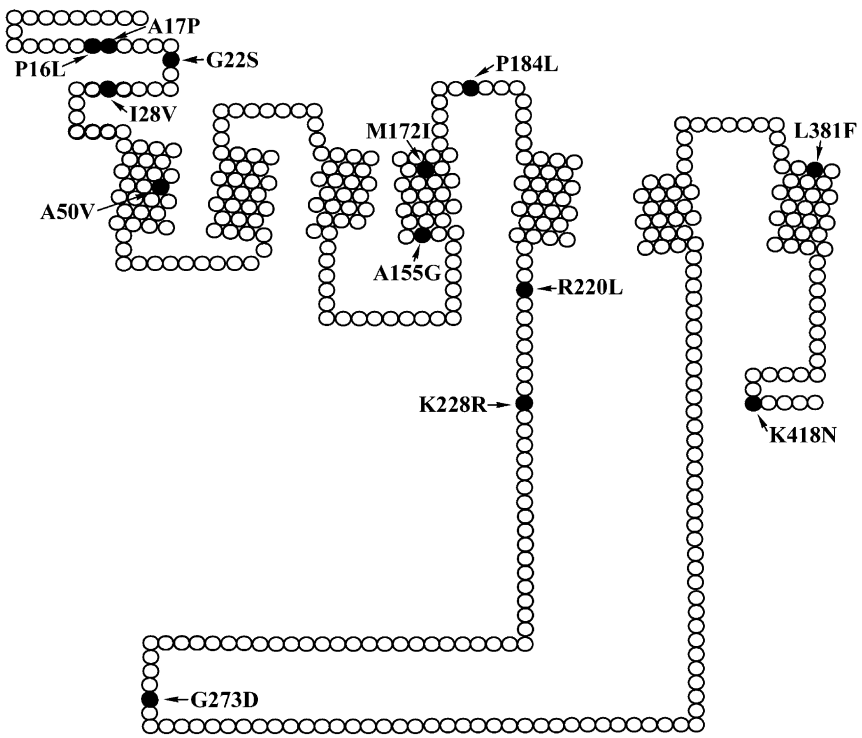

Fig. 1. 5-HT_{1A} receptor variants are distributed throughout the molecule. Putative cSNPs were found in all major domains of the 5-HT_{1A} receptor (see Table 1). Predicted protein topology of the 5-HT_{1A} receptor derived from www.gpcr.org.

Variant clones were initially identified by restriction digest and pharmacologically profiled in R-SAT. Any variant that exhibited differences from wild-type pharmacology (>2-fold EC_{50} shifts, changes in rank order of potency, or >40% change in efficacy) was subjected to further verification. Thus, for the variants A50V, K228R, M172I, L381F, and G273D (control), a small region of DNA surrounding the cSNP was subcloned into a sequence-verified plasmid coding for the wild-type receptor. These subcloned plasmids were then sequence-verified through the polymorphic site, and agonist pharmacology was confirmed by R-SAT in at least two separate additional experiments. For the A50V, M172I, K228R, G273D, and L381F plasmids, DNA quality was controlled for by cotransfection with a plasmid encoding for an unrelated G_i coupled receptor, the somatostatin 5 receptor (SSR5). In these multiplexing experiments, agonist response to somatostatin 14 was not suppressed by the addition of plasmids expressing 5-HT_{1A} receptor variants (data not shown).

2.2. R-SAT (receptor selection and amplification technology)

R-SAT assays were performed as described [23] with minor modifications. Briefly, NIH/3T3 cells were grown in 96-well tissue culture plates to 70–80% confluency in Dulbecco's modified essential media (DMEM) supplemented with 10% calf serum and 1% penicillin/streptomycin/glutamine (PSG). Cells were transfected using Polyfect (Qiagen) as described in manufacturer's protocols with DNA encoding receptor (1 ng/well), chimeric G proteins (5 ng/well) [24], and β-galactosidase (20 ng/well). After overnight transfection, medium was replaced with serum-free DMEM containing 2% Cyto-SF3 (Kemp Biotechnologies). Cells were grown in a humidified atmosphere with 5% ambient CO_2 for 5 days. Medium was replaced with the β-galactosidase substrate, *o*-nitrophenyl-β-D-galactopyranoside (in phosphate-buffered saline with 5% Nonidet P-40). The colorimetric response was measured at 420 nM. Dose–response curves were fit using Excel Fit and GraphPad Prism software. All agonist data are presented as pEC_{50} or EC_{50} values in molar units, while all competitive antagonist data are presented as K_i values that have been corrected by the formula: $K_i = (IC_{50}\text{ observed})/1+([\text{agonist}]/EC_{50}\text{ agonist})$. When $N = 3$ or $N > 3$, paired *t*-test was performed to assess statistical significance.

2.3. Drugs

All compounds for R-SAT studies were solubilized as 10 mM stock solutions in either water or DMSO. Working dilutions were made from 20 to 100 μM solutions in Dulbecco's modified Eagle's medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) and 2% Cyto-SF3. All compounds were obtained from Sigma Chemical Co./RBI, except methiothepin (Tocris), sertindole (Lundbeck A/S), and ebalzotan (a generous gift from Astra-Zeneca Research & Development).

2.4. Radioligand binding assays

NIH/3T3 cells or COS-7 cells (American Type Culture Collection) were grown to 70% confluence in 15 cm^2 cell culture dishes, and transfected with 10 μg of receptor plasmid DNA using Polyfect Transfection Reagent (Qiagen), as per manufacturer's protocols. For HEK293T cells (American Type Culture Collection), cells were grown in 10 cm^2 dishes and transfected with 8 μg of receptor plasmid DNA. Cells expressing the 5-HT_{1A} receptor variants were harvested 48 hr (for HEK293T cells) or 72 hr (for COS-7 or NIH/3T3 cells) posttransfection. Membranes were prepared in 50 mM Tris, pH 7.4 essentially as previously described [25]. Freshly prepared membranes were incubated with [^{3}H]WAY-100635 (83 Ci/mmol) (Amersham Pharmacia Biotech) and competing ligands in 50 mM Tris, pH 7.4 at room temperature for 2–3 hr. The final assay volume was 1 mL. Incubations were terminated by rapid filtration with ice-cold buffer through Whatman GF/B glass fiber filters pretreated with 0.3% polyethyleninmine. Non-specific binding was determined with 10 μM methiothepin, except when competitive binding experiments were performed with methiothepin. In that case, values at 2 μM methiothepin were used to determine non-specific binding. For NIH/3T3 and COS-7 cells, each concentration of [^{3}H]WAY-100635 was tested in duplicate. For HEK-293 membranes, a single saturating concentration of [^{3}H]WAY-100635 (15 nM) was tested in triplicate. Protein content was determined using the Bio-Rad Protein Assay Reagent (Bradford method) (Bio-Rad). For competition curves, 0.8 nM [^{3}H]WAY-100635 was used. K_i values were calculated using the equation, $K_i = (IC_{50})/(1 + [L]/K_d)$, where [L] is the concentration and K_d is the dissociation constant of [^{3}H]WAY-100635. All curves were analyzed using GraphPad Prism software. All binding data were tested at one and two-site curve fitting models. For all competition curves, data was fitted to one-site curve fitting model with Hill coefficient set to 1 using GraphPad Prism® 4.0 data analysis software.

2.5. [^{35}S]-GTPγS binding

HEK293T cells were transfected as described above. Cells were harvested 48 hr post transfection and homogenized using a polytron homogenizer in a buffer containing 10 mM HEPES, pH 7.4 and 10 mM EDTA. Crude cell homogenates were prepared by centrifugation at 15,000 *g* for 10 min followed by two washes. Final cell homogenate was resuspended at a protein concentration of 1 mg/mL and stored at −80° until use. GTPγS binding was performed as described with minor modifications [26]. Membranes (8–50 μg) were incubated with [^{35}S]-GTPγS (Amersham Pharmacia Biotech), 5-CT, and cold GDP

(10 μM) in 10 mM HEPES, pH 7.4, 300 mM NaCl, 10 mM $MgCl_2$ at 30° for 1 hr. The final assay volume was 100 μL. Non-specific binding was determined using cold GTPγS (10 μM). Incubations were terminated by rapid filtration with ice-cold buffer through Whatman GF/B glass fiber filters.

2.6. Genotyping

The sample population for the A50V polymorphism consisted of 48 Asian, 74 African-American, 73 Caucasian, 38 Hispanics, 16 African and 2 non-specified. The sample population for the L381F polymorphism consisted of 23 African-American, 23 Hispanic, 23 Asian, and 24 Caucasian samples. Genomic DNA was purified from blood or cell lines by standard protocols. Details about the DNAs used are available on request. DNA surrounding the polymorphic site was amplified by standard PCR techniques, where the polymorphism would predict a restriction fragment length polymorphism (RFLP). The oligo sequences were as follows: (A50V, sense) CAACACCACATCACCACCGGCTCCCTTTG, (A50V, antisense) CTAAGGCGATGGCAGCCACCACGCACGCATTG, (L381F, sense) CTGCTGGCTGCCCTTCTTCATCGTGGCT, (L381F, antisense) GGAGTAGCCCAGCCAATTGATTATGGGGCC. The PCR amplified DNA was then assayed by restriction digest followed by gel electrophoresis.

3. Results

3.1. Functional screening of 13 putative 5-HT_{1A} polymorphic variants

To identify cSNPs within the 5-HT_{1A} receptor gene, we searched the dbSNP database and surveyed the scientific and patent literature for reports of cSNPs (Table 1). In addition, we looked for nonredundant sequences within sequence databases and the scientific literature. We obtained a total of 13 potential cSNPs, spanning the entire coding region of the 5-HT_{1A} receptor gene (Fig. 1). Six have been reported to exist in humans by single-strand conformational polymorphism analysis, direct DNA sequencing, and/or restriction fragment-length polymorphism analysis: P16L [21,27], G22S, I28V [20,21], R220L [22], G273D [28], and K418N [22]. The P184L polymorphism was identified by searching the dbSNP database. Four variants, A17P, A50V, A155G, and L381F were found in the patent literature. Finally, two putative cSNPs, K228R [29] and M172I [1] were identified as sequencing discrepancies.

Plasmids encoding these 13 5-HT_{1A} receptor variants were generated using PCR-based site-directed mutagenesis of the wild-type 5-HT_{1A} receptor cDNA. The cell-based functional assay R-SAT was utilized to assess the potential biological and pharmacological effects of the wild-type and variant receptors [22,23,25]. This assay is based on the ability of many proteins, including GPCRs, to mediate proliferation of mammalian cells. As shown in Table 2, the rank of order of potency for various agonists at the wild-type 5-HT_{1A} receptor was: lisuride > 5-CT > (+)8-OH-DPAT > buspirone = 5-HT. Only buspirone displayed less than 90% efficacy relative to (+)8-OH-DPAT (Table 2B). Thus, as we have reported for muscarinic and other serotonin receptors [22,25,30] the R-SAT response of the human 5-HT_{1A} receptor to known agonists is similar to that obtained from radioligand binding (http://pdsp.cwru.edu/PDSP.asp) [31] cAMP assays [2], and [^{35}S]-GTPγS assays [32]. Moreover, treatment with pertussis toxin completely abrogated response to 5-HT_{1A} agonists, as expected for a $G_{i/o}$ coupled receptor (data not shown).

Table 1
Identification of putative 5-HT_{1A} cSNPs

AA change	cSNP	Source	Allele frequencies
P16L	C/T	Literature [21]	2.3% in Japanese [27]
A17P	G/C	Patent[a]	ND
G22S	G/A	Literature [21]	0.5% in American and Finnish Caucasians [21,49]
I28V	A/G	Literature [21]	1.2% in Germans [20]; 0.5% in Caucasians and Native Americans [49]
A50V	C/T	Patent[a]	ND
A155G	C/G	Patent[b]	ND
M172I	G/C	Nonredundant sequence [1]	ND
P184L	C/T	dbSNP	ND
R220L	G/T	Literature [22]	Found in Tourette's syndrome patient [22]
K228R	A/G	Nonredundant sequence [29]	ND
G273D	G/A	Literature [28]	3.7% in Japanese
L381F	G/C	Patent[a]	ND
K418N	C/G	Literature [1,22]	Found in Tourette's syndrome patient [22]

Potential cSNPs were found by surveying the dbSNP database (www.ncbi.nlm.nih.gov/SNP) and the scientific and patent literature for reports of cSNPs. In addition, we searched sequence databases and the scientific literature for 5-HT_{1A} receptor cDNA sequences nonredundant with our wild-type 5-HT_{1A} sequence. Known allele frequencies are listed. ND, not determined.

[a] WO 00/58519, 2000

[b] WO 01/10884, 2001

Table 2
5-HT_{1A} receptor agonist pharmacology

	(+)8-HO-DPAT			Lisuride			Buspirone			5-CT			5-HT		
	pEC_{50}	SNP/WT	N	pEC_{50}	SNP/WT	N	pEC_{50}	SNP/WT	N	pEC_{50}	SNP/WT	N	pEC_{50}	SNP/WT	N
(A) Potency															
WT	6.9 ± 0.1	1	31	8.1 ± 0.1	1	14	6.1 ± 0.1	1	14	7.4 ± 0.2	1	14	6.0 ± 0.1	1	14
P16L	6.7 ± 0.1	1	6	8.3 ± 0.1	1	3	6.1 ± 0.3	1	3	7.7 ± 0.1	1	3	5.8 ± 0.1	1	3
A17P	6.8 ± 0.1	1	8	7.7 ± 0.2	1	3	6.4 ± 0.1	1	4	7.1 ± 0.3	2	4	5.9 ± 0.1	1	4
G22S	6.9 ± 0.1	1	5	8.5 ± 0.2	1	3	6.1 ± 0.2	1	3	7.7 ± 0.1	1	3	5.9 ± 0.1	1	3
I28V	6.8 ± 0.1	1	8	8.3 ± 0.1	1	4	6.3 ± 0.3	1	4	7.2 ± 0.1	3	3	5.7 ± 0.5	1	4
A50V	5.8 ± 0.3***	14	16	7.3 ± 0.1***	9	5	5.3 ± 0.2***	10	5	NR		9	NR		6
A155G	6.5 ± 0.3**	3	8	8.0 ± 0.2	2	4	6.5 ± 0.3	1	4	6.8 ± 0.1	5	3	5.6 ± 0.1	4	4
M172I	7.2 ± 0.1****	0.3	9	8.5 ± 0.1*	0.3	5	6.7 ± 0.2**	0.1	5	7.9 ± 0.2	0.2	4	6.4 ± 0.1**	0.3	4
P184L	6.5 ± 0.1	2	8	7.8 ± 0.2	2	4	5.6 ± 0.3	3	4	7.1 ± 0.2	2	4	5.8 ± 0.1	1	4
R220L	6.9 ± 0.2	1	6	8.4 ± 0.1	1	3	6.8 ± 0.4	0.5	3	7.7 ± 0.1	1	3	6.0 ± 0.3	1	3
K228R	6.5 ± 0.2	2	8	7.8 ± 0.2	3	4	6.1 ± 0.6	1	4	7.2 ± 0.4	3	4	5.7 ± 0.2	1	4
G273D	6.7 ± 0.1	1	8	7.9 ± 0.2	1	4	6.4 ± 0.1	1	3	7.3 ± 0.3	1	4	5.9 ± 0.2	1	4
L381F	6.4 ± 0.1****	2	10	7.6 ± 0.2	2	5	5.6 ± 0.5	3	3	6.1 ± 0.3**	20	5	<5.5		4
K418N	6.7 ± 0.1	2	6	8.4 ± 0.1	1	3	6.3 ± 0.3	1	3	7.4 ± 0.5	2	3	5.5 ± 0.1	2	3

	Lisuride			Buspirone			5-CT			5-HT		
	Efficacy	SNP/WT	N	Efficacy	SNP/WT	N	Efficacy	SNP/WT	N	Efficacy	SNP/WT	N
(B) Efficacy												
WT	110 ± 6	1.0	13	39 ± 5	1.0	13	112 ± 5	1.0	14	91 ± 4	1.0	14
P16L	120 ± 20	1.0	3	33 ± 11	1.0	3	118 ± 12	0.8	3	110 ± 8	1.1	3
A17P	103 ± 10	0.9	3	30 ± 8	0.8	4	113 ± 14	1.0	4	93 ± 20	1.0	4
G22S	114 ± 11	0.9	3	45 ± 8	1.3	3	102 ± 3	1.0	3	85 ± 11	0.9	3
I28V	111 ± 16	0.9	4	15 ± 3	0.5	4	136 ± 17	1.2	3	118 ± 15	1.2	4
A50V	126 ± 15	1.1	5	28 ± 6	0.5	5	NR***		9	NR***		6
A155G	103 ± 4	1.0	4	29 ± 8	0.6	4	130 ± 9	1.1	3	78 ± 16	0.8	4
M172I	123 ± 8	1.2	5	55 ± 13	1.1	5	127 ± 13	1.1	4	121 ± 14	1.2	4
P184L	128 ± 12	1.2	4	37 ± 15	0.9	4	112 ± 15	0.9	4	88 ± 22	0.9	4
R220L	101 ± 14	1.0	3	34 ± 8	1.2	3	111 ± 10	0.9	3	107 ± 17	1.1	3
K228R	82 ± 5	0.9	4	36 ± 13	0.7	4	128 ± 17	0.9	4	109 ± 2	1.1	4
G273D	104 ± 13	1.0	4	37 ± 5	0.7	3	117 ± 8	1.0	4	83 ± 1	0.9	4
L381F	107 ± 7	1.0	5	41 ± 12	0.8	3	107 ± 9	0.9	5	ND		4
K418N	101 ± 9	0.9	3	34 ± 5	1.0	3	78 ± 9	0.7	3	72 ± 15	0.7	3

(A) *Potency*: Agonist responses to clinical and reference compounds were determined using R-SAT, with nine-point concentration–response curves performed in duplicate. The average pEC_{50} (median effective concentration, $pEC_{50} = -\log(EC_{50})$) and standard error for the 5-HT_{1A} wild-type (WT) and variant receptors are reported for N experiments. The ratio of variant to wild-type EC_{50} is also shown (SNP/WT), where the values for the wild-type and variant receptors were derived from the same experiments. Paired *t*-test indicates significant differences from wild-type values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NR, no response detected.

(B) *Efficacy*: The average efficacy and standard error for wild-type and variant 5-HT_{1A} receptors are reported. Efficacy is calculated as a percentage of maximum response to that observed for (+)8-OH-DPAT in the same experiment for wild-type or variant 5-HT_{1A} receptors. No significant differences in maximal response to (+)8-OH-DPAT were observed for wild-type or variant 5-HT receptors. Paired *t*-test indicates significant differences from wild-type values: $^{***}P < 0.001$. NR, no response; ND, not determined.

Using R-SAT, we screened the 13 5-HT_{1A} variant receptors against a panel of known 5-HT_{1A} receptor agonists (Table 2). No signal was observed for cells transfected with β-galactosidase alone indicating no detectable contribution of any endogenous 5-HT_{1A} receptor (data not shown). Comparison of the average pEC_{50} and efficacy values (relative to (+)8-OH-DPAT) revealed that most variants clustered at nearly wild-type agonist pharmacology (1–5-fold increases in EC_{50}) (Table 2; see SNP/WT in Table 2A). The only variant tested with an increased potency was M172I, which exhibited only ~3-fold lower EC_{50} values compared to the wild-type receptor for all agonists tested (Fig. 2A; also see Table 1). No significant differences in basal response were observed for any of the variants, indicating no alteration in constitutive activity. Additionally, no significant differences in maximal response were observed for (+)8-OH-DPAT at the wild-type or variant receptors. Accordingly, no significant differences in the fold response (ratio of maximal to basal response) were observed; e.g. while the average fold response for the wild-type receptor at (+)8-OH-DPAT was 15 ± 1, the value was 13 ± 1 for the A50V receptor, with no significant difference in the values by paired *t*-test. Thus, most of the receptor variants tested exhibited small, if any, changes in response to 5-HT_{1A} receptor agonists.

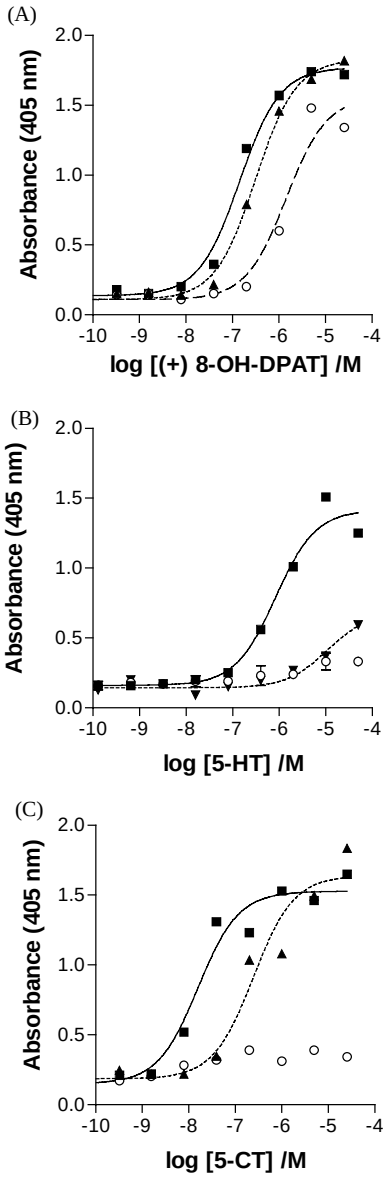

Fig. 2. 5-HT has reduced efficacy for the A50V and L381F receptor variants. R-SAT concentration–response curves for (+)8-OH-DPAT (A), 5-HT (B), and 5-CT (C) are shown for wild-type (■), L381F (▲), and A50V (○) receptor variants. Responses are shown in absorbance units (405 nm). A representative experiment is shown, where each point represents the average of two determinations.

The most severely altered variant was A50V. The natural ligand, 5-HT, and a structurally related compound, 5-CT (10% response of wild-type, $P < 0.01$; also see Fig. 2) displayed no detectable responses at the A50V variant receptor. In contrast, (+)8-OH-DPAT, buspirone, and lisuride had 10-fold lowered potencies at the A50V variant with efficacies similar to the wild-type receptor (Table 2). Notably, the variant L381F also exhibited an altered response to 5-CT in comparison to other compounds (Table 2, also see Fig. 2). While the potency for 5-CT was decreased 20-fold compared to wild-type ($P < 0.01$), the potency for (+)8-OH-DPAT was only decreased 2–3-fold ($P < 0.001$). In addition, the 5-HT displayed reduced potency at the L381F receptor variant (Table 2B, Fig. 2). However, high levels of 5-HT or 5-CT (100 μM) were unable to inhibit (+)8-OH-DPAT (10 μM) induced responses at the A50V receptor variant (data not shown). Thus, functional characterization indicated that while most of the 5-HT_{1A} receptor variants clustered near wild-type agonist pharmacology, the A50V and L381F variants had reduced responses for 5-HT and 5-CT.

3.2. Competitive antagonist pharmacology of 5-HT_{1A} receptor variants

To determine whether antagonist interactions with the 5-HT_{1A} receptor were affected by polymorphic variation, known receptor antagonists were tested for their abilities to inhibit responses induced by (+)8-OH-DPAT at the wild-type 5-HT_{1A} receptor, as well as variants A50V, M172I, K228R, G273D, and L381F (Table 3). The following rank order of competitive antagonist potencies was found for the wild-type 5-HT_{1A} receptor: WAY-100635 > methiothepin = spiperone > pindolol > sertindole. This rank order compares well with that previously reported using radioligand binding assays (http://pdsp.cwru.edu/PDSP.asp; [31]). In addition, when agonist concentration–response curves were obtained in the presence of single concentrations of antagonist, similar K_i values were obtained (data not shown). While the K228R and L381F receptor variants exhibited small decreases in agonist potency, they displayed K_i values similar to that of the wild-type receptor (Table 3, see SNP/WT). Similarly, the G273D receptor variant exhibited wild-type K_i values. In contrast, at the M172I receptor variant, for which agonists had a 3-fold increase in potency, WAY-100635 had a 10-fold increase in K_i.

Among the receptor variants tested, the A50V variant exhibited the most altered responses to competitive antagonists. The A50V variant exhibited a 60-fold increased K_i for pindolol and a 19-fold increase in K_i for methiothepin (Table 3, also see Fig. 3B). In addition, octoclothepin, which is structurally related to methiothepin, also exhibited increased K_i values at the A50V receptor variant (WT: $pK_i = 7.3 \pm 0.1$; A50V: $pK_i = 5.4 \pm 0.4$, for two experiments), as shown by rightward shifts of the concentration–response curves (Fig. 3C). In contrast, methiothepin was able to antagonize agonist responses of the L381F receptor variant with similar potency as to the wild-type receptor (Fig. 3D). Thus, the A50V receptor variant exhibited ligand-specific differences in responses to both agonists and competitive antagonists.

3.3. Radioligand binding studies

The altered functional responses exhibited by the A50V receptor variant could be at least partially due to disparate receptor levels. We therefore conducted radioligand binding experiments to determine relative receptor expression levels (Table 4). [^{3}H]WAY-100635 bound with high affinity

Table 3
5-HT_{1A} receptor competitive antagonist pharmacology

	WAY-100635			Methiothepin			Sertindole			Spiperone			Pindolol		
	pK_i	SNP/WT	N	pK_i	SNP/WT	N	pK_i	SNP/WT	N	pK_i	SNP/WT	N	pK_i	SNP/WT	N
WT	9.9 ± 0.2	1	12	8.0 ± 0.1	1	13	6.1 ± 0.2	1	6	7.8 ± 0.2	1	8	7.0 ± 0.1	1	10
A50V	9.4 ± 0.1**	3	6	6.7 ± 0.1***	19	7	5.9 ± 0.2	3	4	6.9 ± 0.3	12	2	5.3 ± 0.4*	60	4
M172I	10.9 ± 0.3**	0.1	3	8.3 ± 0.03	0.3	2	6.0 ± 0.2	1	2	8.1 ± 0.1	2	2	7.0 ± 0.1	1	2
K228R	9.7 ± 0.3	1	3	7.7 ± 0.1	2	3	6.4 ± 0.3	2	2	7.2 ± 0.2	5	3	6.8 ± 0.3	2	3
G273D	9.8 ± 0.1	2	3	7.9 ± 0.1	1	3	5.9 ± 0.5	3	2	7.5 ± 0.2	2	3	6.8 ± 01	2	3
L381F	9.9 ± 0.1	2	5	8.2 ± 0.3	1	3	6.4 ± 0.2	1	2	7.9 ± 0.3	1	2	7.2 ± 0.1	1	2

The competitive antagonist responses of 5-HT_{1A} wild-type and variant receptors were tested in R-SAT, using nine-point concentration–response curves performed in duplicate. Curves for the A50V, M172I, and K228R receptor variants were obtained in the presence of 10 μM, 7.5 μM, and 260 nM (+)8-OH-DPAT, respectively. All other curves obtained in the presence of 600 nM (+)8-OH-DPAT. The average pK_i (p$K_i = -\log K_i$) and standard error are reported for N experiments. The ratio of variant to wild-type K_i is also shown (SNP/WT), where the values for the wild-type and variant receptors were derived from the same experiments. Paired *t*-test indicates significant differences from wild-type values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and saturability to both wild-type and A50V variant receptors, with a receptor density (B_{max}) more than 10-fold lower for the A50V variant receptor ($P < 0.05$, paired *t*-test) (Table 4A). Similarly, the L381F receptor variant was expressed at levels 3-fold lower than the wild-type receptor (Table 4A). Both the A50V and L381F receptor variants exhibited binding affinities (pK_d) for the antagonist WAY-100635 that were similar to the wild-type receptor ($P > 0.2$, paired *t*-test). Unlike the A50V and L381F receptor variants, the receptor variants K228R and M172I displayed expression levels similar to that of the wild-type receptor (data not shown). No specific [^{3}H]WAY-100635 binding was detected for cells transfected with vector alone (data not shown). It should be noted, however, that it was necessary to increase the amount of DNA transfected (for all receptors tested) to 18-fold the amount used in R-SAT assays to obtain expression levels sufficient for detectable radioligand binding. Thus, the receptor expression levels in cells used for R-SAT are significantly lower than the values inferred by radioligand binding.

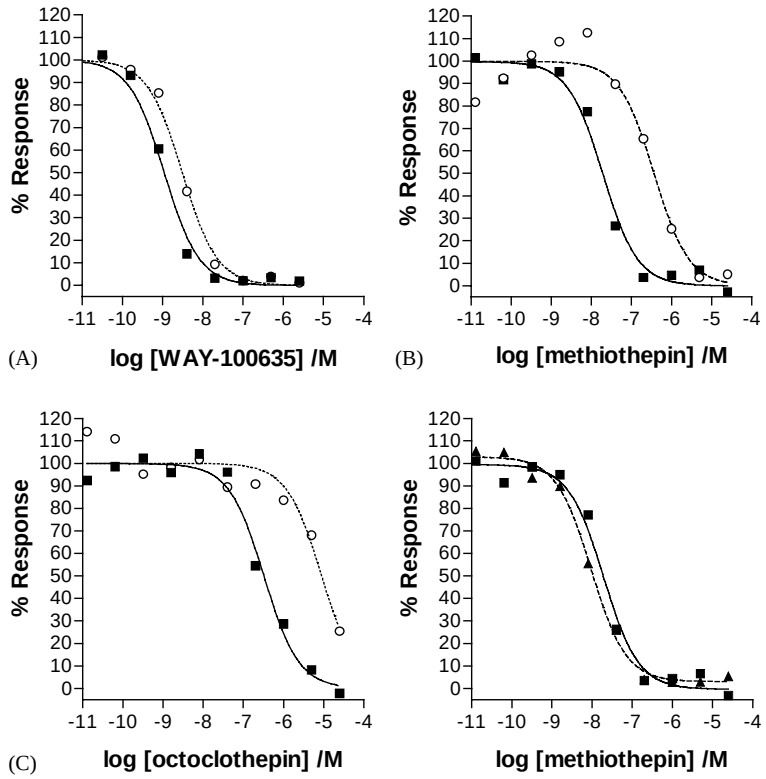

Fig. 3. The A50V variant receptor exhibits altered responses to methiothepin and octoclothepin. The competitive antagonist responses of 5-HT_{1A} wild-type and variant receptors were tested in R-SAT, using nine-point concentration–response curves performed in duplicate. Normalized responses from a representative experiment shown for 5-HT_{1A} wild-type (■), A50V (○), and L381F (▲) receptors are shown for WAY-100635 (A), methiothepin (B, D), and octoclothepin (C). All curves obtained in the presence of (+)8-OH-DPAT, as described in Table 3. For octoclothepin, average pK_i values were as follows for two separate experiments: WT, 7.3 ± 0.1, A50V, 5.5 ± 0.4. For other compounds, average pK_i values are reported in Table 3.

Table 4
Radioligand binding studies

	pK_d (M)	B_{max} (pmol/mg)	N
(A) Saturation binding studies			
WT	10.0 ± 0.2	1.0 ± 0.1	7
A50V	10.2 ± 0.1	0.07 ± 0.02	4
L381F	9.7 ± 0.03	0.3 ± 0.1	2
	Ligand	pK_i	
(B) Competition studies			
WT	5-HT	6.9 ± 0.2	3
A50V	5-HT	6.8 ± 0.3	2
WT	5-CT	8.0 ± 0.1	2
A50V	5-CT	8.1 ± 0.4	2
WT	(+)8-OH-DPAT	7.3 ± 0.1	2
A50V	(+)8-OH-DPAT	7.9 ± na	1
WT	Methiothepin	8.2 ± 0.3	2
A50V	Methiothepin	8.5 ± 0.2	2
WT	WAY-100635	9.7 ± 0.2	2
A50V	WAY-100635	9.6 ± 0.3	2

Radioligand binding experiments were performed for wild-type and variant receptors as described in Section 2. (A) *Saturation binding studies*: K_d and B_{max} values for [^{3}H]WAY-100635 binding to the wild-type and variant receptors are shown for N experiments. (B) *Competition studies*: Competition of [^{3}H]WAY-100635 with unlabeled 5-HT, methiothepin, or WAY-100635 for N experiments. Average pK_i values and standard errors for N experiments each performed in duplicate or triplicate. Radioligand binding studies shown performed with membranes harvested from transfected NIH/3T3 cells.

To determine whether the ligand-specific differences observed in the R-SAT assay resulted from decreased ligand binding affinity for the A50V receptor variant, competition binding studies with 5-HT and methiothepin were performed (Fig. 4B and C). All competition binding curves fitted well to a one binding site model. Both 5-HT and methiothepin were able to compete for [^{3}H]WAY-100635 binding to the A50V variant with similar binding affinity (K_i) as to the wild-type receptor (Table 4).

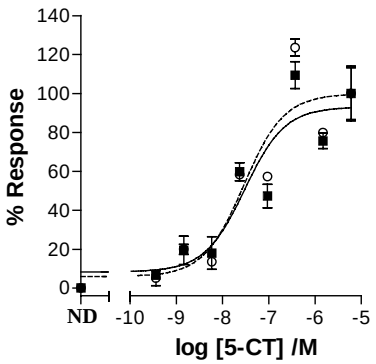

Fig. 4. 5-CT stimulates [^{35}S]-GTPγS binding in cells expressing wild-type and A50V receptors. Concentration–response curves for 5-CT are shown for wild-type (■) and A50V (○) receptor variants. Responses were normalized so that the maximum response at either receptor was 100%. Maximum stimulation of GTPγS binding to either receptor over basal GTPγS levels were approximately 3-fold (WT: from 1219 ± 187 cpm/8 μg protein to 408 ± 21 cpm/8 μg protein; A50V: from 1469 ± 234 cpm/11 μg protein to 503 ± 54/11 μg protein). Data presented are from a representative experiment carried out in triplicate. The experiment was repeated a second time with similar results.

To investigate whether relative expression level differences were specific to mouse NIH/3T3 cells, radioligand binding studies were also performed using transiently transfected African green monkey COS-7 cells. The B_{max} was estimated using a single dose (7.5 nM) of [^{3}H]WAY-100635. As observed for NIH/3T3 cells, in COS-7 cells, the A50V receptor was expressed at lower levels (0.88 ± 0.22 pmol/mg, N = 2) than the wild-type receptor (10.6 ± 0.15 pmol/mg, N = 2), whereas total receptor expression levels were much higher than in NIH/3T3 cells [33]. Similarly, the A50V receptor was expressed at lower levels (1.6 ± 1.0 pmol/mg, N = 1) compared to the wild-type receptor in human HEK-293T cells (4.2 ± 0.5 pmol/mg, N = 1). Thus, in three cell lines from three different species, the A50V receptor was expressed at lower levels than the wild-type receptor.

3.4. [^{35}S]-GTPγS binding studies

One explanation for the altered phenotype of the A50V receptor could be defective coupling to G proteins. To test this hypothesis, we assayed the ability of the A50V and wild-type receptors to bind [^{35}S]-GTPγS upon stimulation with 5-CT. In transiently transfected HEK293 cells, 5-CT stimulated [^{35}S]-GTPγS binding with a pEC_{50} of 7.48 ± 0.05 (N = 2) (Fig. 4). Similarly, in cells expressing A50V receptors, 5-CT stimulated [^{35}S]-GTPγS binding with a pEC_{50} of 7.39 ± 0.21 (N = 2). Similar efficacies were also observed for both receptors.

3.5. Pharmacology of the A50V 5-HT$_{1A}$ receptor variant

We profiled additional clinical compounds for functional responses at the wild-type and A50V variant receptors (Fig. 5). As shown for 5-HT (Fig. 2), the 5-HT analogue, α-methyl-hydroxytryptamine (α-Me-HT), also had no response at the A50V receptor variant and full agonism at the wild-type receptor (Fig. 5). However, like (+)8-OH-DPAT and buspirone (also see Table 1), oxymetazoline, ergometrine, rauwolscine, pergolide, ebalzotan, and terguride exhibited higher EC_{50} values for the A50V receptor variant, as depicted by the rightward shift of the corresponding concentration–response curves (Fig. 4). With regard to efficacy, oxymetazoline, ergometrine, buspirone, and terguride displayed near wild-type responses for the A50V receptor variant. Thus, while 5-HT and related compounds exhibited loss of response at the A50V receptor variant, all other agonists tested, including the partial agonist buspirone, exhibited lowered potencies, with no significant reduction of efficacy. Taken together, these results suggest that the A50V receptor variant does not respond to 5-HT and structurally-related compounds, and exhibits decreased potency with wild-type like efficacy for other 5-HT$_{1A}$ receptor agonists.

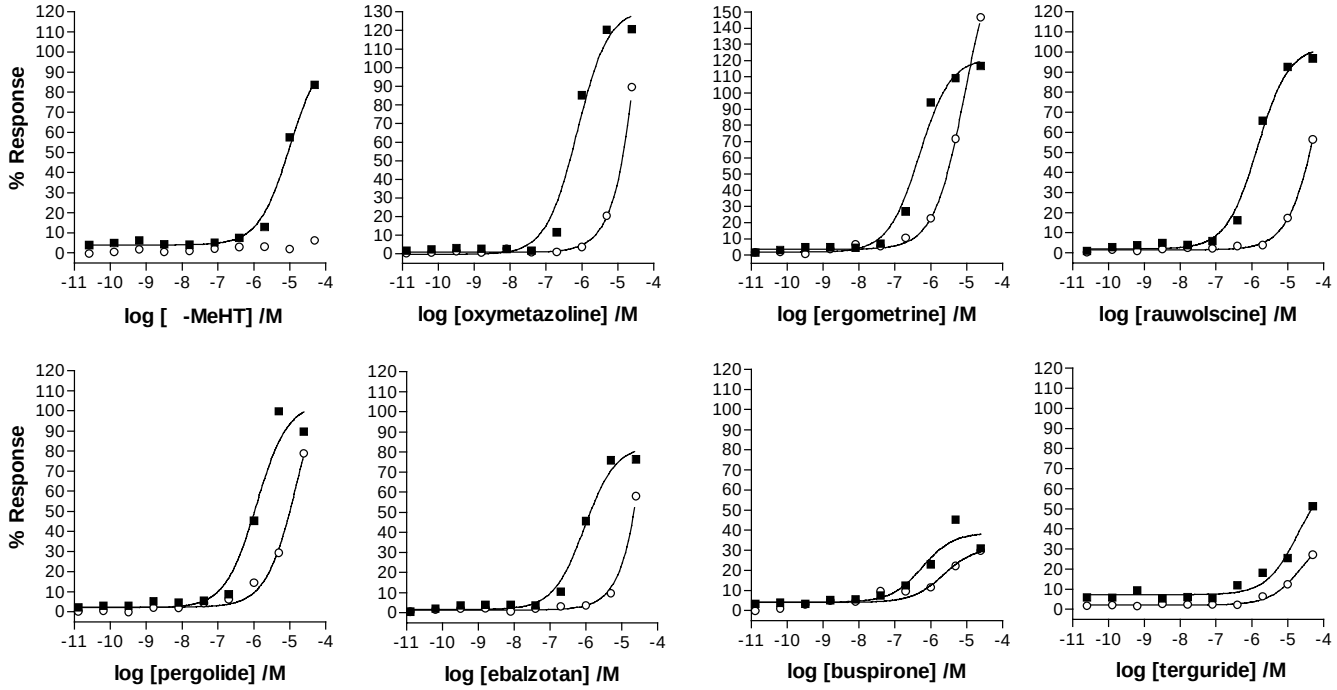

Fig. 5. The A50V variant receptor displays wild-type efficacies for many clinical compounds. Concentration–response curves for clinical and reference compounds at the 5-HT_{1A} wild-type (■) and A50V (○) variant receptors. Data were determined using R-SAT, with nine-point concentration–response curves performed in duplicate. For each compound, a representative concentration–response curve is shown. Responses were normalized so that the maximum response for (+)8-OH-DPAT at either receptor was 100%. Due to cellular toxicity at higher drug concentrations, maximal responses could not be obtained for rauwolscine, pergolide, and ebalzotan at the A50V receptor variant. The average pEC_{50} values for at least three experiments for the wild-type receptor are as follows: oxymetazoline, 6.6 ± 0.3; ergometrine, 6.1 ± 0.1; rauwolscine, 6.9 ± 0.1; pergolide, 5.4 ± 0.2; ebalzotan, 6.1 ± 0.1. All pEC_{50} values for the A50V receptor variant, as well as for α-Me-HT and terguride at the wild-type receptor, were lower than 5.5. The average efficacy values for the wild-type receptor are: oxymetazoline, 130 ± 24; ergometrine, 135 ± 14; rauwolscine, 91 ± 9; pergolide, 73 ± 13; ebalzotan, 65 ± 14. Efficacy values for the A50V receptor, as well as for α-Me-HT and terguride at the wild-type receptor, were not determined. Average pEC_{50} and efficacy values for buspirone are reported in Table 1.

4. Discussion

This study is the most extensive functional characterization of polymorphic variants in a GPCR to date. We report that out of 13 5-HT_{1A} polymorphic receptor variants tested, 11 variants exhibited at or near wild-type pEC_{50} and efficacy values to 5 known 5-HT_{1A} agonists. In addition, the K228R, G273D, and L381F receptor variants responded with wild-type affinity to 5 known 5-HT_{1A} competitive antagonists.

Our data extends previously reported observations of functional activity of 5-HT_{1A} receptor variants. We have previously shown that the R220L polymorphism does not change response to 5-HT in R-SAT [22]. Here we report that this receptor variant also has wild-type responses for other 5-HT_{1A} agonists. Our data also correlates with previous observations that (+)8-OH-DPAT binds to the G22S and I28V receptor variants with the same affinity as to the wild-type receptor, and that cells expressing the wild-type, G22S, or I28V receptors exhibit the same extent of 5-HT inhibition of cAMP synthesis [34,35].

The wild-type pharmacology of many of the 5-HT_{1A} receptor variants would predict a lack of physiological consequences *in vivo* in humans. Indeed, for most of these variants, no correlation with disease has been found, despite extensive association studies (e.g. [36]). For example, the I28V allele has been found to lack association with schizophrenia, bipolar affective disorder, and Tourette's syndrome [20]. Similarly, no association of G273D with either schizophrenia or suicide has been identified [28,37]. The R220L allele has also been reported to lack association with Tourette's syndrome [22]. In addition, the P16, I28V, and G273D alleles have been found to lack association with major depression [38]. Finally, no association between the P16L polymorphism and suicide or alcoholism has been found [27,37]. Thus, our results indicate that the A17P, G22S, A155G, M172I, P184L, K228R, and K418N variants are not likely to be disease-associated, and that efforts to identify disease-association might be better focused on those polymorphic 5-HT_{1A} variants which display altered biological properties *in vitro*.

One such altered variant, A50V, displayed altered pharmacological properties. No response to 5-HT and related compounds was detectable at A50V receptor variant, while all other agonists tested had decreased potencies of greater than 5-fold. Similarly, (+)8-OH-DPAT, lisuride, and buspirone were 2–3-fold lowered in potency at the L381F receptor variant, while 5-CT was 20-fold decreased in potency. The lowered potency of agonists at the A50V

and L381F receptor variants could be partially due to lowered receptor expression levels relative to the wild-type receptor. Classical receptor theory predicts that as receptor levels are depleted, decreases in potency occur [39]. Others have shown that lowered levels of 5-HT_{1A} receptor expression are correlated with decreased potencies for agonist-mediated intracellular calcium release [40].

However, lower expression alone cannot explain the lack of response of the A50V receptor variant for the endogenous ligand. 5-HT displayed full agonism at the wild-type receptor, but had no measurable response at the A50V receptor. Structurally similar compounds (5-CT, α-Me-HT) also had no detectable response at the A50V receptor. In contrast, all other agonists tested, including partial agonists, had similar efficacies at both the wild-type and A50V receptors. Moreover, varying the receptor levels by increasing or decreasing the amount of A50V DNA used for transfection did not alter the phenotype observed for 5-HT or 5-CT (data not shown). Thus, the A50V receptor appears to be altered specifically in response to 5-HT and structurally related compounds.

Consistent with a ligand-specific phenotype, the tricyclic 5-HT_{1A} antagonists, methiothepin and octoclothepin, as well as pindolol, displayed a reduced potency for the A50V receptor, while other antagonists tested displayed no change in potency compared to the wild-type receptor. Interestingly, the results from competitive binding experiments suggest that the binding affinity for either 5-HT or methiothepin was unchanged for the A50V receptor variant. Further studies indicated that that 5-CT stimulated binding of GTPγS is not affected by the A50V substitution. Thus, the ligand-specific differences in potency in R-SAT are likely not due to defects in the ligand binding site of the receptor or its ability to interact with G proteins.

We suggest that 5-HT or 5-CT induces a conformational state of the 5-HT_{1A} receptor not shared by other ligands and that the A50V polymorphism interferes with the generation of that conformational state. In support of such an idea, others have reported that 5-HT activation markedly differs from activation by other ligands. Malmberg and Strange have reported that 5-HT induces coupling to both $G_{i/o}$ and Gs proteins, while buspirone and (+)8-OH-DPAT induce relatively little coupling to Gs protein ([15]). Others have reported 5-HT induced Gs coupling ([41]). Interestingly, Sundaram *et al.* [42] have shown that 5-HT, 5-CT, and (+)8-OH-DPAT bind to receptor coupled to G proteins while lisuride binds to both free and coupled receptor. Indeed, evidence suggests that the 5-HT_{1A} receptor has additional ligand-specific conformational states. Mutation of the asparagine at codon 386 in TM7 results in a 100-fold loss of binding affinity for pindolol and other aryloxypropanolamines, but only a 3–5-fold lower affinity for other antagonists, such as methiothepin [14].

A ligand-specific defect was unexpected for the A50V polymorphism, which is located in TM1. However, sequence alignment of the 5-HT_{1A} receptor with the three-dimensional structure of rhodopsin [43] indicated that A50V is likely to be located near the middle of TM1 (data not shown). Interestingly, this region is in close proximity to the kinked irregular region of TM7, which is important for monoamine receptor activation [44]. Notably, the L381F polymorphism, which also displayed reduced responses to 5-HT and 5-CT, is located in the middle of TM7. Similarly, the muscarinic M1 receptor has recently been shown to have an ectopic activation domain comprised of parts of TM1 and TM7 [45]. Thus, our data suggests the existence of ligand-specific conformational states for the 5-HT_{1A} receptor, and a previously undescribed role for TM1 in mediating responses to 5-HT. An alternative explanation for the A50V phenotype could be the effect of various regulatory pathways, which might be easily observable in a long-term intact cell-based assay such as R-SAT, but not detectable in assays of membranes. We also cannot completely rule out the effects of the differences in G proteins or receptor levels in the cell lines used. Further experiments exploring the molecular mechanisms underlying the altered phenotype of the A50V receptor variant are warranted.

In addition to altered responses to 5-HT, the A50V receptor variant was also found to display 10-fold lower expression levels in two different cell types. Polymorphisms within TM1 of other GPCRs also result in observed defects in receptor expression, which can lead to disease (reviewed in [46]). For example, a C109R substitution in TM1 of the endothelin B receptor results in defects in processing and cell surface transport, resulting in a phenotype associated with Hirschsprung's disease.

Thus, the A50V and L381F polymorphic variant 5-HT_{1A} receptors are likely to result in altered physiology due to their lowered receptor levels and ability to respond to endogenous ligand. Predictions of disease states can be made based on studies in animals with lowered amounts of 5-HT_{1A} receptor. Decreases in 5-HT_{1A} receptor mRNA levels in the brain have been demonstrated in rat models of chronic stress as well as human suicide victims with a history of major depression [47]. The most compelling reports are from three genetic strains of mice null for the 5-HT_{1A} receptor; all three exhibit elevated anxiety-related behaviors (reviewed in [5]). This phenotype is reversible by expression of the gene in the postnatal period but not in the adult, indicating that a functional 5-HT_{1A} receptor is necessary during development to establish normal anxiety-like behavior [48]. In addition, in behavioral tests of depression, 5-HT_{1A} receptor null mice exhibit behaviors similar to wild-type mice that have been dosed with antidepressants. Moreover, these mice show learning and memory deficits [6]. Mice with a single 5-HT_{1A} receptor allele display phenotypes intermediate between wild-type and null mice (reviewed in [5]).

Based on these studies, human individuals harboring A50V or L381F 5-HT_{1A} receptor variants would be predicted to exhibit a heightened susceptibility to anxiety

disorders or depression, coupled with learning or memory problems. As suggested for 5-HT_{1A} receptors in mice, a nonresponsive 5-HT_{1A} receptor might lead to reduced autoreceptor regulation of 5-HT synthesis and release, and thus a hyperactive serotonergic system [5]. However, unlike the mice null for the 5-HT_{1A} receptor, individuals with these polymorphisms would be expected to respond to 5-HT_{1A} agonists such as buspirone. Such individuals might exhibit a supersensitive response, with unregulated suppression of 5-HT synthesis and release, leading to idiosyncratic clinical responses to drugs with 5-HT_{1A} receptor activity.

The A50V and L381F receptor variants were identified from a patent in which no data on allele frequencies or DNA analysis were disclosed [48]. We genotyped over 200 total individuals from Caucasian, Asian, and African-American ethnic groups to detect allele frequencies for these polymorphisms (data not shown). We were unable to detect either polymorphism in this initial survey, suggesting that they either do not exist in humans or are relatively rare. However, the screening procedure used in the original patent is estimated to result in >90% confidence that the variants actually exist in humans [17]. Moreover, most cSNPs identified display an allele frequency below 5% [17,18]. Indeed, for the 13 polymorphisms studied here, the highest reported allele frequency is 3.7% within a group of Japanese individuals (Table 1). Thus, it is likely that the A50V and L381F polymorphisms do exist, but are rare. The described functional alterations caused by the A50V and L381F 5-HT_{1A} receptor polymorphisms argue for a large-scale genotyping effort to determine their prevalence in the human population, as well as their possible association with human affective disease and treatment responses.

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