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# Site-directed mutagenesis of a single residue changes the binding properties of the serotonin 5-$HT_2$ receptor from a human to a rat pharmacology

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Mesulergine displays approximately 50-fold higher affinity for the rat 5-$HT_2$ receptor than for the human receptor. Comparison of the deduced amino acid sequences of cDNA clones encoding the human and rat 5-$HT_2$ receptors reveals only 3 amino acid differences in their transmembrane domains. Only one of these differences (Ser → Ala at position 242 of TM5) is near to regions implicated in ligand binding by G protein-coupled receptors. We investigated the effect of mutating $Ser^{242}$ of the human 5-$HT_2$ receptor to an Ala residue as is found in the rat clone. Both [$^3$H]mesulergine binding and mesulergine competition of [$^3$H]ketanserin binding showed high affinity for rat membranes and the mutant human clone but low affinity for the native human clone, in agreement with previous studies of human postmortem tissue. These studies suggest that a single naturally occurring amino acid change between the human and the rat 5-$HT_2$ receptors makes a major contribution to their pharmacological differences.

Serotonin 5-$HT_2$ receptor; Site-directed mutagenesis; G protein-coupled receptor; Mesulergine

## 1. INTRODUCTION

Many serotonin receptor subtypes have now been cloned from more than one species. This information allows one to examine the properties of isolated receptor subtypes to determine which amino acid sequences impart species-specific pharmacology to various receptors. In many cases, such as the human [1] and rat [2] serotonin 5-$HT_{1A}$ receptors, or human and dog 5-$HT_{1D\alpha}$ receptors [3] similar pharmacological binding properties were reported for transfected clones obtained from either species. In other cases, dramatic pharmacological differences exist for species homologues of the same receptor. For example, the 5-$HT_{1B}$ receptor has been shown to be the rat analog of the human 5-$HT_{1D\beta}$ receptor [4], yet these two homologous genes, which are 92% identical in overall amino acid sequence, display markedly different pharmacological binding properties.

In this report we studied the species differences between the rat and human 5-$HT_2$ receptors. Certain ligands, most notably mesulergine [5, 6], bind with different affinities to the rat 5-$HT_2$ receptor compared to the human 5-$HT_2$ receptor. We cloned the human 5-$HT_2$ receptor and expressed this receptor, and a mutant receptor with a single amino acid substitution, in mammalian cell lines. Comparison of mesulergine binding to these preparations and to native rat and human cortical membranes suggests that the different binding properties of rat and human 5-$HT_2$ receptors are due to changes at a single amino acid residue.

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## 2. MATERIALS AND METHODS

### 2.1. *Isolation of a human 5-$HT_2$ receptor cDNA clone*

The rat 5-$HT_{1C}$ gene was used as a probe for the isolation of the human 5-$HT_2$ gene. The rat 5-$HT_{1C}$ receptor cDNA was obtained by screening a rat choroid plexus cDNA library (in λgt10) using oligomers complementary to the rat 5-$HT_{1C}$ published sequence [7]. The cDNA containing the entire coding region was verified by sequencing and subcloned in the RNA transcription vector pGEM1 (Promega-Biotech; Madison, WI).

A human brain stem cDNA library provided by Stratagene (La Jolla, CA) was screened using a randomly primed probe made by reverse transcription of Sp6 generated RNA made from the rat 5-$HT_{1C}$ receptor gene. Hybridization was performed at 60°C in a solution containing 5× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 2× Denhardt's (0.02% polyvinyl-pyrrvolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 100 mM sodium phosphate pH 8.0, 25mM EDTA, 0.1% sodium dodecyl sulfate (SDS) and 100μg/ml of sonicated salmon sperm DNA. The filters were washed at 60°C in 0.1× SSC containin 0.1% SDS and exposed at −70°C to Kodak XAR film in the presence of an intensifying screen. Lambda phage hybridizing to the probe were plaque purified and plasmid DNA (in the vector pBluescript) self-excised from the lambda vector (lambda ZAP).

One clone from the screening of the human brainstem library displayed high homology to the published sequence of a rat 5-$HT_2$ receptor clone [8]. This clone did not contain the entire coding region.

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$^{32}$P-labelled oligomers complementary to the 5' end of this clone were used to screen a human temporal cortex cDNA library (Stratagene, La Jolla, CA). Four independent clones were isolated, and one, designated 6B, containing the entire coding region was selected for further studies. The coding region from 6B was subcloned into the mammalian expression vector pMO5. The human 5-HT$_2$ receptor has also been cloned by Saltzman et al. [9]. Our sequence is essentially identical to this published sequence.

2.2. *DNA sequencing*

Nucleotic sequence analysis was done by the Sanger dideoxy nucleotide chain-termination method [10] on denatured double-stranded plasmid templates [11] using Sequenase (US Biochemical Corp., Cleveland, OH).

2.3. *Mutagenesis*

Mutagenesis was carried out using PCR primers in which the desired mutation was incorporated into the sequence of the primer. The methods used are essentially the same as that described by Dulau et al. [12]. The region of DNA mutated was flanked by the Sp6 and T7 primers in the vector pGEM1. The mutant fragment (containing the mutating primer) was ligated along with its corresponding unmutated fragment into the expression vector pMO5. The mutation was confirmed by sequencing.

2.4. *Receptor expression in transfected mammalian cells*

To verify that the cDNA encoded a functional 5-HT$_2$ receptor, Cos-7 cells were transiently transfected using the DEAE-dextran technique [13]. Subsequently, stable cell lines were produced by cotransfection of the plasmid PM05 containing the 5-HT$_2$ gene with the plasmid PGCneo (which contains the aminoglycoside phosphotransferase gene) into murine fibrolast L-M(TK$^-$) cells (American Tissue Type Collection, Rockville, MD, Cell Line CCL 1.3). These stable transfections were accomplished using the calcium phosphate technique (Specialty Media; Lavellette, NJ). Stable cell lines were not established for the mutant clones. Cells were selected by resistance to the antibiotic G-418 (Gibco; 1 mg/ml) as previously described [14]. [$^3$H]Ketanserin binding was used to monitor 5-HT$_2$ receptor gene expression in these clones (see below). Three out of sixteen clones displayed specific binding of [$^3$H]ketanserin. No significant specific binding was detected on pseudotransfected or non-transfected L-M(TK$^-$) or Cos-7 cells at 1 or 2 nM [$^3$H]ketanserin (data not shown).

2.5. *[$^3$H]Ketanserin binding assays*

[$^3$H]Ketanserin was used as the radioligand to detect expression of the 5-HT$_2$ gene product in membrane fractions isolated form transfected cells. Radioligand binding assays were done as previously described [15] with slight modification. Briefly, binding assays were conducted in 96-well microliter plates, in a total volume of 0.25 ml of buffer (50 mM Tris-HCl, 0.5 mM EDTA, 10 mM $MgSO_4$, 0.1% ascorbate and 10 $\mu$M pargyline, pH 7.6) containing 1–2 nM [$^3$H]ketanserin (64.9 Ci/mmol; DuPont-NEN, Wilmington, DE). Mianserin at 1.0 $\mu$M was used to define non-specific binding. Assays were initiated by the addition of 50 $\mu$l of membrane homogenate (20–40 $\mu$g protein/well). After a 20 min incubation at 37°C the assay was terminated by filtration. Specific binding was 95% of total binding for the transiently transfected cells and 85% for the stable cell lines at 1 nM [$^3$H]ketanserin. Protein was determined by the method of Bradford [16].

Competition studies for [$^3$H]ketanserin binding were performed by adding increasing concentrations of test drug to the reaction. 10–12 ligand doses were used and spanned the expected $IC_{50}$ range as determined from literature values. Data were analyzed by computer-assisted analysis (Accufit Competition; Lundon Software; Chagrin Falls, OH). $IC_{50}$ values were converted to $K_i$ values using the Cheng-Prusoff equation [17].

2.6. *[$^3$H]Mesulergine binding*

To determine whether the clones human 5-HT$_2$ receptor bound [$^3$H]mesulergine with high affinity, saturation studies were performed according to the method of Closse [18]. [$^3$H]Mesulergine (84 Ci/mmol; Amersham) was tested at concentrations between 2.0 and 400 nM for the human 5-HT$_2$ clone (h5-HT2) and between 0.02 and 3.0 nM for the mutant 5-HT$_2$ clone (h5-HT2mu) and rat cortical membranes. Incubations were performed at 37°C for 60 min and the reaction was terminated by rapid filtration as above.

2.7. *Drugs and chemicals*

Mianserin hydrochloride and mesulergine were purchased from Research Biochemicals, Natick, MA. All other chemicals were of highest purity, purchased from commercial sources.

## 3. RESULTS

### 3.1. *Pharmacological properties of the transfected human 5-HT$_2$ receptor*

Membranes collected from cells stably transfected with a human 5-HT$_2$ receptor cDNA (h5-HT2) were found to bind [$^3$H]ketanserin saturably, reversibly, and with high affinity, as previously reported [15]. Compounds reported to differ in affinity for the human vs the rat 5-HT$_2$ receptor were selected for competition studies. Apparent inhibition constants ($K_i$ values) from the analysis of competition experiments is shown in Table I, in comparison to values obtained in human and rat cortex. These data indicate that the cloned gene encoded a 5-HT$_2$ receptor and that the pharmacological properties of this cloned human receptor assayed in a heterologous expression system match that reported for the 5-HT$_2$ receptor assayed in homogenates of human post-mortem cortical tissue.

The most significant difference between the binding properties of human and rat 5-HT$_2$ receptors has been reported to be their affinities for mesulergine [5]. Mesulergine competition of [$^3$H]ketanserin binding to the

Table I

Competition experiment for [$^3$H]ketanserin labeled human 5-HT$_2$ receptor stably transfected into L-M (TK$^-$) cells as compared with published values for the native human and rat 5-HT$_2$ receptor

| Drug | $K_i$ (nM) | $K_i$ (nM)[a] | |
|---|---|---|---|
| | Human | Human cortex | Rat cortex |
| Spiperone | 1.1 ± 0.07 | 0.92[b] | 0.29[b] |
| 5-HT | 598 ± 52 | 174 | 79 |
| Mesulergine | 129 ± 7.9 | 151 | 4.7 |
| Ritanserin | 1.1 ± 0.16 | 1.3 | 7.2 |
| Cyproheptadine | 2.9 ± 0.10 | 6.3 | 1.8 |
| Methysergide | 3.3 ± 0.50 | 5.2[b] | 0.54[b] |
| (+)-Butaclamol | 2.3 ± 0.38 | 1.2[b] | 2.4[c] |
| 5-CT | 2,866 ± 228 | 8,128 | 21,878 |
| Quipazine | 1,771 ± 85 | 3,802 | 1,549 |
| (−)-Butaclamol | 3,881 ± 868 | 510[b] | 1,831[c] |
| 5-MT | 513 ± 29 | 295 | 240 |

Each value is the mean ± S.E.M. of at least 3 independent trial runs in triplicate.

[a] Hoyer et al. [6].

[b] Schotte et al. [20].

[c] Lyon et al. [21].

| Species | Receptor | Transmembrane V |
|---|---|---|
| h | 5-HT$_{1A}$ | HGYTIYSTFGAFYIPLLLMLVLYG |
| r | 5-HT$_{1B}$ | VLYTVYSTVGAFYLPTLLLIALYG |
| r | 5-HT$_{1C}$ | PNFVLIGSFVAFFIPLTIMVITYF |
| h | 5-HT$_{1D\alpha}$ | ISYTIYSTCGAFYIPSVLLIILYG |
| r | 5-HT$_{1D\alpha}$ | ISYTIYSTCGAFYIPSILLIILYG |
| h | 5-HT$_{1D\beta}$ | ILYTVYSTVGAFYFPTLLLIALYG |
| h | 5-HT$_{1E}$ | VIYTIYSTLGAFYIPLTLILILYY |
| hm | 5-HT$_2$ | DNFVLIGSFVAFFIPLTIMVITYF |
| r | 5-HT$_2$ | DNFVLIGSFVAFFIPLTIMVITYF |
| h | 5-HT$_2$ | DNFVLIGSFVSFFIPLTIMVITYF |
| h | $\alpha_{1A}$ | AGYAVFSSVCSFYLPMAVIVVMYC |
| h | $\alpha_{1B}$ | PFYALFSSLGSFYIPLAVILVMYC |
| h | $\alpha_{1C}$ | PGYVLFSALGSFYLPLAIILVMYC |
| h | $\alpha_2$C10 | KWYVISSCIGSFFAPCLIMILVYV |
| h | $\alpha_2$C2 | AWYILASSIGSFFAPCLIMILVYL |
| h | $\alpha_2$C4 | TWYILSSCIGSFFAPCLIMGLVYA |
| h | $\beta_1$ | RAYAIASSVVSFYVPLCIMAFVYL |
| h | $\beta_2$ | QAYAIASSIVSFYVPLVIMVFVYS |
| h | $\beta_3$ | MPYVLLSSSVSFYLPLLVMLFVYA |
| h | $D_1$ | RTYAISSSVISFYIPVAIMIVTYT |
| h | $D_2$ | PAFVVYSSIVSFYVPFIVTLLVYI |
| h | $D_3$ | PDFVIYSSVVSFYVPFGVTVLVYA |
| h | $D_4$ | RDYVVYSSVCSFFLPCPLMLLLYW |
| h | $D_5$ | RTYAISSSLISFYIPVAIMIVTYT |

Fig. 1. Amino acid sequence of the putative fifth transmembrane domain of serotonin, adrenergic and dopamine receptors. Receptor subtypes are shown from the following species: h, human; r, rat; hm, hamster. Comparison of residue 242 (Ser$^{242}$ of the human 5-HT$_2$ receptor is shown by shading to the corresponding residue found in other serotonin receptors (alanine) and to adrenergic and dopamine receptors (serine).

cloned human receptor (h5-HT2) displayed low affinity, in agreement with previous studies in human cortex (Table I). We also used [$^3$H]mesulergine as a radioligand to directly measure the affinity of this drug for the cloned human and native rat 5-HT$_2$ receptors. In contrast to the native rat 5-HT$_2$ receptor, which exhibits high affinity binding of [$^3$H]mesulergine, we were unable to detect any high affinity binding to the cloned human receptor. Using increasing concentrations of [$^3$H]mesulergine ranging from 2.0 to 400 nM, only a low affinity, high capacity binding site was detected.

### 3.2. *Sequence comparison*

We were interested in determining which specific amino acid residues were responsible for the divergent pharmacology between the rat and human 5-HT$_2$ receptors. We reasoned, by analogy to the model for ligand binding to adrenergic receptors [19], that the amino extracellular and carboxy cytoplasmic sequences of the rat and human 5-HT$_2$ receptors were unlikely to significantly affect ligand binding specificities. The transmembrane domains of these receptors exhibit only 3 amino acid sequence differences: at residue 82 (Thr → Ala), at residue 150 (Ile → Val), and at residue 242 (Ala → Ser). Residue 242 appeared to be significant because it occurred in a transmembrane region (TM 5) at a site previously implicated in ligand binding to adrenergic receptors [19], and involved a change from a non-polar amino acid to a polar amino acid. Furthermore, this specific position contains a Ser residue in both the human 5-HT$_2$ receptor and in all adrenergic and dopamine receptors reported to date (Fig. 1). In these catecholamine receptors, this serine residue is believed to form a hydrogen bond to a ring hydroxyl of the catechol neurotransmitter [19]. Since the indole ring of serotonin does not contain a ring hydroxyl in the corresponding position, the role of this serine residue in the human 5-HT$_2$ receptor is unclear. Nevertheless, this serine is located near to the ligand binding site and was expected to contribute to its ligand binding properties.

### 3.3. *Mutagenesis results*

We made a site-specific mutation at residue 242 of the human serotonin 5-HT$_2$ receptor to convert the human residue (Ser) to the residue found in the rat receptor (Ala). The nucleotide and deduced amino acid sequence comparison between the rat, human (h5-HT2) and mutant receptor (h5-HT2mu) genes is depicted in Fig. 2. The human 5-HT$_2$ mutant receptor in which Ser$^{242}$ was

**Sequence comparison of 5-HT$_2$ receptor sequence in transmembrane V.**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **h5-HT2 (NORMAL HUMAN)** | S<br>TCT | F<br>TTT | V<br>GTG | S<br>TCA | F<br>TTT | F<br>TTC | I<br>ATT | P<br>CCC | L<br>TTA |
| **h5-HT2mu (MUTANT HUMAN)** | S<br>TCT | F<br>TTT | V<br>GTG | A<br>GCA | F<br>TTT | F<br>TTC | I<br>ATT | P<br>CCC | L<br>TTA |
| **r5-HT2 (NORMAL RAT)** | S<br>TCT | F<br>TTT | V<br>GTG | A<br>GCA | F<br>TTT | F<br>TTC | I<br>ATC | P<br>CCC | L<br>CTA |

Fig. 2. Nucleotide and deduced amino acid sequences of the human, rat and mutant 5-HT$_2$ receptors in transmembrane domain V. h5-HT2=normal human receptor sequence; r5-HT2=normal rat receptor sequence; h5-HT2mu=human receptor sequence mutated from Ser$^{242}$ to Ala$^{242}$.

Table II

Kinetic parameters of the [$^3$H]mesulergine binding to the cloned human 5-HT$_2$ receptor expressed in L-M(TK$^-$) cells, comparison with the human mutant 5-HT$_2$ receptor expressed in Cos-7 cells and to the native rat 5-HT$_2$ receptor

| Membranes | $K_d$ (nM) | $B_{max}$ (pmol/mg protein) |
|---|---|---|
| Human 5-HT$_2$ receptor (h5-HT2) | 174 ± 26 | 5.7 ± 0.82 |
| Human 5-HT$_2$ mutant (h5-HT2mu) | 2.6 ± 1.1 | 1.6 ± 0.81 |
| Rat cortex | 3.0 ± 0.96 | 1.1 ± 0.23 |

Values for the dissociation constant ($K_d$) and site density ($B_{max}$) are reported. Each value is the mean ± S.E.M. for 3 independent trial runs in triplicate. Saturation curves, each containing 12 different concentrations of radioligand, were run in parallel. Parameters were calculated by computer-assisted non-linear regression analysis (Accufit, Lundon Software).

changed to Ala (h5-HT2mu) displayed at 60-fold higher affinity for [$^3$H]mesulergine than the native human receptor (h5-HT2) (Table II). The affinity of mesulergine for the mutant receptor was very close to that determined for rat cortical membranes in this study (Table I). Similarly, when unlabelled mesulergine was used to compete for [$^3$H]ketanserin binding, the human 5-HT$_2$ clone h5-HT2 displayed approximately 30-fold lower affinity for mesulergine compared to the mutant h5-HT2mu clone or the rat cortical membranes. Mean $K_i$ values (apparent dissociation constants) for unlabelled mesulergine were as follows: 5-HT$_2$ clone: 129 ± 7.9 nM ($n$=4); mutant h5-HT2mu clone: 4.2 ± 2.0 nM ($n$=3); rat cortex: 4.5 ± 0.22 nM ($n$=3). These data indicate that changing a single key amino acid residue of the human 5-HT$_2$ clone to the residue contained in the rat clone is sufficient to change its pharmacological binding properties for mesulergine to that of the rat receptor, even though all other amino acid differences between the clones remain unaltered. This suggests that Ser$^{242}$ is the most important determinant of rat vs. human pharmacological binding properties in this receptor.

## 4. DISCUSSION

We have cloned and expressed the gene encoding a human 5-HT$_2$ receptor. Binding studies on transfected cell membranes support the interpretation that this clone is a 5-HT$_2$ receptor, with properties that match previous binding studies on post-mortem human cortical tissues. Several different chemical classes of compounds displayed affinities for the transfected human receptor which were close to human cortex values and clearly different from values in rat cortical membranes. This species difference in 5-HT$_2$ receptor binding properties is particularly evident for mesulergine, which displays high affinity for the rat but not for the human receptor [6]. As has been reported for the native human 5-HT$_2$ receptor [5], we observed that [$^3$H]mesulergine could not be used to label the transfected human receptor, and that unlabeled mesulergine displayed low affinity in competition binding experiments. It has been speculated that species-specific drug binding differences such as this could be due to factors ranging from the lipid composition of the membrane environment of the receptor, to possible post-mortem degradation. Our data strongly suggest that the species differences in pharmacological binding properties of the human vs. rat 5-HT$_2$ receptor arise from amino acid differences in the protein rather than from differences in its membrane environment or cellular processing.

The human and rat 5-HT$_2$ receptor sequences are 92% identical at the amino acid level. In the transmembrane domains, the regions which appear to contain the ligand binding site [19] only three amino acid differences occur between the rat and human receptors. Our study shows that the single transmembrane amino acid difference that occurs within a proposed ligand binding region (Ser$^{242}$) is a critical determinant of the species-specific pharmacological binding properties of the 5-HT$_2$ receptor. We have found that mutating this residue of the human receptor to the amino acid found in the rat sequence (from Ser → Ala) alters mesulergine binding such that the mutated human 5-HT$_2$ receptor now resembles the rat 5-HT$_2$ receptor. The other 40 amino acids which differ between the rat and human receptors do no appear to be critical in determining the species-specific binding properties of the 5-HT$_2$ receptor for mesulergine. The fact that this single amino acid substition increased rather than decreased the affinity of the receptor for mesulergine seems to rule out nonspecific effects (such as a delocalized conformational change) as a mechanism and stongly suggests that this residue forms a component of the serotonin and drug binding sites.

In comparing our results to the original ligand binding model of Strader et al. [19], it is interesting to note that the presence of a Ser residue at position 242 of the human 5-HT$_2$ receptor would have led to the prediction that the human 5-HT$_2$ receptor should bind catechols with high affinity, since this residue is in the position proposed for interactions with the ring hydroxyl of a catechol neurotransmitter. We were unable to detect any high affinity binding of norepinephrine to the transfected human 5-HT$_2$ receptor (data not shown). In addition, the pharmacological binding properties of the human 5-HT$_2$ receptor clearly distinguish it from catecholamine receptors. This suggests that the presence of a Ser residue at position 242 (such as is found in all catecholaminergic receptors) is not of itself a sufficient condition to impart high affinity for catecholaminergic ligands, and that additional factors, such as critical conformational positioning of this residue, may play important roles in the ligand binding properties of the receptor. Recently, a group of insect serotonin receptors were reported which further illustrate this principal.

The dro-1 receptor, like the human 5-$HT_2$ receptor, contains a Ser residue at an analagous position in TM5, while the dro-2A and dro-2B receptors contain a homologous residue, Thr, in this position [22]. All three of these *Drosophila* serotonin receptors display serotonergic rather than catecholaminergic binding and response properties.

In summary, our results suggest that residue $Ser^{242}$ of the serotonin 5-$HT_2$ receptor forms a component of the ligand binding site and exerts a strong influece on drug binding affinities. The fact that the human 5-$HT_2$ receptor displays human pharmacological binding properties when expressed in a rodent cell line suggests that the amino acid sequence of a receptor is more important than the environment in which a receptor is more important than the environment in which a receptor is expressed in determining drug binding affinities. Finally, these site-specific mutagenesis results imply that the pharmacological differences between the rat and human receptors are due to a very small change in the sequence, most of which can probably be attributed to a single amino acid.

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