(51) International Patent Classification 6: C07K 14/705, C12N 15/10, 15/12

(21) International Application Number: PCT/US98/03991
(22) International Filing Date: 27 February 1998 (27.02.98)

(30) Priority Data:
   60/039,465 27 February 1997 (27.02.97) US
   60/061,268 7 October 1997 (07.10.97) US

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(54) Title: CONSTITUTIVELY ACTIVATED SEROTONIN RECEPTORS

(57) Abstract

Mutations have been discovered in mammalian G protein–coupled serotonin 5-HT2A and 5-HT2C receptors which render the mutated receptors constitutively active. An alignment methodology based on the highly conserved sixth transmembrane domain has been discovered for the monoamine receptors which accurately predicts the amino acid position in the third intracellular loop which, when mutated, produces constitutive activation of the receptor. Constitutive activation of the G protein–coupled serotonin receptors has been shown by the demonstration of an enhanced affinity and potency for serotonin, by increased basal activity of the second messenger system in the absence of agonist, and by reduction of the basal second messenger activity by inverse agonists.
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CONSTITUTIVELY ACTIVATED SEROTONIN RECEPTORS

The benefit of U.S. Provisional Application No. 60/039,465 filed February 27, 1997, and U.S. Provisional Application No. 60/061,268 filed October 7, 1997 is claimed for this application.

BACKGROUND OF THE INVENTION

Field Of The Invention

The present invention relates generally to the field of transmembrane receptors, more particularly to seven segment transmembrane G protein-coupled receptors, and most particularly to the serotonin (5-HT) receptors. Through genetic mutational techniques, the amino acid sequences of the native 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have been modified so that the receptors exist in a constitutively activated state exhibiting both a greater response to agonists and a coupling to the G Protein second messenger system even in the absence of agonist. A method for constitutively activating G protein-coupled 5-HT receptors in general is also disclosed.

Description Of Related Art

The research interest in G protein-coupled cell surface receptors has exploded in recent years as it has been apparent that variants of these receptors play a significant role in the etiology of many severe human diseases. These receptors serve a diverse array of signalling pathways in a wide variety of cells and tissue types. Indeed, over the past 20 years, G protein-coupled receptors have proven to be excellent therapeutic targets with the development of several hundred drugs directed towards activating or deactivating them.

G protein-coupled receptors form a superfamily of receptors which are related both in their structure and their function. Structurally the receptors are large macromolecular proteins embedded in and spanning the cell membrane of the receiving cell and are distinguished by a common structural motif. All the receptors have seven domains of between 22 to 24 hydrophobic amino acids forming seven $\alpha$ helixes arranged in a bundle which span the cell membrane substantially perpendicular to the cell membrane. The transmembrane helixes are joined by chains of hydrophilic amino acids. The amino terminal and three connecting chains extend into the extracellular environment while the carboxy
terminal and three connecting chains extend into the intracellular environment. Signalling molecules are believed to be recognized by the parts of the receptor which span the membrane or lie on or above the extracellular surface of the cell membrane. The third intracellular loop joining helixes five and six is thought to be the most crucial domain involved in receptor/G protein coupling and responsible for the receptor selectivity for specific types of G proteins.

Functionally, all the receptors transmit the signal of an externally bound signalling molecule across the cell membrane to activate a heterotrimeric transducing protein which binds GDP (guanosine diphosphate). Upon activation, the bound GDP is converted to GTP (guanosine triphosphate). The activated G protein complex then triggers further intracellular biochemical activity. Different G proteins mediate different intracellular activities through various second messenger systems including, for example, 3’5’-cyclic AMP (cAMP), 3’5’-cyclic GMP (cGMP), 1,2-diacylglycerol, inositol 1,4,5-triphosphate, and Ca²⁺. Within the human genome, several hundred G protein-coupled receptors have been identified and endogenous ligands are known for approximately 100 of the group. While the seven transmembrane motif is common among the known receptors, the amino acid sequences vary considerably, with the most conserved regions consisting of the transmembrane helixes.

Binding of a signalling molecule to a G protein-coupled receptor is believed to alter the conformation of the receptor, and it is this conformational change which is thought responsible for the activation of the G protein. Accordingly, G protein-coupled receptors are thought to exist in the cell membrane in equilibrium between two states or conformations: an "inactive" state and an "active" state. In the "inactive" state (conformation) the receptor is unable to link to the intracellular transduction pathway and no biological response is produced. In the altered conformation, or "active" state, the receptor is able to link to the intracellular pathway to produce a biological response. Signalling molecules specific to the receptor are believed to produce a biological response by stabilizing the receptor in the active state.

Discoveries over the past several years have shown that G protein-coupled receptors can also be stabilized in the active conformation by means other than
binding with the appropriate signal molecule. Four principal methods have been identified: 1) molecular alterations in the amino acid sequence at specific sites; 2) stimulation with anti-peptide antibodies; 3) over-expression in in vitro systems; and 4) over-expression of the coupling G proteins. These other means simulate the stabilizing effect of the signalling molecule to keep the receptor in the active, coupled, state. Such stabilization in the active state is termed "constitutive receptor activation".

Several features distinguish the constitutively activated receptors. First, they have an affinity for the native signalling molecule and related agonists which is typically greater than that of the native receptors. Second, where several known agonists of varying activity (to the native receptor) were known, it was found that the greater the initial activity of the agonist, the greater was the increase in its affinity for the constitutively activated receptor. Third, the affinity of the constitutively activated receptor for antagonists is not increased over the affinity for the antagonist of the native receptor. Fourth, the constitutively activated receptors remain coupled to the second messenger pathway and produce a biological response even in the absence of the signalling molecule or other agonist.

The importance of constitutively activated receptors to biological research and drug discovery cannot be overstated. First, these receptors provide an opportunity to study the structure of the active state and provide insights into how the receptor is controlled and the steps in receptor activation. Second, the constitutively active receptors allow study of the mechanisms by which coupling to G proteins is achieved as well as how G protein specificity is determined.

Third, mutated constitutively active receptors are now recognized in disease states. Study of constitutively activated receptors has demonstrated that many mutations may lead to constitutive activation and that a whole range of activation is possible.

Fourth, the existence of constitutively active receptors provides a novel screening mechanism with which compounds which act to increase or decrease receptor activity can be identified and evaluated. Such compounds may become lead compounds for drug research. Finally, studying the affect of classical antagonists
(compounds previously identified as, in the absence of agonist, binding to the receptor but causing no change in receptor activity, and, in the presence of agonist, competitively decreasing the activity of a receptor) and other drugs used as treatments on the constitutively active receptors has led to the discovery that there are compounds, inverse agonists, which decrease the constitutive activity of the active state of the receptors but which have no or little affect on the inactive state. The difference between antagonists, which act on the inactive state, and inverse agonists, which act on the active state, is only discernable when the receptor exhibits constitutive activity. These inverse agonists, identifiable with constitutively active receptors, present an entirely new class of potential compounds for drug discovery.

About 10 years ago, it was recognized that neurotransmitter receptors can be divided into two general classes depending on the rapidity of their response. Fast receptors were identified with ion channels and mediate millisecond responses while slower receptors were identified with G protein-coupled receptors. These G protein-coupled receptors include certain subtypes of the adrenergic as well as the muscarinic cholinergic (M1 - M5), dopaminergic (D1 - D5), serotonergic (5-HT1, 5-HT2, 5-HT4 - 5-HT7) and opiate (δ, κ, and μ) receptors. Each of these G protein-coupled neurotransmitter receptors has been associated with profound changes in mental activity and functioning, and it is believed that abnormal activity of these receptors may contribute to certain psychiatric disorders. Consequently, the elucidation of the mechanism of action of these receptors has been the focus of vigorous research efforts.

Serotonin receptors are of particular importance. Serotonin-containing cell bodies are found at highest density in the raphe regions of the pons and upper brain stem. However, these cells project into almost all brain regions and the spinal column. Serotonin does not cross the blood-brain barrier and is synthesized directly in neurons from L-tryptophan. In the CNS serotonin is thought to be involved in learning and memory, sleep, thermoregulation, motor activity, pain, sexual and aggressive behaviors, appetite, neuroendocrine regulation, and biological rhythms. Serotonin has also been linked to pathophysiological conditions such as anxiety, depression, obsessive-compulsive disorders,
schizophrenia, suicide, autism, migraine, emesis, alcoholism and neurodegenerative disorders. Presently several drugs are used to modify serotonin receptors: 1) 5-HT1: sumatriptan for treatment of migraine, ipsapirone and buspirone for treatment of anxiety; 2) 5-HT2: clozapine and risperidone for treatment of schizophrenia; and 3) 5-HT3: odanefetrone for the prevention of emesis in chemotherapy.

To date, fourteen serotonin receptors have been identified in 7 subfamilies based on structural homology, second messenger system activation, and drug affinity for certain ligands. The 5-HT₂ subfamily is divided into 3 classes: 5-HT₂A, 5-HT₂B, and 5-HT₂C. 5-HT₂A and 5-HT₂C receptor antagonists are thought to be useful in treating depression, anxiety, psychosis, and eating disorders. 5-HT₂A and 5-HT₂C receptors exhibit 51% amino acid homology overall and approximately 80% homology in the transmembrane domains. The 5-HT₂C receptor was cloned in 1987 and led to the cloning of the 5-HT₂A receptor in 1990. Studies of the 5-HT₂A receptor in recombinant mammalian cell lines revealed that the receptor possessed two affinity states, high and low. Both the 5-HT₂A and 5-HT₂C receptors are coupled to phospholipase C and mediate responses through the phosphatidylinositol pathway. Studies with agonists and antagonists display a wide range of receptor responses suggesting that there is a wide diversity of regulatory mechanisms governing receptor activity. The 5-HT₂A and 5-HT₂C receptors have also been implicated as the site of action of hallucinogenic drugs.

Much of the knowledge about the structure of G protein-coupled receptors has come from the study of the β₂-adrenergic receptor. Over the last several years, site-directed mutagenesis has been used to try to determine the amino acid residues important for ligand binding in both the β₂-adrenergic and 5-HT₂A receptors. In addition, studies have suggested that in a native (inactive) state of G protein-coupled receptors, the third intracellular loop is tucked into the receptor and is not available for interaction with the G protein. A change of receptor conformation (active) results in the availability or exposure of the C-terminal region of the third intracellular loop.

In 1990 Cotecchia et al.¹ were studying the G protein specificity determining characteristics of the third intracellular loop by creating chimeric
receptors in which the third intracellular loops had been exchanged between the 
α₁-adrenergic receptor and the β₂-adrenergic receptor. The specific G protein 
coupled activation was essentially switched between the two receptors. While 
attempting to determine which portions of the loop were responsible for the 
specificity, Cotecchia et al. discovered an unexpected phenomena; namely that 
the modification in the third intracellular loop of the α₁-adrenergic receptor of 
three residues, Arg288, Lys290, and Ala293, created a mutant receptor with two 
orders of magnitude greater affinity for agonist and which coupled to the second 
messenger system even in the absence of agonist. These modifications were 
made in the carboxy end of the third cytoplasmic loop adjacent to the sixth 
transmembrane helix. The changes responsible for this increase were isolated to 
either a Ala293 → Leu or a Lys290 → His mutation. Thus, a constitutively active 
state of a G protein-coupled neuroreceptor had been created. Subsequently, 
Kjelsberg et al.² demonstrated that mutation of the amino acid at position 293 in 
the α₁β-adrenergic receptor to any other of the 19 amino acids also produced a 
constitutively active state. Subsequently, mutations in the β₂-adrenergic receptor 
near the carboxy end of the third cytoplasmic loop have also been shown by 
Samama et al.³ to constitutively activate this receptor.

When foci resulting from constitutively active α₁β-adrenergic receptors were 
injected into nude mice, tumor formation occurred. Over the past 5 years, since 
the discovery that several thyroid adenomas contained mutations of the thyroid 
stimulating hormone (TSH) receptor, constitutively activated receptors have been 
found associated with several human disease states. The mutations responsible 
for these disease states have been found in the transmembrane domains and 
intracellular loops. For the TSH receptor, mutations at 13 different amino acid 
positions have been found in the transmembrane domain, the third intracellular 
loop, and the second and third extracellular loops. Clearly, constitutively 
activating mutations are not limited to the third intracellular loop and the critical 
site for constitutive activation varies with each G protein-coupled receptor. The 
importance of the initial observations was well stated in Cotecchia et al.¹: "Such 
mutations might not only help to illuminate the biochemical mechanisms involved 
in receptor-G protein coupling but also provide models for how point mutations
might activate potentially oncogenic receptors."

In light of the above referenced discoveries, the importance and utility of discovering other constitutively activated neuronal receptors cannot be understated. However, the hope that other neuronal receptors could be easily and readily mutated to a constitutively active form by mutations in the third cytoplasmic loop was destroyed by the report of Burstein et al. in 1995 of a comprehensive mutational approach to the G protein coupled M5 muscarinic acetylcholine receptor. In that approach, Burstein et al. had randomly and comprehensively mutated the C-terminal region of the third intracellular loop of the M5 muscarinic acetylcholine receptor, but no constitutive activating mutations were found.

Definition: CONSTITUTIVELY ACTIVATED RECEPTOR shall mean a G protein-coupled receptor which: 1) exhibits an increase in basal activity of the second messenger pathway in the absence of agonist above the level of activity observed in the wild type receptor in the absence of agonist; 2) may exhibit an increased affinity and potency for agonists; 3) exhibits an unmodified or decreased affinity for antagonists; and 4) exhibits a decrease in basal activity by inverse agonists.

SUMMARY OF THE INVENTION

Constitutively active forms of the rat 5-HT$_{2A}$ and 5-HT$_{2C}$ serotonin receptors have been obtained by a site-directed mutational method that will permit the constitutive activation of all mammalian G protein-coupled serotonin receptors. An amino acid position that will lead to a successful mutation in the serotonin receptor may be identified by alignment of the serotonin receptor against the amino acid sequence of the $\alpha_{1B}$-adrenergic receptor. Mutating the amino acid in the serotonin receptor which corresponds to the most sensitive position in the $\alpha_{1B}$-adrenergic receptor, alanine 293, yields a constitutively active serotonin receptor. A strongly constitutively active serotonin receptor is achieved when the mutation in the serotonin receptor is to one of the amino acids which produces the highest level of basal activation in constitutively activated $\alpha_{1B}$-adrenergic receptors. Successful constitutive activation of the serotonin receptor can be shown by increased high basal levels of second messenger activity in the absence of agonist, increased affinity and potency for agonists, and an unmodified or
decreased affinity for antagonists. While standard methods of site-directed mutagenesis may be employed, the careful placement of restriction sites in the primer permits the more rapid and direct determination of the clone containing the desired mutated receptor.

5 It is the object of this invention to provide a general methodology for obtaining constitutively active forms of the G protein-coupled mammalian monoamine receptors.

It is a further object of this invention to provide a general methodology for obtaining constitutively active forms of the G protein-coupled mammalian serotonin receptors.

10 It is another object of this invention to provide a constitutively active 5-HT$_{2A}$ serotonin receptor.

It is a further object of this invention to provide a constitutively active 5-HT$_{2C}$ serotonin receptor.

15 Yet another object of this invention is to provide a method for rapidly identifying the clone containing the desired mutated receptor.

These and other achievements of the present invention will become apparent from the detailed description which follows.

**DESCRIPTION OF THE FIGURES**

20 Figure 1A shows the full DNA sequence for the rat 5-HT$_{2A}$ serotonin receptor including the 5' and 3' untranslated regions with the translated codons underlined. Figure 1B shows the translated amino acid sequence for the rat 5-HT$_{2A}$ receptor.

Figure 2A shows the full DNA sequence for the rat 5-HT$_{2C}$ serotonin receptor including the 5' and 3' untranslated regions with the translated codons underlined. Figure 2B shows the translated amino acid sequence for the rat 5-HT$_{2C}$ receptor.

Figure 3A shows the full DNA sequence for the rat $\alpha_{1B}$-adrenergic receptor including the 5' and 3' untranslated regions with the translated codons underlined. Figure 3B shows the translated amino acid sequence for the rat $\alpha_{1B}$-adrenergic receptor.

Figure 4 shows the amino acid sequences for part of the C-terminal third
intracellular loop and transmembrane domain VI for the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors aligned opposite the corresponding part of the $a_{1b}$-adrenergic receptor with numerals representing the amino acid positions in each receptor.

Figure 5 shows a schematic outline of the 5-HT$_{2A}$ site-directed mutagenesis procedure.

Figure 6 shows a schematic outline of the 5-HT$_{2C}$ site-directed mutagenesis procedure.

Figure 7 shows the competition curves of 5-HT for $^3$H-ketanserin labeled native and mutant 5-HT$_{2A}$ receptors. 0.5nM $^3$H-ketanserin was used to label the native and mutant receptors transiently transfected in COS-7 cells.

Figure 8 shows the radioligand binding data of $^3$H-ketanserin labeled native and mutant 5-HT$_{2A}$ receptors in the presence of agonists and antagonists. 0.5 nM $^3$H-ketanserin was used to label the native and mutant 5-HT$_{2A}$ receptors expressed in COS-7 cells.

Figure 9 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT$_{2A}$ receptors. IP production assays were performed using anion-exchange chromatography. The data are expressed as percent of maximal IP stimulation produced by 10 $\mu$M 5-HT.

Figure 10 shows the basal activity and 5-HT stimulation of the native and mutant 5-HT$_{2A}$ receptors. IP levels were measured in COS-7 cells with vector alone, native 5-HT$_{2A}$ receptors, or mutant 5-HT$_{2A}$ receptors. The data are expressed as dpm's of IP stimulation minus basal levels of IP produced by vector. Basal activity of vector alone was typically 400 dpm's.

Figure 11 shows a saturation analysis of $^3$H-ketanserin labeled native and cys $\rightarrow$ lys mutant receptors. Bmax values were determined by a BCA assay.

Figure 12 shows the competition curves of 5-HT for $^3$H-mesulergine labeled native and mutant 5-HT$_{2C}$ receptors. 1 nM $^3$H-mesulergine was used to label the native and mutant receptors transiently transfected in COS-7 cells.

Figure 13 shows the radioligand binding analysis of native and mutant 5-HT$_{2C}$ receptors. Native and mutant 5-HT$_{2C}$ receptors expressed in COS-7 cells were labeled with 1 nM $^3$H-mesulergine. 5-MT = 5-methoxytryptamine.

Figure 14 shows the 5-HT stimulation of IP production in COS-7 cells
transfected with the ser → lys or ser → phe mutated receptors. Cells were labeled with \(^3\)H-myoinositol and challenged with 5-HT (0.1 nM - 10 nM). Total IP production was measured by anion exchange chromatography.

Figure 15 shows the EC\(_{50}\) values for the 5-HT stimulation of IP production in COS-7 cells transfected with native, mutant ser → lys receptor, and mutant ser → phe receptor. Figure 15 also shows the results of \(^3\)H-mesulergine saturation analyses. Saturation experiments were performed using \(^3\)H-mesulergine (0.1 nM - 5.0 nM).

Figure 16 shows the effect of the ser → lys and ser → phe mutations on basal levels of IP production by the mutated 5-HT\(_{2c}\) receptors. IP levels were measured in COS-7 cells with vector alone, native 5-HT\(_{2c}\) receptors, or mutant 5-HT\(_{2c}\) receptors. The data are expressed as dpms of IP stimulation minus basal levels of IP produced by vector.

Figure 17 shows the inverse agonist activity of spiperone and ketanserin on the mutated constitutively active 5-HT\(_{2a}\) cys → lys receptor. Parallel transfections with the native 5-HT\(_{2a}\) receptor were performed to determine native basal activity which was then subtracted from the mutant receptor basal activity to determine constitutive stimulation.

Figure 18 shows the inverse agonist activity of chlorpromazine, haloperidol, loxapine, spiperone, clozapine and risperidone on the mutated constitutively active 5-HT\(_{2a}\) cys → lys receptor.

Figure 19 shows the inverse agonist activity of mianserin and mesulergine on the mutated constitutively active 5-HT\(_{2c}\) ser → lys receptor both in the presence and absence of 5-HT.

Figure 20A sets forth the full DNA sequence for the human 5-HT\(_{2a}\) serotonin receptor with the translated codons underlined. The sixth transmembrane domain conserved sequence of WxPFFI is indicated with block letters. Figure 20B shows the translated amino acid sequence for the human 5-HT\(_{2a}\) receptor.

Figure 21A sets forth the full DNA sequence for the human 5-HT\(_{2c}\) serotonin receptor with the translated codons underlined. The sixth transmembrane domain conserved sequence of WxPFFI is indicated with block
letters. Figure 21B shows the translated amino acid sequence for the human 5-HT$_{2c}$ receptor.

Figure 22 is the amino acid sequence of the 5-HT$_{2a}$ cys $\rightarrow$ lys mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 23 is the DNA sequence of the 5-HT$_{2a}$ cys $\rightarrow$ lys mutant receptor including the 5’ and 3’ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 24 is the DNA sequence of the 5-HT$_{2a}$ cys $\rightarrow$ lys mutant receptor including the 5’ and 3’ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters and are indicated with arrows.

Figure 25 is the amino acid sequence of the 5-HT$_{2a}$ cys $\rightarrow$ arg mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 26 is the DNA sequence of the 5-HT$_{2a}$ cys $\rightarrow$ arg mutant receptor including the 5’ and 3’ untranslated regions with the translated codons underlined. The bases specifying the #322 arginine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 27 is identical to Figure 26 since the AGG mutation introduced for arginine creates an Mnl1 restriction site by itself at #319.

Figure 28 is the amino acid sequence of the 5-HT$_{2a}$ cys $\rightarrow$ glu mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 29 is the DNA sequence of the 5-HT$_{2a}$ cys $\rightarrow$ glu mutant receptor including the 5’ and 3’ untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 30 is the DNA sequence of the 5-HT$_{2a}$ cys $\rightarrow$ glu mutant receptor
including the 5' and 3' untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the additional base which was mutated to create the Rsa1 site is shown as a larger outlined letter and is indicated with an arrow.

Figure 31 is the amino acid sequence of the 5-HT₂C ser → lys mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 32 is the DNA sequence of the 5-HT₂C ser → lys mutant receptor including the 5' and 3' untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 33 is the DNA sequence of the 5-HT₂C ser → lys mutant receptor including the 5' and 3' untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the base which was mutated to create the Sca1 site is shown as a larger outlined letter and is indicated with an arrow.

Figure 34 is the amino acid sequence of the 5-HT₂C ser → phe mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 35 is the DNA sequence of the 5-HT₂C ser → phe mutant receptor including the 5' and 3' untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 36 is the DNA sequence of the 5-HT₂C ser → phe mutant receptor including the 5' and 3' untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the base which was mutated to create the Sca1 site is shown as a larger outlined letter and is indicated with an arrow.
DETAILED DESCRIPTION OF THE INVENTION

Despite the disappointing results obtained by Burstein in mutating positions in the third intracellular loop of the M5 muscarinic acetylcholine receptor, the present inventive efforts focused on finding mutations at the carboxy end of the third intracellular loop near the sixth transmembrane helix in the serotonin receptors. DNA and amino acid sequences for rat 5-HT$_{2A}$ and 5-HT$_{2C}$ serotonin receptors were obtained from GeneBank as was the DNA and amino acid sequence for the $\alpha_{1B}$-adrenergic receptor. Figures 1, 2, and 3 list the full DNA and translated amino acid sequences for these receptors.

Receptor Alignment:

As noted above, Cotecchia et al. had identified amino acid position number 293 in the third intracellular loop adjoining the sixth transmembrane domain in the $\alpha_{1B}$-adrenergic receptor as a critical position, mutation of which lead to constitutive activity. However, the length of the serotonin receptors is different than the $\alpha_{1B}$-adrenergic receptor, and even had they been the same, matching the ends would not necessarily provide a structural or functional match. What was important was to find an alignment method which made sense in terms of locating the equivalent functional site to position 293 of the $\alpha_{1B}$-adrenergic receptor in the serotonin receptors.

A meaningful alignment method has been discovered based upon the fact that the transmembrane domains are highly conserved in G protein-coupled receptors. A series of conserved amino acid positions were identified in the sixth transmembrane domain which permit alignment of the transmembrane domain and the adjacent third intracellular loop between receptors. In Figure 5 the conserved sixth transmembrane domain amino acid sequence WxPFFI (x may be variable) has been used to align the three receptors. Alignment using this sequence also aligns the LGIV sequence found at the intracellular beginning of the sixth transmembrane domain which is connected to the third intracellular loop. This alignment indicates that in the 5-HT$_{2A}$ receptor the cysteine at position #322 corresponds to the alanine at position #293 in the $\alpha_{1B}$-adrenergic receptor. In the 5-HT$_{2B}$ receptor, the corresponding amino acid is a serine at position #312.

It should be noted that position 293 is not the only position in the $\alpha_{1B}$-
adrenergic receptor which, when mutated, produced a constitutively active receptor. While Cotecchia et al.\textsuperscript{1} reported that the A293L mutation produced the greatest constitutive activation, they also noted that the K290H mutation also induced dramatic constitutive activity. There are clearly other sites in the third intracellular loop of each of these receptors that can be mutated. In the future, other sites on other receptors may be reported. However, the alignment methodology presented above should serve to permit the structural correlation between different receptors so that information gleaned from one receptor may be utilized to mutate another receptor. However, the evidence presently available suggests that the third position removed from the beginning of the transmembrane domain represented by position 293 in the $\alpha_{1b}$-adrenergic receptor seems to play a crucial role in the binding and activation of the coupled G protein, and that mutations introduced at that position alter the tertiary structure of the region.

As noted earlier, Kjelsberg et al.\textsuperscript{2} further demonstrated that substitution of any of the 19 amino acids at position 293 of the $\alpha_{1b}$-adrenergic receptor produced constitutive activity. However, the relative activity increased in the following order of amino acids: S, N, D, G, T, H, W, Y, P, V, L, M, Q, I, F, C, R, K, and E. In that study, replacing the native amino acid with amino acids having long basic or acidic side chains produced the greatest degree of constitutive activity, while amino acids with aromatic substituents produced an intermediate degree of constitutive activity. It is proposed that this order, with minor variations, exists for most G protein-coupled receptors due to the importance of the third position removed from the beginning of the transmembrane domain. A reasonable starting place for mutating receptors should therefore involve mutation to one of the amino acids at the most active end of the above list. Further, the tertiary structure of the region may be significantly altered by substituting an amino acid with longer side chains or of different polarity from the native amino acid.

**Efficient Screening of Mutant Receptors:**

When performing site-directed mutagenesis, it is common (and necessary) laboratory practice to fully sequence the cloned receptor to confirm that the mutation has been incorporated. However, because colonies containing the
mutant receptor cannot be distinguished from those that do not, it is necessary to sequence each colony. A method, outlined schematically by way of example in Figure 5 for the 5-HT$_{2A}$ cys → lys receptor mutation and in Figure 6 for the 5-HT$_{2C}$ ser → lys and ser → phe receptor mutations, has been devised that rapidly and easily eliminates most non-mutated colonies, and from those remaining, identifies the mutant colony so that unnecessary sequencing is avoided. A two-pronged approach is used. The first prong is designed to prevent non-mutated vector from being incorporated during the first transformation by digesting the vector. E coli will only incorporate uncut (circular) plasmid DNA. Recognizing the limitations of the first prong, namely, that all restriction digests are not 100% complete so that some of the colonies at the end of the procedure will contain native DNA instead of mutant DNA, the second prong is designed to easily identify among the remaining colonies, those colonies containing the desired mutation after a second transformation.

To begin, a unique restriction site, not occurring in the native amino acid sequence, is incorporated into the mutant. It is possible to introduce the unique restriction site because of the degeneracy of the genetic code. The unique restriction site is ideally located within or near the amino acid(s) which specify the structural mutation which is being introduced into the mutant. Thus, the restriction site can be located on the same mutagenic primer as the structural mutation.

In addition, during the initial annealing, a second primer is used to remove a restriction site specific to the vector being used. When the second strand is synthesized with polymerase and ligase, only the second strand of the vector (the one not containing the mutations) will contain the original vector restriction site. Subsequently, after transformation, the colonies can be treated with the restriction enzyme specific for the vector site and only those resulting from the wildtype vector will be digested. Digested (cut) DNA will not be taken up by E. coli during the second transformation step. The colonies containing the mutated vector will not be digested and will be taken up by E. coli during the final transformation step.

Each resulting colony can be tested to see whether the restriction enzyme,
which recognizes the unique site introduced by the mutated primer, digests the DNA. Only samples from colonies containing the desired mutation will be digested. These colonies can then be sequenced to confirm the insertion of the mutated amino acid. It is unnecessary to sequence colonies whose DNA is not digested by the restriction enzyme. This procedure yields a much more highly efficient method by saving both time and expense of sequencing every colony which results from the transformation experiment.

Measurement of Receptor-Coupled Second Messenger Activation:

In order to measure the stimulation produced through the 5-HT$_{2A}$ and the 5-HT$_{2C}$ receptors, an assay was utilized which measures the accumulation of inositol phosphates, the product that is formed when phosphatidylinositol 4,5-bisphosphate is hydrolyzed to DAG and IP. This assay was established by Berridge and coworkers (1983) in studies of the blowfly salivary glands, and found to be an accurate measurement of the stimulation of phospholipase C through receptor activation. $^3$H-myoinositol is incorporated into the cell membrane by conversion to phosphatidylinositol 4,5-bisphosphate and upon receptor activation, is cleaved by phospholipase C to yield two products: diacylglycerol and $^3$H-inositol 1,4,5 triphosphate (IP$_3$).

Inositol-free media must be used for this assay because unlabeled inositol, which is normally found in many commercially available media, can result in less than maximal incorporation of radiolabeled inositol into the cell membrane, resulting in a reduction in the amount of $^3$H-IP that would be detected. The $^3$H-IP is recovered by anion-exchange chromatography in which IP is separated from anion-exchange resin using washes of increasing concentrations of formate.

IP$_3$ is rapidly hydrolyzed to IP$_2$ by an inositol triphosphatase which is then converted to IP by inositol bisphosphatase. Because IP$_3$ is hydrolyzed so quickly, accumulation of IP would be hard to measure unless the cycle of IP to inositol and phosphate is blocked. Lithium is used in this assay to block the enzyme which converts IP to inositol and phosphate (myo-inositol monophosphatase). This ensures that IP levels can accumulate and be experimentally measured and are not undergoing the normal rapid degradation pathway. These experiments are also performed in serum free media in order to remove serotonin that can be found in
serum which would complicate experimental results.

The total IP levels were measured in order to obtain an accurate measurement of the total amount of stimulation that occurred. The actual experimental conditions and concentrations of reagents used in this assay are set forth in the methods and materials sections under each example below.

Example 1: Constitutive Activation Of The 5-HT₂A Receptor:

Three separate mutations of the 5-HT₂A receptor were made. The cysteine at position 322 was mutated to lysine, glutamate, and arginine.

Materials and Methods For Site-directed Mutagenesis:

The rat 5-HT2A receptor cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen) using EcoR1 (GIBCO). This construct served as the native template for site-directed mutagenesis performed using Clontech's transformer kit. Mutagenic primers (Midland Certified Reagent Company) were designed as follows: the C322K primer was complementary to amino acid nos.

318-329 of the native 5-HT2A cDNA, while changing amino acid no. 322 from cysteine (TGC) to lysine (AAG). The same primer was designed to incorporate a Sca1 restriction site using amino acid nos. 323 and 324 by changing the third base in amino acid no. 323, lysine, from AAG to AAA and the third base in amino acid no. 324, valine from GTG to GTA. The C322E and C322R were designed complementary to amino acid nos. 319-330 of the native 5-HT2A cDNA, while changing amino acid no. 322 from cysteine (TGC) to glutamate (GAG) and arginine (AGG). In the C322E primer, an Rsa1 site was introduced by changing the third base in amino acid no. 324, valine, from GTG to GTA. The C322R mutation in the primer created an Mnl1 site, by itself, at amino acid no. 319. The selection primer, complementary to bases 4,871-4,914 of the pcDNA3 vector, was designed to remove a unique PVUI site by changing base G to T at location 4891. Phosphorylated primers were annealed to 10 ng of alkaline-denatured plasmid template by heating to 65°C for 5 min and cooling slowly to 37°C. Mutant DNA was synthesized using T4 DNA polymerase and ligase (Clontech) by incubating for 1 hr at 37°C, followed by digestion with PVU1 (GIBCO) and transformation of BMH71-18mutS E. coli (Clontech). Plasmid was purified using the Wizard miniprep kit (Promega), digested with PVU1, and used to transform
DH5( E.Coli (GIBCO). Individual colonies were isolated and plasmid DNA was
digested with SCA1, Mnl1 or Rsa1 to screen for C322K, C322E and C322R
mutations, respectively (GIBCO). DNA sequencing (Sequenase version 2.1
kit,USB, $^{35}$Sd-ATP, New England Nuclear) was performed to confirm the
incorporation of lysine, glutamate, or arginine at amino acid no. 322. Sequencing
reactions were run on a 5% acrylamide/bis (19:1) gel (Bio-Rad) for 2 hr at 50°C,
dried for 2 hr at 80°C, and exposed on Kodak Biomax MR film for 24 hr at -80°C.

In Figure 22 is shown the amino acid sequence of the 5-HT$_2$A cys $\rightarrow$ lys
mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 23 shows the resulting DNA sequence of the 5-HT$_2$A cys $\rightarrow$ lys mutant
receptor including the 5′ and 3′ untranslated regions with the translated codons
underlined. The bases specifying the #322 lysine mutant are shown as larger
outlined letters, and the starting and ending locations of the primer are also
indicated. In addition to showing the mutated DNA sequence of the 5-HT$_2$A cys $\rightarrow$
lys mutant receptor, Figure 24 shows the two bases, which were mutated to
create the Sca1 site, as larger outlined letters and are indicated with arrows.

In Figure 25 is shown the amino acid sequence of the 5-HT$_2$A cys $\rightarrow$ arg
mutant receptor with the mutated amino acid shown as a larger outlined letter.
Figure 26 shows the resulting DNA sequence of the 5-HT$_2$A cys $\rightarrow$ arg mutant
receptor including the 5′ and 3′ untranslated regions with the translated codons
underlined. The bases specifying the #322 arginine mutant are shown as larger
outlined letters, and the starting and ending locations of the primer are also
indicated. Figure 27 showing the added restriction site is identical to Figure 26
since the arginine mutation to AGG creates, by itself, an Mnl1 restriction site at

#319.

In Figure 28 is shown the amino acid sequence of the 5-HT$_2$A cys $\rightarrow$ glu
mutant receptor with the mutated amino acid shown as a larger outlined letter.
Figure 29 shows the resulting DNA sequence of the 5-HT$_2$A cys $\rightarrow$ glu mutant
receptor including the 5′ and 3′ untranslated regions with the translated codons
underlined. The bases specifying the #322 glutamic acid mutant are shown as
larger outlined letters, and the starting and ending locations of the primer are also
indicated. Figure 30 shows the additional base mutation introduced in amino
acid 324 to create an Rsa1 site. The base mutation is indicted by a larger outlined letter and an arrow.

**Cell culture and transfection:**

COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) with 10% fetal bovine serum (Sigma) in 5% CO₂ at 37°C and subcultured 1:8 twice a week. Twenty-four hours before transfection, cells were seeded at 30% confluence in 100-mm dishes for radioligand binding assays or at 10⁵ cells per well in 24-well cluster plates for IP production assays. Cells were transfected with native or mutant 5-HT2A cDNA using Lipofectamine (GIBCO). This was accomplished by combining 20 μl of Lipofectamine with 2.5 μg of plasmid per 100-mm dish or 2 μl of Lipofectamine with 0.25 μg of plasmid per well. Transfections were performed in serum-free DMEM for 4 hr at 37°C.

**Radioligand binding:**

Thirty-six hours after transfection, membranes were prepared from COS-7 cells by scraping and homogenizing in 50mM Tris-HCl/5mM MgCl₂/0.5mM EDTA, pH 7.4 (assay buffer), and centrifugation at 10,000xg for 30 min. Membranes were resuspended in assay buffer, homogenized, and centrifuged again. After resuspension in assay buffer, 1-ml membrane aliquots (approximately 10 μg of protein measured by bicinchoninic acid assay) were added to each tube containing 1ml of assay buffer with 0.5nM [³H]ketanserin and competing drugs. 10μM spiperone was used to define non-specific binding. Saturation experiments were performed by using [³H]ketanserin (0.1-5.0nM). Samples were incubated at 23°C for 30 minutes, filtered on a Brandel cell harvester, and counted in Ecoscint cocktail (National Diagnostics) in a Beckman liquid scintillation counter at 40% efficiency.

**Phosphatidylinositol hydrolysis:**

Inositol phosphate (IP) production was measured using a modified combination of the methods of Berridge et al. (1982) and Conn and Sanders-Bush (1985). In brief, 24 h after transfection, cells were washed with phosphate-buffered saline (PBS) and labeled with 0.25 μCi/well of myo-[³H]inositol (New England Nuclear) in inositol free/serum-free DMEM (GIBCO) for 12 h at 37°C. HPLC analysis of this culture medium, after incubation, has been reported to
contain <10^{-10} M 5-HT (Barker et al. 1994). After labeling, cells were washed with PBS and preincubated in inositol-free/serum-free DMEM with 10mM LiCl and 10μM pargyline (assay medium) for 10 min at 37^{0}\text{C}. When antagonists were used, they were added during the 10-min preincubation period. 5-HT (Sigma), or assay medium alone, was added to each well and incubation continued for an additional 35 min (Westphal et al., 1995). Assay medium was removed and cells were lysed in 250 μl of stop solution (1 M KOH/18mM sodium borate/3.8mM EDTA) and neutralized by adding 250 μl of 7.5 % HCl. The contents of each well were extracted with 3 volumes of chloroform/methanol (1:2), centrifuged 5 min at 10,000xg, and the upper layer loaded onto a 1-ml AG1-X8 resin (100-200 mesh, Bio-Rad) column. Columns were washed with 10ml of 5 mM myo-inositol and 10ml of 5 mM sodium borate/60mM sodium formate. Total IPs were eluted with 3ml of 0.1 M formic acid/1 M ammonium formate. Radioactivity was measured by liquid scintillation counting in Ecoscint cocktail.

**Demonstration of Constitutive Activation:**

Constitutive activity of the mutated 5-HT_{2A} receptors is demonstrated by the fact that the mutated receptors exhibit all the hallmark characteristics established for constitutive activation: a showing of increased agonist affinity, increased agonist potency, and coupling to the G protein second messenger system in the absence of agonist.

Figure 7 shows the competition curves of 5-HT for ^3H-ketanserin labeled native and mutant 5-HT_{2A} receptors. 0.5nM ^3H-ketanserin was used to label the native and mutant receptors transiently transfected in COS-7 cells. While the native receptor demonstrated a relatively low affinity for 5-HT (K_i = 293 nM), the three mutant receptors displayed a high affinity for 5-HT with the cys→lys mutant exhibiting a 12-fold increase in affinity for 5-HT (K_i = 25 nM), the cys→arg mutant exhibiting a 27-fold increase in affinity for 5-HT (K_i = 11 nM). and the cys→glu mutant exhibiting a 3.4-fold increase in affinity for 5-HT (K_i = 86 nM).

To determine whether other agonists would display a similar increase in affinity for the mutant receptors, two known agonists (DOM and DOB) were tested with both the native and cys→lys mutant. Figure 8 shows the radioligand binding data of ^3H-ketanserin labeled native and mutant 5-HT_{2A} receptors in the
presence of agonists and antagonists. 0.5 nM \(^3\)H-ketanserin was used to label the native and mutant 5-HT\(_{2A}\) receptors expressed in COS-7 cells. The DOM and DOB agonists show increased affinity for the mutant receptor, as is seen for 5-HT. The \(K_i\) for DOM shows a 5-fold increase, while the \(K_i\) for DOB shows a 7.4-fold increase.

To determine if the mutant 5-HT\(_{2A}\) receptors would exhibit an increase in agonist potency relative to the native 5-HT\(_{2A}\) receptor, 5-HT stimulation of the native and mutant 5-HT\(_{2A}\) receptors was measured using an IP production assay. Figure 9 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT\(_{2A}\) receptors. Both the cys → lys and cys → glu mutant receptor curves exhibit a leftward shift away from the native curve in the 5-HT dose-response indicating that there was an increase in 5-HT potency at the mutant receptors. The cys → lys and cys → glu mutant receptors displayed EC\(_{50}\) values of 25 nM and 61 nM, respectively, as compared to the native 5-HT\(_{2A}\) receptor which had an EC\(_{50}\) value of 152 nM.

Figure 10 shows the basal activity and 5-HT stimulation of the native and mutant 5-HT\(_{2A}\) receptors. As can be seen, both the cys → lys and the cys → glu mutant 5-HT\(_{2A}\) receptors show dramatic increases in basal intracellular inositol phosphate (IP) accumulation compared to the native receptor. The cys → lys mutant receptor produced a 345% (8-fold) increase in IP levels over the vector control. The cys → glu mutant receptor produced a 158% (3.7-fold) increase in IP levels over the vector control. Upon the addition of 10 \(\mu\)M 5-HT, both the native and mutant receptors produced an additional increase in IP production. The basal activity of the cys → lys mutant was 48% of that of the maximally stimulated native 5-HT\(_{2A}\) receptor. The basal activity of the cys → glu mutant was 31% of that of the maximally stimulated native 5-HT\(_{2A}\) receptor.

In order to determine whether the above results were due to an increase in the number of expressed mutant receptors rather than to a change in the properties of the mutated receptors, saturation curves were generated. Figure 11 shows a saturation analysis of \(^3\)H-ketanserin labeled native and cys → lys mutant receptors. \(B_{\text{MAX}}\) values were determined by a BCA assay. For the native receptor the \(B_{\text{MAX}} = 193 \pm 37\) fmol/mg, while for the cys → lys mutant receptor, the
$B_{\text{MAX}} = 218 \pm 31 \text{ fmol/mg.}$ There is no significant difference in the $B_{\text{MAX}}$ values for the native and mutant receptors. The $K_p$ of $^3$H-ketanserin also did not differ between the native and mutant receptors. These data demonstrate that the results were not due to an increase in number of expressed mutant receptors compared to expressed native receptors.

Thus, the mutated 5-HT$_{2A}$ receptors meet all the criteria for constitutively activated receptors; they show a higher affinity for agonists; they show a higher potency for 5-HT; and they show activation (coupling) of the G protein second messenger pathway (IP production) even in the absence of agonist.

**Example 2: Constitutive Activation of 5-HT$_{2C}$ Receptor**

**Materials and Methods For Site-directed Mutagenesis:**

The rat 5-HT$_{2C}$ receptor cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen) using BamH1 (Gibco). This construct served as the native template for site-directed mutagenesis performed using Clonetech's Transformer kit. Mutagenic primers (Midland Certified Reagent Company) were designed complementary to amino acids #308-317 of the native 5-HT$_{2C}$ cDNA, while changing amino acid #312 from serine (TCC) to lysine (AAG) or phenylalanine (TTC). The same primers were designed to incorporate an Sca1 restriction site at amino acid #314 by changing the third codon in valine from GTC to GTA. The selection primer, complementary to bases 2081-3017 of the pcDNA3 vector, was designed to remove a unique Sma1 site by changing glycine at base 2093 from GGG to GGA. Phosphorylated primers were annealed to 10ng of alkaline denatured plasmid template by heating to 65°C for 5 minutes and cooling slowly to 37°C. Mutant DNA was synthesized using T4 DNA polymerase and ligase (Clonetech) by incubating for 1 hour at 37°C, followed by digestion with Sma1 (Gibco) and transformation of BMH71-18mutS E. coli (Clonetech). Plasmid was purified using the Wizard miniprep kit (Promega), digested with Sma1, and used to transform DH5α E. coli (Gibco). Individual colonies were isolated and plasmid DNA was digested with Sca1 to screen for S312K and S312F mutants (Gibco). S312K and S312F mutant plasmids contain an additional Sca1 site and appear as two bands (2.3Kb and 7.6Kb) when run on a 1% agarose gel. DNA sequencing (Sequenase version 2.1 kit USB, $^{35}$Sd-ATP NEN)
was performed to confirm the incorporation of lysine or phenylalanine at amino acid #312. Sequencing reactions were run on a 5% acrylamide/bis (19:1) gel (BioRad) for 2 hours at 50°C, dried for 2 hours at 80°C, and exposed to Kodak Biomax MR film for 24 hours at -80°C.

In Figure 31 is shown the amino acid sequence of the 5-HT\textsubscript{2c} ser → lys mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 32 shows the resulting DNA sequence of the 5-HT\textsubscript{2c} ser → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT\textsubscript{2c} ser → lys mutant receptor, Figure 33 shows the base, which was mutated to create the Sca1 site, as a larger outlined letter indicated with an arrow.

In Figure 34 is shown the amino acid sequence of the 5-HT\textsubscript{2c} ser → phe mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 35 shows the resulting DNA sequence of the 5-HT\textsubscript{2c} ser → phe mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT\textsubscript{2c} ser → phe mutant receptor, Figure 36 shows the base, which was mutated to create the Sca1 site, as a larger outlined letter indicated with an arrow.

Cell culture and transfection:

COS-7 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) with 10% fetal bovine serum (Sigma) in 5% CO\textsubscript{2} at 37°C and subcultured 1:8 twice a week. Twenty-four hours prior to transfection, cells were seeded at 30% confluence in 100mm dishes for radioligand binding assays or at 10\textsuperscript{6} cells/well in 24 well cluster plates for PI assays. Cells were transfected with native or mutant 5-HT\textsubscript{2c} cDNA using Lipofectamine (Gibco). This was accomplished by combining 20 μl of lipofectamine with 2.5 μg plasmid per 100mm dish or 2 μl lipofectamine and 0.25 μg plasmid per well. Transfections were performed in serum-free DMEM for 4 hours at 37°C.
Radioligand binding:

Thirty-six hours after transfection, membranes were prepared from COS-7 cells by scraping and homogenizing in 50mM Tris-HCl / 5mM MgCl₂ / 0.5mM EDTA pH 7.4 (assay buffer) and centrifugation at 10,000xg for 30 minutes. Membranes were resuspended in assay buffer, homogenized and centrifuged again. Following resuspension in assay buffer, 1 ml membrane aliquots (approximately 10 µg protein measured by BCA assay) were added to each tube containing 1ml of assay buffer with 1nM ³H-mesulergine and competing drugs. 10µM mianserin was used to define non-specific binding. Saturation experiments were performed using ³H-mesulergine (0.1nM-5.0nM) or ³H-5-HT (0.1nM-30nM) in the absence of presence of 10µM GppNHp (RBI). Samples were incubated at 37°C for 30 minutes, filtered on a Brandel cell harvester, and counted in Ecoscint cocktail (National Diagnostics) in a Beckman liquid scintillation counter at 40% efficiency.

Phosphatidylinositol hydrolysis:

Inositol phosphate (IP) production was measured using a modified combination of the methods of Berridge et al., 1982 and Conn and Sanders-Bush 1985. Briefly, 24 hours after transfection, cells were washed with PBS and labeled with 0.25µCi/well of ³H-myoinositol (NEN) in inositol-free/serum-free DMEM (Gibco) for 12 hours at 37°C. Following labeling, cells were washed with PBS and preincubated in inositol-free/serum-free DMEM with 10mM LiCl and 10µM pargyline (assay medium) for 10 minutes at 37°C. When antagonists were used they were added during the 10 minute preincubation period. 5-HT (Sigma), or assay medium alone, was added to each well and incubation continued for an additional 35 minutes (Westphal et al., 1995). Assay medium was removed and cells were lysed in 250 µl of stop solution (1M KOH / 18mM NaBorate / 3.8mM EDTA) and neutralized by adding 250µl of 7.5% HCl. The contents of each well were extracted with 3 volumes of chloroform:methanol (1:2), centrifuged 5 minutes at 10,000xg, and the upper layer loaded onto a 1ml AG1-X8 resin (100-200 mesh, BioRad) column. Columns were washed with 10mls of 5mM myoinositol and 10mls of 5mM NaBorate / 60mM NaFormate. Total IPs were eluted with 3mls of 0.1M formic acid / 1M ammonium formate. Radioactivity was
measured by liquid scintillation counting in EcoScint cocktail.

**Stable Transfection:**

Although not yet fully characterized, it has been found possible to create a stable cell line expressing mutant receptors by the following method. The rat 5-HT<sub>2c</sub> cDNA (edited VSI isoform) was used as a template for site-directed mutagenesis to convert amino acid 312 from serine to lysine as previously described. Native and S312K 5-HT<sub>2c</sub> cDNAs were ligated into the BamHI/EcoRI site of the pZeoSv2 + mammalian expression vector (Invitrogen) containing the zeocin resistance gene. NIH3T3 cells (ATCC) were stably transfected using the high efficiency BES method. Briefly, cells were seeded at 5x10<sup>5</sup> cells/100mm culture dish in complete medium (DMEM/10%FBS) and grown in 5% CO<sub>2</sub> at 37<sup>0</sup> overnight. Twenty micrograms of pZeoSv2/5-HT<sub>2c</sub> DNA (linearized with BglIII) was mixed with 500µl of 0.25M CaCl<sub>2</sub> and 500µl of 2x BES solution (50mM N,N-bis-2-hydroxyethyl-2-aminoethanesulfonic acid; 280mM NaCl; 1.5mM Na<sub>2</sub>HPO<sub>4</sub>; pH to 6.95) and incubated at 25°C for 20 minutes. The solution was added dropwise on top of the cells. The cells were incubated for 20 hours at 35°C in 3% CO<sub>2</sub>, washed twice with PBS, complete medium replenished, and incubated for 48 hours at 37°C in 5% CO<sub>2</sub>. Cells were split 1:4 into complete medium containing 500µg/ml zeocin. Individual colonies were isolated and tested for 5-HT<sub>2c</sub> receptor expression by <sup>3</sup>H-mesulergine binding.

**Demonstration of Constitutive Activation:**

Constitutive activity of the mutated 5-HT<sub>2c</sub> receptors is demonstrated by the fact that the mutated receptors also exhibit all the hallmark characteristics established for constitutive activation: a showing of increased agonist affinity, increased agonist potency, and coupling to the G protein second messenger system in the absence of agonist.

Figure 12 shows the competition curves of 5-HT for <sup>3</sup>H-mesulergine labeled native and mutant 5-HT<sub>2c</sub> receptors. 0.5nM <sup>3</sup>H-mesulergine was used to label the native and mutant receptors transiently transfected in COS-7 cells. As shown in Figure 12, the 5-HT competition isotherms for <sup>3</sup>H-mesulergine labeled ser → lys and ser → phe mutant receptors display a marked leftward shift compared with native receptors. The affinity of 5-HT for ser → lys mutant receptors increased
almost 30-fold from 203 nM in the native to 6.6 nM in the ser → lys mutant. Similarly, on a smaller scale, the ser → phe mutation resulted in a 3-fold increase in 5-HT affinity to 76 nM.

To determine whether other agonists would display a similar increase in affinity for the mutant receptors, two known agonists, 5-methoxytryptamine and DOB were tested with the ser → lys mutant. Figure 13 shows the radioligand binding analysis of native and mutant 5-HT$_{2c}$ receptors in the presence of agonists and antagonists. Native and mutant 5-HT$_{2c}$ receptors expressed in COS-7 cells were labeled with 1 nM 3H-mesulergine. The 5-MT and DOB agonists show increased affinity for the mutant receptor, as is seen for 5-HT. 5-methoxytryptamine and DOB display an 89-fold and 38-fold increase, respectively, in affinity for the ser → lys mutant receptors.

To determine if the mutant 5-HT$_{2c}$ receptors would exhibit an increase in agonist potency relative to the native 5-HT$_{2c}$ receptor, 5-HT stimulation of the native and mutant 5-HT$_{2c}$ receptors was measured using an IP production assay. Figure 14 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT$_{2c}$ receptors. Both the ser → lys and ser → phe mutant receptor curves exhibit a leftward shift away from the native curve in the 5-HT dose-response indicating that there was an increase in 5-HT potency for the mutant receptors. The shifts were similar in magnitude to the shifts in the 5-HT competition binding isotherms. Figure 15 shows the 5-HT stimulation of IP production in COS-7 cells transfected with the ser → lys or ser → phe mutated receptors. As shown in Figure 15, the EC$_{50}$ value for 5-HT mediated stimulation of IP production increased from 70 nM in cells transfected with native receptors to 2.7 nM in the ser → lys mutant and 28 nM in the ser → phe mutant.

Figure 16 shows the effect of the ser → lys and ser → phe mutations on basal levels of IP production by the mutated 5-HT$_{2c}$ receptors. Cells transfected with native 5-HT$_{2c}$ receptors displayed a small increase (9%, 225dpm) in basal IP production over cells transfected with vector alone. Transfection with ser → lys and ser → phe mutant 5-HT$_{2c}$ receptors resulted in 5-fold and 2-fold increases, respectively, in basal levels of IP production when compared with cells expressing native 5-HT$_{2c}$ receptors. Basal levels of IP stimulated by ser → lys mutant
receptors represented 50% of total IP production stimulated by native receptors in the presence of 10 μM 5-HT. 5-HT stimulated IP production 10 fold over basal levels in cells transfected with native receptors and 2-fold over basal levels in cells transfected with ser → lys mutant receptors. However, 5-HT elicited the same maximal IP response in cells transfected with native or mutant receptors.

Since receptor density can influence agonist binding affinity and potency in stimulating second messenger systems, saturation curves were generated. Therefore, ^3^H-mesulergine saturation analyses and Scatchard transformations were performed in parallel to control for variations in transfection efficiency and receptor expression levels. As shown in Figure 15, the 5-HT_{2c} receptor density was greater in cells transfected with native receptors than in cells transfected with either the ser → lys or the ser → phe mutant receptors. These data indicate that the increase in agonist binding affinity and potency of the mutated receptors did not result from increased receptor expression, but directly resulted from the mutations.

Thus, like the mutated 5-HT_{2a} receptors, the mutated 5-HT_{2c} receptors meet all the criteria for constitutively activated receptors; they show a higher affinity for agonists; they show a higher potency for 5-HT; and they show activation (coupling) of the G protein second messenger pathway (IP production) even in the absence of agonist.

**Inverse Agonism at Constitutively Activated Serotonin Receptors**

As noted above, the discovery and elucidation of the mechanisms of action of constitutively activated receptors has led to the recognition of a new class of receptor antagonists, identified as inverse agonists. The mutated 5-HT_{2a} and 5-HT_{2c} receptors of this invention were used to test the activity of known serotonin receptor antagonists. Figure 8 shows the binding affinities of four known 5-HT_{2a} antagonists to the native and cys → lys mutant 5-HT_{2a} receptors. There is an apparent decrease in the binding affinity of methysergide and mianserin at the mutant 5-HT_{2a} receptors, but no change in binding affinity for spiperone and ketanserin. However, as shown in Figure 17, both spiperone and ketanserin reversed the constitutive stimulation of IP production in cells expressing the mutant 5-HT_{2a} receptor. Ketanserin and spiperone decreased the constitutive IP
stimulation by 80% and 58% respectively.

Several antipsychotic drugs presently in use are thought to act at the 5-HT$_{2A}$ receptor. As shown in Figure 18, all these drugs, chlorpromazine, haloperidol, loxapine, clozapine, and risperidone as well as spiperone reduce the constitutively activated IP basal activity of the mutated 5-HT$_{2A}$ receptor.

The constitutively active ser $\rightarrow$ lys mutated 5-HT$_{2C}$ receptor of this invention can also be used to screen compounds for inverse agonist activity. Figure 19 shows that two classical 5-HT$_{2C}$ receptor antagonists, mianserin and mesulergine, exhibit inverse agonist activity by decreasing basal levels of PI hydrolysis associated with the constitutively active 5-HT$_{2C}$ mutant receptor. The inverse agonism of these compounds is apparent both in the presence and absence of serotonin.

The demonstration of inverse agonism at the mutated 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors further characterizes the mutated serotonin receptors of this invention as being constitutively active. Not only have the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors been mutated to a constitutively active form, but a method has been disclosed for mutating all mammalian G protein-coupled monoamine receptors, including serotonin receptors, to a constitutively active form. Unlike the case of the M5 muscarinic acetylcholine receptor where mutations in the third cytoplasmic loop do not produce constitutive activation, the present invention clearly demonstrates that mutations in the third cytoplasmic loop of G protein-coupled serotonin receptors may be used to induce constitutive activation. Previously, third intracellular loop mutations near the transmembrane region had only been found to produce constitutively active receptors of the adrenergic type. With the present discoveries, it is now recognized that the alignment and positional mutation method of this invention is applicable to the general class of monoamine receptors of which the adrenergic and serotonin receptors are major subclasses. Further, based upon the present discoveries, it is expected that mutations may be introduced at other sites in the third cytoplasmic loop which will constitutively activate the G protein-coupled monoamine receptors including the serotonin receptors.
Additional Advances Enabled By The Discoveries Of The Present Invention:

Figures 20A and 20B show the DNA and amino acid sequences for the human $5\text{-HT}_{2A}$ receptors. In Figure 20A, it can be seen that the sixth transmembrane domain has the same WxPFFI conserved sequence (outlined type) as seen in the rat receptors. Figures 21A and 21B show the DNA and amino acid sequences for the human $5\text{-HT}_{2C}$ receptors. In Figure 21A it can be seen that the sixth transmembrane domain also has the same WxPFFI conserved sequence (outlined type) as seen in the rat receptors. Both of these human receptors may, therefore, be similarly aligned with the rat $\alpha_1$-adrenergic, $5\text{-HT}_{2A'}$ and $5\text{-HT}_{2C}$ receptors to identify the amino acid positions which may be mutated to produce constitutively active human receptors following the methodologies of this invention.

Having identified mutations which constitutively activate the $5\text{-HT}_{2A}$ and $5\text{-HT}_{2C}$ serotonin receptors, it is now possible to create transgenic mammals incorporating these mutations using techniques well known in the art. This will provide an opportunity to study the physiological consequences of constitutive receptor activation and may lead to the development of novel therapeutic agents.

Those skilled in the art will recognize that various modifications, additions, substitutions and variations of the illustrative examples set forth herein can be made without departing from the spirit of the invention and are, therefore, considered within the scope of the invention.

References


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5. The native rat 5-HT$_{2A}$ receptor cDNA was generously donated by Dr. David Julius of the University of California, San Francisco.

6. The native rat 5HT$_{2C}$ receptor cDNA was generously donated by Dr. Beth Hoffman of the National Institutes of Health.
CLAIMS

What is claimed is:

1. A method of constitutively activating targeted G protein-coupled mammalian monoamine receptors comprising the following steps:
   a. aligning a conserved amino acid sequence occurring in the sixth transmembrane domain of the targeted monoamine receptor with the conserved amino acid sequence in the sixth transmembrane domain of a second monoamine receptor for which a constitutively activated form having a mutation in the third intracellular loop is known;
   b. identifying in the aligned receptor sequences the amino acid position in the targeted monoamine receptor which corresponds to the amino acid position in the third intracellular loop which produced constitutive activation in the second monoamine receptor; and
   c. mutating, by site-directed mutagenesis, the identified amino acid position in the targeted monoamine receptor so that a different amino acid is substituted for the amino acid occurring in the native targeted receptor.

2. The method of claim 1 in which the targeted monoamine receptor is a G protein-coupled serotonin receptor.

3. The method of claim 2 in which the G protein-coupled serotonin receptor is the 5-HT\textsubscript{2A} receptor.

4. The method of claim 2 in which the G protein-coupled serotonin receptor is the 5-HT\textsubscript{2C} receptor.

5. The method of claim 1 in which the conserved amino acid sequence within the sixth transmembrane domain used for the alignment is WxPFFI, where x represents that any amino acid may occur at that position.

6. A method of constitutively activating G protein-coupled mammalian serotonin receptors comprising the following steps:
   a. aligning a conserved amino acid sequence occurring in the sixth transmembrane domain of the serotonin receptor with the conserved amino acid sequence in the sixth transmembrane domain of the \(\alpha_{1b}\)-adrenergic receptor for which a constitutively activated form having
a mutation in the third intracellular loop is known;

b. identifying in the aligned receptor sequences the amino acid position
in the serotonin receptor which corresponds to the amino acid
position in the third intracellular loop which produced constitutive
activation in the $\alpha_{1B}$-adrenergic receptor; and

c. mutating, by site-directed mutagenesis, the identified amino acid
position in the serotonin receptor so that a different amino acid is
substituted for the amino acid occurring in the native serotonin
receptor.

7. The method of claim 6 in which the G protein-coupled serotonin receptor is
   the 5-HT$_{2A}$ receptor.

8. The method of claim 6 in which the G protein-coupled serotonin receptor is
   the 5-HT$_{2C}$ receptor.

9. The method of claim 6 in which the conserved amino acid sequence within
   the sixth transmembrane domain used for the alignment is WxPFFI, where x
   represents that any amino acid may occur at that position.

10. The constitutively active 5-HT$_{2A}$ receptor in which the amino acid at
    position number 322 has been mutated from the cysteine found in the native
    receptor to an amino acid selected from the group consisting of lysine, glutamic
    acid, and arginine.

11. The constitutively active 5-HT$_{2C}$ receptor in which the amino acid at
    position number 312 has been mutated from the serine found in the native
    receptor to an amino acid selected from the group consisting of lysine and
    phenylalanine.

12. The DNA encoding the constitutively active 5-HT$_{2A}$ receptor in which the
    amino acid at position number 322 has been mutated from the cysteine found in
    the native receptor to an amino acid selected from the group consisting of lysine,
    glutamic acid, and arginine.

13. The DNA encoding the constitutively active 5-HT$_{2C}$ receptor in which the
    amino acid at position number 312 has been mutated from the serine found in the
    native receptor to an amino acid selected from the group consisting of lysine and
    phenylalanine.
14. A method of efficiently minimizing the number of full DNA sequencings, which must be performed on the colonies resulting from site-directed mutagenesis employing vectors, by eliminating most colonies not containing the desired mutation and by tagging colonies containing the desired mutation for easy identification comprising the following steps:

a. creating two primers, the first of which will remove a restriction site occurring in the original form of the vector and the second of which will introduce the desired mutation as well as a second mutation which specifies a unique restriction site not found in the primer;

b. annealing the primers to the vector;

c. synthesizing the second strands;

d. exposing the double stranded DNA to the restriction enzyme for the restriction site which occurs on the original vector thereby digesting the DNA containing the restriction site so that it cannot be taken up during a subsequent transformation;

e. transforming the test organism with the remaining double stranded circular DNA; and

f. testing the resulting colonies to see if they contain DNA which can be digested by the restriction enzyme for the unique site introduced by the second primer

whereby only DNA from those colonies which have incorporated the desired mutation will be digested with the restriction enzyme for the unique restriction site and the presence of such digestion indicates that that colony contains the desired mutation.

15. The method of claim 14 in which the following additional steps are performed after step e and before step f of claim 14:

e'. repeating a restriction digest using the restriction enzyme for the restriction site which occurs on the original vector; and

e'''. transforming the test organism with the remaining double stranded circular DNA.

16. The constitutively active 5-HT$_{2a}$ receptor coded by the DNA sequence specified in Figure 24 which DNA also contains a mutation creating a unique
restriction site.

17. The constitutively active 5-HT$_{2A}$ receptor coded by the DNA sequence specified in Figure 27 which DNA also contains a mutation creating a unique restriction site.

18. The constitutively active 5-HT$_{2A}$ receptor coded by the DNA sequence specified in Figure 30 which DNA also contains a mutation creating a unique restriction site.

19. The constitutively active 5-HT$_{2C}$ receptor coded by the DNA sequence specified in Figure 33 which DNA also contains a mutation creating a unique restriction site.

20. The constitutively active 5-HT$_{2C}$ receptor coded by the DNA sequence specified in Figure 36 which DNA also contains a mutation creating a unique restriction site.

21. The use of the constitutively activated mammalian G protein-coupled monamine receptor to screen for agonists, inverse agonists, and antagonists not previously identified as such at the native receptor.

22. The method of claim 21 where the mammalian G protein-coupled monoamine receptor is a serotonin receptor.

23. A transgenic mammal having incorporated and expressed in its genome a constitutively activated monoamine G protein-coupled receptor.

24. The transgenic mammal of claim 23 wherein the constitutively activated monoamine G protein-coupled receptor is a serotonin receptor.

25. The method of constitutively activating G protein-coupled receptors as described and illustrated in the specification.

26. The method of efficiently minimizing the number of full DNA sequencings as described and illustrated in the specification.

27. The constitutively activated receptors as described and illustrated in the specification.

28. DNA encoding constitutively activated receptors as described and illustrated in the specification.

29. The invention as described and illustrated in the specification.
Rat 5-HT$_{2A}$

1  cccaggctat gaaccctctag tcttccaca cttcatctgc tacaactcc ggcttagaca
61  ttgaaatctt ttgtaagacg aatctctctc tagctcaatc tccaaactcc ttaatgcaat
121  taggtgatgg cccgaggtcct taccataao tgcctcauct cagagatgct aacacttccg
181  aagcactgaa cttgacacat gatgtctgaa aacagaaccca cctctctgtg gaaaggtacc
241  tccaccggac atgcctctcc attctctacg tccagagaaa aacacttctt gctttattga
301  caacctgtctt gattttctct accattgctg gaaatatatact ggtcatcagtc gcagttgcc
361  tagaaaaaaa gctgcagaat gccaccaact attctctgat gtcactgacc atagctgata
421  tgcctgctggg tttctttctc atgcctgtgt ccagtttaac cattctgtat ggtaccgggt
481  ggcctttcgc tagcaagctgt tctgcgtatct ggtttaaccc gatgtgctgc ttttctacgg
541  catcccatct gcaccctgctt ggcactcttcctt tgcgcgtact cgcacaccctt ccaccccttc
601  ttccacccag cccgcttcaac tccagacacc aagctttctcctt gaaatatatt gcagttgagga
661  ccaatatctt agtgatatcc atgcacaccc ctgcttttgtt gctacaggat gattcgaagg
721  tctttaagga gggaggtgtc ctgctttcgc atgacactt tttttctcata ggccttttgg
781  tggcatttt tcatcccccta acacatcag tggatcaccct tccctctact atcaagctcag
841  ttgagaaag cggccaccttg ttgatgagtg acctcagcac tgcagccaaat ctagcctctt
901  tcagcttcct cccctcagatg tcctctctat cagaaagact cttccacccg tccatccaca
961  gagagcgcgg ctcctcagca ggcgcgaagga cgatgcagtc cacagcgaat gaggaaagg
1021  cgctcaggt ggtggccact tgggtctctc tgggttgggt cattgtgcc
1081  tcaccaaatc atgggctgtc atctggcagag aatcctgcaa tggaaatgtc atgggacacc
1141  tgctcaagt gttttgtcgg atgggttatac tcgcctcagc tgctgaactcc cttgtatatata
1201  cgttctctca taaaacctcat agttccgctt cttcaaggtc cattcagtc ctgtaaagg
1261  aaacacagaa ggcactcgcag ttaaatttatg tgaacactat accagcattg gcctacagaag

FIGURE 1A
FIGURE 1A - CONTINUED
FIGURE 1B
Rat 5-HT$_2c$

ORIGIN 23 bp upstream of HindIII site.

1 ggcgcctcgg tgcctactga ggaagctcc ttaggtgtac cgatcttaat gattgagccc
61 tggagcagc aagattgta atcttgggtg ctccttgggc ctgtctatcc cttacctccc
121 tattacatat gaaccttttc tctttctgca catcgatttg cgtgcgccgc tggagatcg
181 tctgtgtgtct cccgtggttg tcctctgtcg cttgagatag tggagagtctc ttcctatgct
241 caagaagaga agaagaagcg atggcgcgg cagagatgcgt gagggtcgctt ttctctatgc
301 agagtagggt agtgaaacaa tccccagcca aaccccttcc gggggcgcag gttgccccaca
361 ggaggtcgc acgctcgcgg cgcggcgctgc tgcctcgtct gcgctgcccc cttctctgtct
421 gctgacgcag cagttgtccgg cgcgcacgct gcgcagcacac ctcagctccgg cgggggctccg
481 tggggcagtt cagccagacgc ctgtctgtcgc ctcagctccgg cggcggcgcac cgtgccttggtc
541 ttctcctcgg acgcgtcggg gttgtcaact tacctaagca agcataaggg cacaagacacc
601 ttctctcagaa attaattgga atgaaacaat tctgttaact ctctaaactt cagttgaaaa
661 ctctgtggtc ttaagcctga agcaatacctg ctggacccctg gccaacggcggt ccgctgcgctc
721 ctgatgacc taatcggcct attggttggc caattcgata tttccataag tcagtagaca
781 gctataagta ctgcacactt taattcctcc gatggttggac gcttggttca atttcgggac
841 ggtgtacaaa actggcagcc gacattcatac gtcgtgatta taatcatgac ataggggggccc
901 aacattccttg ttaatcaggg acgaaagcatg gagaagaaac tccacaatgc aaccaattacc
961 ttcttaatgtt cccgtagcatc tgcgtatagtgc tggttgggcac tacctgtcatt gccccctgtccc
1021 tgcctgtgctaatttatgcttatgttcgg cttttacctataatatgggccccgtctggtg
1081 atttcactag atgtgtcatt tcaactgcgc tcaatagctgc acctctgcgc catatcgcgtg
1141 gacccggtarg tagcatacg tagcctatt gacgcatagcc gttcaattcg cggcagctag
1201 gcccattgca agatgtgcatc cggtttggcca atataaatag gatgttcatg tcctatcctct

FIGURE 2A
FIGURE 2A - CONTINUED
MVNLGNVRSLLMLHLIGLLWQFDISISPVAIVTDFTNSSDGG
RLFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVMSEMKLHNATNYFLMSLAIADML
VGLLVMPLSLLAITALYVVWPLPRLYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNP
IEHSRFNSRTKAIMKIAIVWAISIGVSVPIVIPRAGLRESKVFNNTTCVLNDPNFVLI
GSFVAFFIPLTMVITYFLTIYVLRRQTLMLLRGEHTEELANMSLNFLNCCKKNNGGE
EENAPNPNDQKPRRKKKEKPRGTMQAINNEKKASKVLGIVFFVFLIMWCPFFITNI
LSVLCGKACNQKLMKLLNVFVWIGYVCSONPLVYTLFNIYRRAFSKYLRCDFKPD
KKPVRQIPRAATALSGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS
NVVSRISSV

FIGURE 2B
Rat α₁₈-adrenergic

MNPDLDTGHNTSAPAHWGEKLDDNFTGPNQTTSSNSTLPQLDVTR
AISVGLVLGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLPFS
ATLEVLGYWVLLSFFCDIWAAVDVLCCCTASILSLCAISIDRYIGVRYSLQYPTLVTRR
KAILALLSVVVLSTVISIGPLLGWKEPAPDDEKCEGVTEEPFYALFSSLGSFYIPLAV
ILVMYCRVYIVAKRTTKNEAGVMKEMSNSKELTIRIHSKNFHEDTLSSTKAKGHNPR
SSIAVKLFSREKKAATLGVVGMFILCWLPFFIALPLGLSFSTLKPPDADVFKVVF
WLGYNNSCLNPIYPCSSKKEFKRAFMRLGQCQCRGGRRRRRRRRRLGACAYTPWTRG
GSLERSQSRKSDLDDGSCMSGTQRTLPSASPSPGYLGRGTQPPVELCAFPEWKPGAL
LSLPEPPGRRGRLDSGPLFTFKLLGDNPESPGTEGDTSNGGCDTTDLANGQPGFKSNM
PLAPGHF

FIGURE 3A
Rat $\alpha_{1b}$-adrenergic

1 gggcggaatt taaaatgaat cccgatcttg acaacccgcca caacacatatca gcacctgcc
61 acctggggaga gttgaaagat gacaacatta ctcgccccca aaccagctctag agcaaactcc
121 cactgccccc gctggagcttc accagggcca tcctctgtgg ccctggtgtg ggccgcctta
181 tcctcttggc ctcgctgggg aacatctcgg tcactcctgc gctggcctgc aaccggtaccc
241 tcgccgacgcc caacacactc ttatcgctca acctggccat tcctgacctg ctgttgaat
301 tcacagctact gcctcttcct gcctacctag aagtgctttgg ctcagctgtgc ctgttgaat
361 tcctcttgga ccatcgggga gggtgtatag tgcctgttcg tggcgtctcc accctgagcc
421 tattgtccat ctccattgcg cgctacattg gggtgcgata ctctctgcag tacccccaccg
481 tggctccccc cggagggcct aacgctggtg tcctcgatgt gctgggtctg tccagctgta
541 tcctccatcgg gcctctctcc ggatggaag acacgtcggcc caatgatgac aaaaagatgg
601 gggtcaccga aacacccctc tacgccctct ttctctctct ggctcctcct tacatccgcc
661 tcgctggctat cctgtggcatg tactgcgggg tcctacatgt gcgcaagagg accaccaaga
721 atctggaggc ggagtctagt aaggaatgt ccaacctcag ggagctgacc ctgagatcc
781 aacctcaagaa cttctcatag gcacacccc gcaagtaccc gcacgaaggg ccacacccca
841 ggagttcctg agctgtgcctt ttttttaagtt tctcagggaa aaaaagagga gccaaacact
901 tggggctttgt agtgcggaatg ttcctcctat gtgggtcctc cttcttcact gcctctccgc
961 ttggctccct gttctccacc ctaagccccg cggacgctgt gttcaagggtg gttgcttgc
1021 tgggctacct gcaacagctc gctaatccca ctaatcacc gcctactcgc gttgctccacg aaggagttca
1081 aggcgacgctt catgctgtac ccggggtctcc agttcggccgg tcgccccgcc gcggcgcggcc
1141 gttcgccgtct aggccgctgc gcctacacct accgccccgt gacccggggt ggctcgcggg
1201 agagatcaca ctcgccaag aacctcttgtg atgcagcggc cgctgctagc agccggtaccc
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FIGURE 3B - CONTINUED
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<td></td>
</tr>
</tbody>
</table>

Transmembrane Domain VI

c-terminus

**FIGURE 4**
Restriction Site → pcDNA3 → 5-HT2A insert

Selection Primer
- removes
Pvu1 site

Synthesize second strand with T4 DNA polymerase & ligase
Digest with Pvu1

Anneal Primers

Mutagenic Primer
- creates Sca1, Rsa1, Mnl1 sites
- C322K, C322E, C322R

Transform mutant E. coli
Grow in LB/AMP
Isolate plasmid DNA

Wildtype DNA

Digest DNA with Pvu1

Mutant DNA

- transform mutant E. coli
- grow in LB/AMP
- isolate plasmid DNA

Mutant DNA

- digest with Sca1, Rsa1, and Mnl1 to confirm mutation

FIGURE 5
Site-directed mutagenesis procedure for the 5-HT2C receptor.

Sma1 Restriction Site → pcDNA3 5-HT2C insert

Anneal Primers

Selection Primer
-removes Sma1

Mutagenic Primer
-creates SCA1 site
-S312K, S312F

-synthesize second strand with T4 DNA polymerase & ligase
-digest with Sma1

-wildtype

Digest DNA with Sma1

mutant

-transform E.coli
-Isolate DNA

mutant

-digest with SCA1 to confirm mutation

FIGURE 6
<table>
<thead>
<tr>
<th></th>
<th>Native 5-HT_{2A}</th>
<th>Cys→Lys Mutant</th>
<th>Cys→Arg Mutant</th>
<th>Cys→Glu Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>293±3.0</td>
<td>25±2.1*</td>
<td>10±1.7</td>
<td>86±2.9</td>
</tr>
<tr>
<td>DOB</td>
<td>17±1.4</td>
<td>2.3±0.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOM</td>
<td>144±52</td>
<td>28±0.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiperone</td>
<td>1.1±0.1</td>
<td>2.4±1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methysergide</td>
<td>0.3±0.1</td>
<td>6.0±0.7*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketanserin</td>
<td>1.0±0.3</td>
<td>1.0±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mianserin</td>
<td>3.9±22</td>
<td>13±2.0*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 8**
FIGURE 9
FIGURE 10
$B_{MAX} = 193 \pm 37 \text{ fmol/mg}$

$B_{MAX} = 218 \pm 31 \text{ fmol/mg}$

FIGURE 11
FIGURE 12

% Specific Binding vs Log 5-HT [M]

- K Mutant
- F Mutant
- Native
<table>
<thead>
<tr>
<th>Agonists</th>
<th>Native</th>
<th>K Mutant</th>
<th>F Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>203 +/-10</td>
<td>6.6 +/-1.2</td>
<td>76 +/-7.1</td>
</tr>
<tr>
<td>5-MT</td>
<td>519 +/-104</td>
<td>5.8 +/-1.1</td>
<td>ND</td>
</tr>
<tr>
<td>(+/-)DOB</td>
<td>256 +/-38</td>
<td>6.7 +/-0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Native</th>
<th>K Mutant</th>
<th>F Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesulergine*</td>
<td>0.6 +/-0.1</td>
<td>1.2 +/-0.1</td>
<td>1.3 +/-0.2</td>
</tr>
<tr>
<td>Mianserin</td>
<td>1.7 +/-0.2</td>
<td>3.0 +/-0.7</td>
<td>ND</td>
</tr>
<tr>
<td>Methysergide</td>
<td>0.5 +/-0.1</td>
<td>0.9 +/-0.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

FIGURE 13
FIGURE 14
<table>
<thead>
<tr>
<th>5-HT&lt;sub&gt;2C&lt;/sub&gt; Receptor</th>
<th>5-HT EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; (nM)</th>
<th>Bmax (pm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>70 +/- 18</td>
<td>0.6 +/- 0.1</td>
<td>1.5 +/- 0.2</td>
</tr>
<tr>
<td>F Mutant</td>
<td>28 +/- 2.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.3 +/- 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.6 +/- 0.1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>K Mutant</td>
<td>2.7 +/- 1.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.2 +/- 0.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.4 +/- 0.2</td>
</tr>
</tbody>
</table>

FIGURE 15
FIGURE 16
FIGURE 17
FIGURE 19
Human 5-HT2A

1  gaattcgggt  gagccagctc  cgggagaaca  gcagctacac  cagcctcagt  gttacagagt
61  gtgggtacat  caaggtgaat  ggtgagcaga  aactataacc  tgttagtctt  tcctacccctc
121  atctgtcaca  agttctggct  tagacatgga  tattctttgt  gaagaaaaata  ctctttgtgag
181  ctcaactacg  aacctcctaa  tgcaatattaa  tgatgacacc  aggctctaca  gtaatgacctt
241  taactctgga  qaagctaaaca  cttctgatgc  attaaactgg  acagtcgact  ctgaaaatcgc
301  aaccaaccccctc  ttctgtaag  gggtgcccttc  acgctgtcgtt  cttctctttac  ttcatactcca
361  ggaaaaaacac  tgtctctgctt  tactgacacgc  ctgatgagtattcttaacta  tgtctgaaaaa
421  catactcgtc  atcatggccac  tgtctccata  gaagaagctg  cagaaatcggca  ccaactattt
481  ccttgatctca  cttggccataag  cttgatgcttctc  gttggttttc  cttgatcgtcc  cggttgtctcat
541  gttaaccacatct  tgtatgcttc  accgggtgcc  tcggccggagc  aagtttttqgtg  cagttctggtat
601  ttacctgaggtctgttttctc  ccaacggtccgtc  catcatgcac  ctctgctgcaca  ttcgcttgaga
661  ccgctacggtc  gccatccaga  atcccatcca  ccacagccccg  ttcaactccca  gaactaaggc
721  atttctgaaa  atcattcttg  tttggacact  atcaagtatgt  atatacagcac  caaatacagt
781  ctttgggcta  caggacgatt  cgaaggtttc  taaggagggg  agttgtttaac  tcggccgatqa
841  taacctttgc  cttggcggtct  cttttgggtc  atttttcattg  cccttaacca  tcagttggtat
901  cacctacttttctaatctaca  agtcatctcaca  gaaaaagact  actttttgtctg  taagtgtatctt
961  tggcacacgqg  cccaaaaggtc  cttcttccagtct  cagagtttctt  tgtcttcgaga
1021  aagccttttcc  cagcccgcagtc  tccataaggq  gccaggtcc  tacacaggca  gaggactat
1081  gcagtcctctc  acgcatgtgac  aaaagccatg  caaggtctg  gccatgcttct  ctctctgtttt
1141  tgtgtgtatgc  tggctttctctc  tcttcatcact  acacatcatg  gcgcgcactct  gcaagagct
1201  ctgcaatgaagatgtcttacg  gggcccctct  caatgtgtttctg  tttggatcgc  gttatctctc
1261  ttcaagcagtc  aacccactagt  tctacactc  qtgaacaaaga  acctatacgt  cagccttttctc

FIGURE 20A
FIGURE 20A - CONTINUED

2701 ccttcaaacg aaatggctcg gccaggcacg gaggctcggtg catgtaatcc tagcaaccttg
2761 ggaggctgag atgggaggat cacttgaaggc caagagtttg agaccaacct gggtaacaaa
2821 gtgagacctc cctgtctcta caaaaaaaaaat caaaaaaatta tctgatcttt gtagaacaca
2881 actgtggtcc cagctacagg ggaggctgag acgcaaggat cacttgagcc cagaagctca
2941 aggctgcagt gagcccaagtt cacaccactg ccatttcctc ctgggaacaacagagtgagacc
3001 cttatcaccccc gaattc
Human 5-HT2A

MDILCEENTLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFN
WTVDSENRTNLSCSCEGLSCLPSSLHLQEKNSALLTAVVIILTIAGNILVIMAVSLE
KKLQNATNYFLMSLAIADMMLLGFLVMPVSMILYGYRWPLPSKLCAYAVWYLDVLFST
ASIMHLCAISLDRYVAIQNPIHHSRFNRTKAFLKIIAVWTTISVGISMPIPVFGOQDD
SKVFKEGSCLLLADDNVVLGFSVSSFIPLTIVTYFTIKSLQKEATLCVSDLGTRA
KLASESFLPQSSLSEKLFQRSIHPGSYTGRRTMQSISNEQKACKVGLIVVFLFVV
MWCFFITNIMAVICKEVNEDVIGALLNVFWIGYLSSAVNPLVYTLFNKYRSAFS
RYIQCQYKENKPLQILVNTIPALAYKSSQLQMGQKNSKQDAKTDNDCSMVALGK
QHSEEASKDNSDGVNEKVSCV

FIGURE 20B
Human 5-HT₂c

1 gaattcggga ggcgcctcag atgcacccgac cttccccgata ctgcccccttgg agccggcctaa
61 ttgctagctt ttgctgccct attggccctgc cttgccccctt acctgcgctg tgtatgatat
121 ctcctctctt ctctgtacat cgttgctgct gcaggcgtgct cgaatgcgtct gcgcgtctgtg
181 tgcattccct cgtccggttta gatactgtga gttatgtagg ggccaacgaa gagaaggaag
241 gacgcgattt gctcgagagac gctggaggttc gtcggtactt aagtagagttta aagatagcgg
301 agcgaaagaa gcgcaacacta ggcggggggc gcaggtggctc ccaagagagg tcacattgcc
361 ggccgtctcct tattgcgcag gctctctctta ttctctctctc tgggagcgaggtt ggagggggtt
421 cgcgacgcag cgcgacgcag ctcagcgcac gcagcgcgcgc ggccgcgctgc ggcgggttgc
481 agcccgagctc gttttctgcag tgcgtgcgcgc cgcggcgaggtc gctgcgctggtt ctctctctccg
541 gacgcgagaag ggtattcgcac taacacccgc gcggctacat aacatagacc aacgtgacgcc
601 atcctttcaaa aacaactgtcc tgggaaaaag gaaataaaa aaggtgtgag agcagaaaaac
661 gtaggtggag cacgaccaatt cttctctcag tgggaaaggg tgggaaaaag gggatgatat
721 gtagaacccta gcctgttaaat ttcgctttcctt ctaatttaaa ctttgggtgct ttaagactga
781 agcaatcattg tgaacccctgt cgaatgcgctct gcataatttc cttgctgac ccacagagctct
841 atggggttg ccaatgtgatttt tttcttctggag cccagagagtc cccggcagaactaattccaa
901 cacatactcc gatggttggcct gctctggacat cccagacggg gtatcataaa gacatgacagt
961 ttcaatcctc atcataaaatg tcatgacacat ggttgacacat ttctttgtgattgtgcaggt
1021 aacatggaga agagactcag acaatggccac caattttctc ttaattgtgcc ttagccatgtc
1081 ttggatgtctt ccgagactc cggctctgtcct cttgcaatcc ttagatgattatagttcagtc
1141 tggatgcaacc ctcctctagatt ttggtgcccc ccccttggttat ctccttttccgctggttttaag
1201 aacagcgttcc atcatgccact tggctgctat atcgctggtct cggaggttaga taatacggtaa
1261 tcctcagttt ccaatttccg gactaagggc atcatgaaga ttgctatggagttt

FIGURE 21A
1321 ttgggcaatt tctataggtg tacagttgcc tatcctctgtg attggactga gggacgaaga
1381 aaaggttgtc gtgaaacaaca gcagctcagctg gctcaacgcac ccacaatttc tctttattg
1441 gtccttcgta gttctctcca tacgctgac gattatgttg attacgtatt gccgaccat
1501 ctacgttctg ggcggcaaca cttttgatgtt actgacaggg cacacggagg aacgctcctgg
1561 actaaatctg gattttcaga aagttcctcag gccggagcaag agaactctgcc
1621 aaacccataac caagaccaca agccacagtgg aagaaaggaag aagagagagc gtcttaaggg
1681 caccatgcag gctatcaaca atgaaagaaa agcttctgaaa gtccttgaga tttttgcttt
1741 tgggtttttg atcatgtgtgt gcccattttt cattaccaat attctgtctg tctttttgta
1801 gaagtcctgt aacccaagac tcatgaaaaa gctttcaagat gtgttttgtt ggtttgtctta
1861 ttggttcctca ggaatcaga ctcgctggtg tacctgcttc aacaaattt accaaagacc
1921 attctcaacat aattttgctg gcaattataaa ggttagagaa aagctcctcg tcaagcagat
1981 tccagaggtgccgccactg ccctgtcctg gaggagctt aagtttaaca tttatcggca
2041 taccaatgaa cccgtgtatcg agaagacgag cagcaatagcg cccgttatag agatgcaagt
2101 tgagaattttagttaccag taaatccccctccagttggtgctt agccggaaaggttagcaggt
2161 gtgagagaga acagcacagt cttttctacg gtacaagcta catatgtagg aaaaatttct
2221 tctttataatt ttcgtttgtt ctaactaat gtaaatattg ctgtctgaaa aagttttttt
2281 acatatagtctgcaacctt tcaacttaca atcatgctca cattagctag atttagggtt
2341 ctatatttac tgtttataaat aggctggagac taacctttt tgaatgttgt aagtaataaa
2401 tgttttttttgtcttcct gcccctccttc cttctcttctt cttctctttctct cttcctctttc
2461 cttctctttctgtgtcatatg ccaacgttca ttctctcttc agttggtcatt tgcaggtgac
2521 cagatgagg cacatcgacgt tgtttatttt tcaaccaacac ctaattaatc aatctcagtg
2581 gacatgtgctgggcttaac agtaataata cttaacctatctgtgtgcttg ctcatctaca
2641 ctataaaacacagtaaggata gttctgtcct ttgctgactct ctgtcagtgac gtcagaggca

FIGURE 21A - CONTINUED
2701 gaacctagtc ttgttgtca tatagggaa ttc

FIGURE 21A - CONTINUED
Human 5-HT<sub>2C</sub>

MVNLRNAVHSLVHLIGLLWVQCDISVSPVAIVTDIFNTSDGG

RFKFPDGVQNPALSIVIIIIMTIGGNILVIMAEMSMEKLHNATNYFLMSLAIADMLV

GLLVMPSSLLAILYDVWPLPRLCPVWISLDVLFLSVMASIMHLCAISLDRYVAIRNPI

EHSRFNSRTKAIMKIAIWASIGVSVVIPVIGLRDEEKVFVNNNTCVLNDPNFVLIG

SFVAFFIIPLTIMVITYCTLTVRLRQALMLHGHTEEPGLSLDFLKCCKRNTAEEEN

SANPNQDQNARRKRRKERRPRGTMQAINNERKASKVVLGVFFVFLIMWCPFFITNILS

VLCEKSCNQKLMKLLNVFVWIGYVCSGINPVLVYTLFNKIYRRAFSNYLRCNYKVEKK

PPVRQPVRVAAATALSGRELNVNIYHRTNEPVIEKASDNSPGIEMQVENLELPVNPSSV

VSEIRISSLV

FIGURE 21B
Rat 5-HT$_{2A}$ Cysteine → Lysine Mutant

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDNFSRDANTSEASN

WTIDAENRTNLSEQYLPPTCILSLHQLQKNWSALLTTVVIILTIAIGNILVIMAVALSE

KKLONATNYFLMSLAIADMLLGFLVMPVSMLTILGYRWPPLPSKLCAIWYLDVFST

ASIMHLCAISLDRYVAIQNPISHSFSRTKAFKLIIAVWTSVGISMPIPVFGQLDD

SKVFKEGSCCLADDNFSVLIGSFVAFIPLTMVYFLIKSLQKEATLCVSDLSTRA

KLASFSLPQQSLSEKLFRFRPSHYAGRRTMQISNEQKASKVLGIVFFLVV

MWCPFFITNIMAVICKECNENIGALLNNFVFWIGYLSAVNPLVYTFLNKTYRSAFS

RYIQCQYKERNRPQILNLVTIPALAYKSSLQVVGQKNSQDEAEQTVDCSMVTGK

QQSEENCTDNIETVNEKVSVCV

FIGURE 22
Rat 5HT$_{2A}$ Cysteine → Lysine Mutant

1 cccaggctat gaacccctag ttctccccca ctctctcctg tcaacactcc ggtttagaca
61 tggtgattct ttgtagagac aatattcttc ttagtctcat tccaaactcc ttaattgcaat
121 tagtgtaggg cccgaggctc taccataatg aactcaactc cagagatgct aacacttccg
181 aagcatcgaa ctggcagaat gatgctgaaa acagaacccaa cctctcctgt qaaggtgtacc
241 tccccaccgc atgccttcctc atctttcactc tccagggaaa aaactgctgt gctttattga
301 ccaactctct gattattctc accattctgt qaataatact ggtcatcatg gcaggttccc
361 tagaaaaaa gctgcagaaat gccaccaact atttctctag atcacttggcc atagctgata
421 ttgctgctgg ttctttgtgc atgcctgtgt ccattttaac actcctgat ggttagcggt
481 ggcccttgcc tagcagacgc ttgctgacat gqatattacq gqatggtgcct tttcttacgg
541 cctccatct gcaccttgcgg cccatcctcc tggccggtta cntgccccct cagaacccccca
601 ttcaccagag cccgtttaac ttcagagcct aagcctttct gaaatctatt gcccgtttgga
661 ccaatctgt aggtatatac atggtacatcc cagtctttgg actacaggt gatgcgaagg
721 tcttaagga ggggaagctgc ctgctttgcc gatgacaactt ttgtctcata ggccttttttgg
781 ttggcatgctt catccccctta accatcatgg tgatcaacca cttcctgact atcaagtcac
841 ttccagaaaga agccaccttg ttgtgagtg acctcagcag tcagccaaaa ctagctctct
901 ttggcctctct cccctcagaqt tctctgctat cagaaaaagct ctcccaacggg cccatccaca
961 gagagccgaag gttctcagga gcgccaagg gaatgcagtc ctcagcagat gqacaaaaagg
1021 gggggaggg cttggtccttct gtttgtgtt gatgtgttgc ccattcttcca
1081 ttccacatag ctggccccgc tctctgcctg gatgtgtgtgtg atctctgctac atcggagccc
1141 tctcattgct ttgtctttgg atgttttact ctcttcagct gtagatccttca ctgtatataa
1201 cttttgagta taagactttt gatggtcctt cttctcagta cttcactggta cagtacaagg
1261 aaaaagaaaa gccacagcag ttaattttg tgaacactat accagccttg gccttcaggt

FIGURE 23
1321 ctagtcagct ccaagtgqga cagaaaaaga actcaacagga agatgcgtgag caggacagtgg
1381 atgactgtctc catggttaca cttgggaaac aacagtgcgga agagaattgt acagacaata
1441 ttgaacccgt gaatgaaag attagctgta tgtatgaac tggtatctat ggcaattgcc
1501 cagggcatgt gcacaagtt atacccatgt gttgtggggc gggataaggga ggtgcaaca
1561 aattag

FIGURE 23 - CONTINUED
Rat 5HT$_2A$ Cysteine → Lysine Mutant with Restriction Site

1
cccaggctat gaacccctag tctctccaca ctctcattgc tacaacttcc ggcttagaca
ttgaaattct ttgtaaagac aatatcctc tgaacatc tctcaacctcc ttaatgcaat
121
tagttgatgg cccaggctc taccataagc acttcaacctc cagagactct aacacttccg
181
aagcatgcaat gattgctgaa aacagaacca ccttctcgtg gaaggtgtacc
tccacccgac atgctctcct cattcctctc tccagagaa aaactcggtg gcttattgga
241
cacatctgct gattatgctt cacactgcttg gaaatatct ggtcatctag gcaagtgtccc
301
tagaaaaaa gctgcaagat gccaccaact atttcggtat gttcacttccc atagctgata
tgctgcggg ttctctggtc atgcgtgtgt ccctgatttc catcttgatg ggtaccggt
361
ggcccttgcc tagcaagctc tggcgtatct ggaattacct gggtctgctc tttctacgg
421
catccatcat gcacctctgc gcctatcctc tgggaggctga tgctgccatc cagacccaca
ttcaccacag ccgccctcct ctcacagaca aagctttcct gaaattcatt ggcgtttgga
481
ccatactgt agtgatatcc atggccttctt cagtctttgg actacaggat qattcgaagg
tccttaagga ggggagctgc ctgctgtcgc tggcagacct tggctttttg
tgcattttt catccccttc ataaccatgg tgtcactctc ctctctctct
ttcagaaaga agccaccttg tgttgtgagtg acctcagcag tgcagccaaa ctggctctct
541
tcagctctct cccctcagatg tctctgtcat cagaaagct tctccacagg tccatccaca
961
Start C322K primer
gagagcaggg ctcctacgca gccggaagga cgattcagtc cttcagcaat cagacaaaaaggg
End C322K primer
1021
Mutations to create Sca1 site
tccaccatat catggccgtc atctgcaagag atctcctgca aagaaatgtc atggagccccc
1081
tgctcaagt gttggtctgg attggttacg tcgctcctagr tgcaatccaa cgattatata
1141
cgattccttc aaaaatctc cccaagctct tctcaaggtc catctcaggt caatcaggg
1201
cggctgcaag cagagctatat acagttcagtct cctcaaggtc caatcaggt gcattacaggt
1261
aaacagaaaa gcacggcgag ttaatattag tgaacactat accagcatgg gcctacaaggt

FIGURE 24
FIGURE 24 - CONTINUED
Rat 5-HT$_2A$ Cysteine → Arginine Mutant

MEILCEDNISLSSIPNSLMLQGDPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTLSILHLQEKNWSSLTTTVIILTIAAGNILVIMAVAL

KKLQNATNYFLMSLAIADMLLGFVLMPVSMILTYGYRWPLPSKLCAIWIYLDVLFS

ASIMHLCAISLDRYVAINPICHRSFNSRTKAFLKIAVWTISVGISMPIPVFLQDD

SKVFKEGSCLLLADDNVFLSFGFVAFFIPLTMIVITYFLTIKLQKEATLCVSDLSTRA

KLASFSLFPQSSLSSEKLFQRSIHREPGSYAGRRTMQISNEQKAKVGLIVFFLFVV

MWCPCFFTINAVICKESEENAVGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQILTVNIPALAYKSSLQVGKKNSEQEADAEQTVDCCSMVTLGK

QQSEENCDNiETVNEKVCV

FIGURE 25
Rat 5HT$_{2A}$ Cysteine → Arginine Mutant

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaaccttc ggcttagaca
61 tggaaatctc ttgtagaacac aatactctctg tgaagctcag tcacaaacctc ttaatgcaat
121 taggtgtatg cccaggctc tccataagtg aacctcaactc cagagagtct aacactctcc
181 aagaattcagag ctggcaakaat gatgctgaaa acagaccaaa cctctctccg caggggtacc
241 tccacaccgc atgctctctcc attctctcatc tccaggaaga aaactgtgtct gctttattga
301 caacctctct catgtttcctg accattctctg gaaatatcct ggtctcatct aaggttgccc
361 tagaaaaaa gctgcagaat gcaccacaact attctctcg aatcactctcc atagctgata
421 tggctctggg tttctgtgtc atgtctgctgt ccatgtttaa cactctgtat ggtctacggg
481 gcctttggcc ttagaacgct tggcctgtct ggttgttac ccggtttgcc ggtgttgcct tttttatacg
541 catccatcat gcaccttccc gccatctccc tggaccgcta tggctcctcgc aagacaacca
601 ttcaccacag ccgctctcag tccaggaacc aagctctctct gaaatctatt ggcgttggga
661 ccctatgtct ggtttatatc atggctcaacc caagttttttg acctacaggt gattcgaagg
721 tctttagga gggagctgct cttgctttcct gtaaaccatt tttttccaca ggtctttttt
781 tggcatttttt cattccccctg aacattcagc tgactaccta cttctctgtct atcaagtcac
841 ttcaggaaga agccaccttg tgtgatagtg acctcagcac tgagccaaac ctagccctct
901 tcagcttct tccctcaaggt tctctgtctc cagaaaaagct tctcacaacgg tccatccaca
961 gaggacggg ttccttccga ggcggcagag cagatgactc catgagcaat gaccagaaag
1021 cgg@aqaggt gctggtctcc ttgatgtttgct aatgtggtgc ccttcttca
1081 tcaccaatgt catggccgctc atctgcaaaq aatcctgcaaa ctgaaaaatgct atcggagcgc
1141 tcctcatatgt gtttttttgg atgggtttac tttctctcag tgcgtctcaca ttggttatata
1201 ctgtgttcaaa taaaacttat agatccgctt cctcagaaga atctcaggtc cattacaagg
1261 aaaaacgaaa gccactctcag ttaattttag tgaacactat accacacttg gcttacaagt

FIGURE 26
1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgcctgag cagacagttg
1381 atgactgctc catggttaca ctggggaac aacagtcgga agagaattgt acagacaata
1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgc
1501 cagggcatgt gaacaagggt atacccatgt gtgtggggcg gggataagga ggctgcaaca
1561 aattag

FIGURE 26 - CONTINUED
Rat 5HT$_2A$ Cysteine → Arginine Mutant with Restriction Site

1 cccaggttat gaacccttag tctctcaca gaatcatgctg tcaaccttc gcgcttagcag
61 tggaaatcttt tttgtaagac aatatctctct tagacctcaat tccaacctcc ttaatgcaat
121 tagtgtagcgg cccgagggtc taccataatg actctcaact cagagatgct aacaacctcg
181 aagcatcgaa ctggcaactt gatgttgaaa acaagaacca cctctcctg gtgggttacc
241 tccccaccgac atgcctctcc atctctctat tccaggaaaa aacactgtct gctttatgga
301 caacctttcg gattacctc accatgtctg gaaatataac ctggctcagct gcaggttccc
361 tagaaaaaaa gctgcagaa gccaccaact attctctgat gcacttggcc atagctgata
421 tggctctggg ttctttgtgc atgctctgat ccagtttaac cctctgtat gggtacccgt
481 ggctttggcc tagcaagctc tggctgcctgt gattttacct ggatgtgctc ttttctaggg
541 catcccatcg gccacctcgc gccctttcctc gggcaccgcta tggccgctaag caragcctcca
601 ttcaccacag cccgcttcaac cccgaaacca aagcctttcct gaaatattt gccgtggtgga
661 ccatatcttg aggttatatcc atgcacaatcc cagcctttgg actacaggg gattcgaggg
721 tctctagaagggagagctgc gctgctggcc atgacaactt tgtctgata ggcctttgttg
781 tggcatttttt cattcccccta aaccatcaggg tatgacacca tctctctact atcaagtcac
841 ttcagacaag aaccaccttg tttggtggttg acctcagcac tggacgaaaa ctgccttctct
901 tcagctctc ccctcagatgt tctctctcat cagaaaaagct ccctcagcagg tccatccaca
961 gagagccagg ctctcagcga ggccgagagga cgatgcaagtc ctacgcaact ganclaaagg

Start C322R primer

1021 cg@aggtgcgtggcctcggtttttttgt aatgtgtggtgc ccattcttca
1081 tccaccaaat catggccgctc atctgcaag agatcctgcaaa taagaatgtc atccgagccc
1141 tgctcatgtg tttgttgctg attgattatc ttctctcagc tgcaatcaca ctggtatatata
1201 tagttgtaaa taaaacttat aggctccgctc tctcaaggtat attcagatgt cagtacaagg
1261 aaaaacaaaa gccacatcag ttaatattta gcaacaactat accagctgg gcctacaagtt

FIGURE 27
FIGURE 27 - CONTINUED
Rat 5-HT2A Cysteine → Glutamic Acid Mutant

MEILCEDNISLSSIPNSLMQLGDPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTRLSCILHLOEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMMLGFLVMPSMLITYGYRWPPLPSKLCAIWYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRNFSRTKAFLKIIAVWTISVGISMIPVFGLODD

SKVFKEGSCLLADDNFLIGSFVAFFIPLTMIVYFLTIKSLQKEATLCVSDLSTRA

KLASFSLPQSSLSSEKLFRHQRSTYAGRTMQSISNEQAKEVGLIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKSQEDAEQTVDCCSMVTLGK

QQSEEENCTDNIETVNEKVSCV

FIGURE 28
Rat 5HT$_2A$ Cysteine → Glutamic Acid Mutant

1 cccaggctat gaaccccttag tctctccaca ctctctctgc tacaacttcc ggcttagaca
61 tggaaatttt ctggagaaac aatatcttc tggagctcaat tccaaacttc ttaatgcaat
121 taggtgatgg ccggagggc tc taccataatg acttcaactc cagagatgtc aaccacctgg
181 aagcatcqaa ctggacaatt gatgctgaaa agacaaccaaa cttctcttct gaaaggtacc
241 tccaaccgac atgcctctcc atctctccat cccagaaaaa aaaccttcct tgtttatgta
301 caactctctt gattttctcc accatgctg aatataatact gtctcatcatg gcaaggttccc
361 tagaaaaaaa gctgcagaat gcaccaacact atttcctgat gtcatctgccc atagctgata
421 tgctgctggg ttctctgtcc atgcctctgt ccaagttacc cacctctgtat ggttaccggt
481 ggctctggcc tagcaagctc tggctgatct ggtcttacct ggtgtcgcttt tttctacgg
541 catccatcat gcacctctgc gcacatctcc tggaccacca tagtgccaca tcagaacccca
601 ttcacacag ccgcttcaac tccagaacca aagacctcct gaaatcatt ggcgtggtgga
661 ccatatcct tcaattatccc atgcacatcc cagctttggg actacaggat gatccgaagg
721 tcttaagaga gccgagctcg ctgcctgcccg ctagcacaact ttctctcata ggtcttttgt
781 tggcattttt catcccccta accatcat gg tagcagcal cttctctgct atcaagtac
841 ttcagaaaga agccaccttg ttgtgagtg acctcagcac tggagccaaac ttgagcctctt
901 tcagctctct ccctcagagt ttctctgcat cagaaaaagct ccctcagccct tcataaccca

Start C322E primer
961 gagagccagg ctctctacgc ggccgaaggg cgaagcagtc ctcagccaaat gacaaaggg

End C322E primer
1021 cgq8qaqaggt gctgggcatc gtgctttctc tgggtgtggt gtacggtgct ccattcctca
1081 tcaccaatat cagggccgtc atctgcaagag aatctctgcaaa tggaaatggtc atcggaagccc
1141 tctctcaagt gtgtgctggg attggtttatc tcctctcagc tgtcaatcca ctgtatatata
1201 cgtgttcaaa taaaacttat aggtccggct tcctcaaggt cattcaaggt cgtcacaaggg
1261 aaaaacagaa ggcacgcaag ttaatittag tgaacactat accagcatgt gcttacaagt

FIGURE 29
FIGURE 29 - CONTINUED
Rat 5HT<sub>2A</sub> Cysteine → Glutamic Acid Mutant with Restriction Site

1 cccaggctat gaacccctag tccttccaca ctctatctgc tacaacctgg gcgttagacg

61 tggaaatctt ttgtgaagac aatatcttcg tgaagccatgt tccaaactcc ttaatgcaat

121 taggtgatgg cccgagggc tcaccataatg aaattcaactc cagagatgct aacactggcg

181 aagcacgga ctcgacaaatt gatgtgaaat acagaaacaa cctctctcgt ggaaaggtacc

241 tccacgccac atgccctctcc atctctcttc tccagaaaaa aaactggtct cgttatattga

301 caactgtcgt gattattcct accattgtcg gaatatata ggtctcatag gcagttctcc

361 tagaaaaaag gctgcgagaatgcc accaaactc attctctgtat gtcactgccc atagctgata

421 tgtgtgctggg ttctcttctg atgctctgtat cctgtttaaac catctcgtat ggattcaggt

481 ggctcttcgcc tagcaagctct tggcgcagct ggattctctg ggtctctctcc ttctctcaggg

541 catcccaatcg acactctctcg ccacatctcc tggaccgcta tgtgcacgtc atccacccca

601 tgtcaccacag ccgctttcaac tcctcagaaaccagcttctct cggatctgga taatatcatt gccgttgtga

661 ccatactgtaggtatatcct atgcaaccttccc gcgagttggg gattacaatt gattcaggg

721 tcttaaggg gggagctctgctgtgcctgcc atgacaactt tttctctgtcctgtcagttggtctttgg

781 tggcatcttt catcccccta accatcatag tgatcactta cttcttgaact attgaagctc

841 tgtcagaaaga agccacccctt gttgctggatc acctcagcac tggagccaaa ctgacccctct

901 tcagctttcc cctccagaga tctctgctat cagaaagacct ctctccgagaacctc tccatccaca

961 gaggaccagg ctcctacgca ggcggagagga gcagtagctg ctcagcagct gcagaaaaag

End C322E primer

1021 cgctcagaggt atcgtggtcatc ctgccttcctct tttttttt gatgggtctgc ccattctttca

Mutation to create Rsa1 site

1081 tcaccataat cagggcgccttc atctggaaag atcctgtgcc tggaaagatgc atggagggcc

1141 tgtctcaatgt gtttgtctgg attgggtatc tctcttcagcg tggatctccca ctgatgatata

1201 cgggtctcagg aatagctcgct tcctcaaggt cattctgtcct cagtcaggg

1261 aaaaagaaaa ggcctgtcag ttaattttag tggacactat accagcattg gcctacaagt

FIGURE 30
FIGURE 30 - CONTINUED
Rat 5-HT_{2C} Serine → Lysine Mutant

MVNLGNAVRSLLMLHLIGLLVWQFDISISPVAIAVTDTFNSSDG

RLFQFPGVQVIPALSIVVIMTITGNILVIMAVSMEKKLHNATNYFLMSLAIADML

VGLLVMPSSLAILVYVWPLPRYCPVWISLDFSTASIMHLCAISLDRYVAIRNP

IEHSRFNRTKAIMKIAIWASIIGSVVIPVIGLRDESKVFVNNTTCVLNDFPFLVLI

GSFAVFIPLTVMITYFLTIYVRQRQLMLRGEHTEELANMSLNLNCCKKKNGGE

EENAPNPNPDQKPRKKKEKPRGTMQAINEKKA[\text{K}]VGLGIVFFVFLIMWCPFFITNI

LSVLGCACNQLMLEKLLNVFWIGYVCNSGINPLYTLFNKIYRRAYSHEYLRCDYKPD

KKPPVRQIRPAATALSGRELNVNIYRHTNERVARKANDPEGIEMQVENLELPVNP

NVVSERISSV

FIGURE 31
Rat 5HT$_{2C}$ Serine → Lysine Mutant

ORIGIN 23 bp upstream of HindIII site.

1 ggccgctctgg tgctcactga ggaaagctccc tttaggtgtac cgatcttaat gattgagcccc
61 ttggagacgc agaattgtta atctttggtg ctcttgggcc ctgtctatcc cttaccttcc
121 tattacatat gaaccttttc tcgttctgca catgcagttg cgtcgccgctgc ggagagatcg
181 tcgtgtgtcgct cgggtgggttg cttccgctcc cttgagaatg ttagttagt taggggcctt
241 caaagaagaa aagaagaagcg atgagcgcggtt agagagctgtt gaggtgctag tttctatgct
301 agagtagggtt agtgaacaa caccccagccca aaccttttgc ggggcgcagc gttgcccaaca
361 ggaggtcagc ttgccccgccc tgcctttcgc gcggagctcc cttccatcctt cttccgctct
421 gctgagagcgc aaggttgccg cgccgacgcct gacgagtcgc ctggcttccccg cgggcttccgc
481 tgggccccgatt cagcggagtc gcggctttctgct ctgggctgccc cccgcgggcagc cttgcttggct
541 ttctccccgag ccgctagcgg ttggtcacta ctattcgtgca aacgataaggcc aacgaacacc
601 ttctttccaa attaattgga atgaaacaaat ccgattcttact tctgtaact ttctaatctt cagtttggaa
661 ctctgtgttgc ttaagcctgta agcaatcagt qtaaacccggt gcaacgccgt gcggcttgctc
721 ctgatgcacc taatcgccgct attggctttccg caatcctgtg aaccgata cttccataag tccagtaacca
781 gctataagta ctgacacttt taattctcgc ctgtggtggac gctttctttca attcccggcc
841 ggggtaaaaa actggtcgcagc actttcaattactgctgtg tttattatcgt aatcgtgtgca
901 aceatcccttg tattcagggc agagagatag gagaagaaac tgcacaatgc aaccaattacc
961 ttcttatggt cccctacgcat cgctgtatag ctgggtggcag tacttttcct tccctgttccc
1021 ctgctgtctgaa cttcttatag cgctgtacgg gctttgacta gatattttgt cccctgttcc
1081 attcctctcg atgtgtcatt ttaacctccg tccatcatgc acctctgccc catatctgcgt
1141 gaccgcgtatag tgaacatcag ccatacatgct gggctcaattg ggggaactaag
1201 accatcatga aagttccat cgtttggcga ataataaag gattttcagt ccttatccctct

FIGURE 32
FIGURE 32 - CONTINUED
Rat 5HT$_{2C}$ Serine → Lysine Mutant with Restriction Site

ORIGIN 23 bp upstream of HindIII site.

1 ggcgcctcgg tgctcactga ggaagcttcc ttaggtgtac gcatcttaat gattgagccc
61 tggagcagc aagatttta atcttggttg ctcttctggc ctgtctatcc cttacccctc
121 tattacatat gaacattct tcgttctgac catcggatttg cgtcgggcgtc gtggagatcg
181 tcgtggtcgcc cgggtgtggc ttctgtcgc cttagaatag tgtagttagtt taggggccctt
241 caaagaagaa agaagaaggc atggcgcggg agagatgtcg gaggtgtcag tttctatgtct
301 agagtagggt agtgaacacaa tccccagccca aaccttccgc ggggggcagc gttgcccaca
361 ggaggtcgcg tcgcggcgcgc tcgtcttccgc gcgcaggtcc ctcctacttct cttccgtctct
421 gcgagacgc cagtggtgagc gcgcacgcgt gacgcacgcga ctcgaactgccc ggaggctgcc
481 cggcgcattgc ccgctagcgt cgttcgtgct cttcttccgc cgcggcgcgtc ctcctctcgct
541 ttccccggag cgcctagccgc gtgtccaatc tacctgcaag cagataggcc aacgaacacc
601 tcttttccac aataattggg atgaacacat ctcgtttatct tcttaattt ctattttgaa
661 ctctggtgtgc ttaagccgtga agcaacatctg gtgaacctttg gcaacgcccgt gcgcctcgctc
721 ctgatgcacc taatcggcct attgottttg caattcgata ttccataag tcgcgaatgca
781 gctataggtg ctgacacttt tcattctcct gatggtgagc gcttttttca attccccggac
841 ggggtacaaa actgccccagc acctttaatc gtcttgatta taatcatgac aataggggggc
901 aacattcttt tcctcatggc agtaagcagc gagaagaaac tgcacaatgc aaccaattac
961 tttatttaat cctagcccat tctgctatag cttcgggtgagc taacgatctt gcccctgcctcc
1021 ctgccttgcta ttctttatgga tatgtgtctgg cctttacata gatattttgt gcgcgtccttg
1081 atttcacttg atggctatttt ccatctggcg tccatctgac accttctggc gcatactcgtg
1141 gacccggtatg tagcataatcg taattcctatt gacgatacgg gttcataattc gcggacattag
1201 gctcatcggag actgggcatg ctttttggcca atatcaataag gattttcagct tccttatcctct

FIGURE 33
1261  gtgattggac tgaggagcga aagcaaaagtg ttctgtaata acaccagctg cgctgctcaat
1321  gaccccaact tctttctcat cggttctccc ttgcgttctc tcatccccgt ttacagattatg
1381  gtgatcacct acttttaaac aatctacgtc ctgcgcccgtc aaaaacttgat gttacttcca
1441  ggtcacacgg agagagaact gcttaataatg agccctgaact ttctgaactg ctgctgcaag
1501  aagaagtgtg tctggagaaga gaaagcctcc cagaccttgca cagacgctca aacccctcta acatcaccaga
1561  aagaagaaag aaaaagcgtcc cagaggccacct atgcaagctat acaacaacgga aaaaagagct
1621  GGGGaaagtgc ttgacattgt atctttgttg ttctgatca ttgtggtccc gtttttcatc
       —Mutation to create ScaI site
1681  accaatatcc ttgctggtttct ttggtgggaag gctctgaacc aaaaagtaat ggagaagctt
1741  ctcattgttg ttgtgtggtat tggctatgtg ttgctgagca tcaacccctct ggtgtacact
1801  ctctttaata aaatttaccc aaggggttcct tctaaatatt tgcgtgccga ttataagcca
1861  gacaaaaagc ctctgtttcg acagatcct caggttctgt ccactgcttt gttctggagg
1921  gaggctcaatg ttaacattta tggcatatcc aatgaacgtg tggctaggaat gcttaatgac
1981  cctgagcccgt gcatagagat gcaagtggaag aacatcgagc tgccagttca aacccctctat
2041  gtggctagcc agaggattag taggtgttaa gcaagagca ggcgcagactt ctctacggaa
2101  agttcctgta ggaagtcct ccccaccccc gctgtttttc ctggtaatca taactaatgt
2161  aatattgct gtgtgacaag acagtgtttt tataaatagc ttgcacgcttt gctactttta
2221  atcatgcgtt aatagtgaga ttggg

FIGURE 33 - CONTINUED
Rat 5-HT$_{2c}$ Serine → Phenylalanine Mutant

MVNLGNARLSLMHLIGLLVWQFDISIPVAAIVTDFTNSDDGG

RLFQFDGVQNWPSIVVIIITIGGNIVIMAVSMEMKHLNATNYFLMSLAIAADMIL

VGLLMPLSLSLALYDYYWPLLPRYLCPVWISLDVLFSASIMHLCALISLDRYVAIRNP

IEHSRFNSRTKAIMKIAIVWAISIGVSVPVPIVGLRDESKVFNNTTVCVNLDPNFVLI

GSFAFIPLTIMVITYFTIYVLRLQRTLMRLRGTTEELANMSLNFNLNCCCKNGGE

EENAPNPDQKPRKKKEKRPRGTMQAINNEKAFKVLGVFFVFVFLIMWCPFIFTI

LSVLGKACNQKLMKLLNVFWIGYVCSSGPQLVYTLFNIYRRAFSKYLRCDCYKPD

KKPPVQIPVRAATALSAGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS

NVVSERISSV

FIGURE 34
Rat 5HT₂C Serine → Phenylalanine Mutant

ORIGIN 23 bp upstream of HindIII site.

1  ggccgccttg  tgcctactga  ggaagcttcc  ttagggtgtac  cgatcttaat  gatggagccc
61  ttaggagcagc  aagattgtta  atcttggttg  ctctcttggc  ctgctcatcc  cttaccttcc
121  tattacatat  gaacttttct  tcgttctgca  catcgattgt  cgtcgccgctc  gtgagatcgc
181  tcgtggtgtgc  ccggtggtgct  ttctcgccgc  cttaagaattag  ttgtagtgat  ttagggcctt
241  caaagaagaa  agaagaagccg  atttgccgccg  agagatgctg  gaggtgtcag  tttctatgct
301  agagtagggt  agtgaaacaa  tccccagccca  aacctttcgcg  gggggcgcag  gttgccccaca
361  ggaggtcgac  ttgcgcgctgc  ttctctctgcg  gcgcagctcc  tcccatccttt  cttccctgtc
421  gctgagacgc  aaggtgctggc  cgccgacgcg  gacaacgcagc  ctaagctcgcg  cgggctccgc
481  ttggtgcattg  cagccgagtc  ttcatctggc  ctgatctgctg  ccggcgccgctc  ctgcgctggc
541  ttctcctccgg  aclctgacggg  ttggctcact  ttaacctgca  agcatagggc  aacgaacacc
601  ttctttccaa  attaatttga  atgaacactc  tcttgtaact  tcctatattct  cagttgaaa
661  ctcttggttcg  ttaagcctgta  agcaatcagt  gtgaacctttc  gcaacgccccgt  gcgtgctgtc
tcgtgacc ccagcgacctt atgccgcttg ccattccgata tttccataag tccaatagca
781  gctataatgaa ctagacatttt taatctctcc gatggtgggag cttggtttcca attccccggac
841  ggggtacaaa actggccgac actttcaatgc cttcggtatta taatcatgac aataggggggcc
901  aacattctgt ttatctagcc  agtagccatg  gagaagaaac  tgcacaatgc  aaccaatattac
961  ttcttaatgt ccctagcatc tcgggtatag tcgggctggac tcattgtcat gcctctggtcc
1021  ctggctgctg tattttatga ttatgtctgg cctttaccta gatattttat gccggtcttg
1081  atttcactag atgtgtcat ttcattctcc gctctcaatgc cttgctagcc catatctcgtg
1141  gaccgggtcg tagcaatatgc atatcctatt gacgcatagcc gtttaatctt ccgactaag
1201  gcccatactg agatgtcctc ctttggccga atatcaatag g atrtttcatct ctctatccct

FIGURE 35
FIGURE 35 - CONTINUED
Rat 5HT2c 312Serine → Phenylalanine Mutant with Restriction Site

ORIGIN 23 bp upstream of HindIII site.

1  ggccgctcgcg ttgctcactgga ggaagcttcc ttgaagtgtac cgatcttaat gatgagccc
61  ttggagcagc aagattgta atcttggttg ctcctttgac gttgcaatcc cttacctcct
121 taattatatc gaacctttctct gcgtcgtcga cattggtgtg cgtcggtgcgt gcggagatcg
181 tctggtgtgc cccggtgtgg ttcctgtgcgt cttagaatag ttagctgatgtaggttattcct
241 caaagaaga agaagacgcg attgggccgcg agagatgtcg gacgtgtcag tttctatgct
301 agagtagggt agtgaacaca tccccagcga aaccctttccg ggggcccagc gttgcccaaca
361 ggaggtgcac ttcgcccgccgc gttgctctcc gcggagctcc ctccacatct cttctccgtct
421 gctgagacgc aaggtgctgg gcgcgacgcgt gcacgcgcgc ggggctccgc
481 tggtggatgtg cagccgagtc cttgctcgtc gtaacggtccg cgcgcgagcgc cgtgcctggtc
541 ttctctcccg acgctagcgcg gtgtcaacct tattacctgc aacataggcc aacgaaacacc
601 ttctttccaa attaattgga atgaacaaat tctgttaacct tcctaaactc caggttsgaa
661 tctctggtgct taagcctgga agcaactagtg ggacacgaggt gcggctccggtc
721 ctgatgcaacc taactccgccct attgggtttgg caattctgata tttccataag gctcaatgca
781 gcttataagta ctgacacttt taattccctcc gatggtggagc gcttggctca atttccggac
841 gggatcacaactgctggg accctttaatc gtctgtgatta tattctgatg gataggggcc
901 aacattcttg tttaaccggag agtaggctag gagaagaaac tggcacaatgc aaccaattac
961 ttcttaatgt cctctgcgtat tcctgtgattc gttggtgac gcttgctctgct accttgctgcc
1021 ctgcttctctc ttctttatga ttattgtgctg cctttacaata cattatggtg cccggtgtgg
1081 atttacagt atttcatatt tcaactctgc tcatcagcgt acctctcgggc ctatactgcc
gacgggtatag tagcaataacg taatcctatt gagcataagcc gttcataatc gacgactaag
1141 ggcctcgtga agattgctcgt cttttgggca ataataatag gaatttcgtc tcttatactct

FIGURE 36
1261  gtagttggac tgagggacga aagcacaagtq ttctgtgaata acaccacactg cgctgctcaat
1321  gacccccact ctgctctcat cgggtccttc gttgctattct tcatcccggt tacgattatq
1381  gtagctacct actcttaacq gatctacgtc ctgcccggctc aaactctgat gttacttcga
1441  ggtcacaacgg aggaggaact gqctataatag agcctgaaact ttctgaaactg ctgctgcaag
1501  aagaatggtg tgagggaga gaaggtcgtcg aacccctaact cagatcagaa accagctgca
Start S312F primer
1561  aagaagaaag aaaaaggttcc angaggcacc atgcaagcctc tcaacatccga aaagaagact
End S312F primer
1621  5'Caaagtctc ttggcattgt attcttttgt ttctgatcga tgtgtgaccc qttttctc
Mutation to create Sca1 site
1681  accaatccg tgtctggctct ttggtgggaag gcctgttacaa aaaaagcataat ggagaagctt
1741  ctcaatgtgt tgtgtgtggat tgtgctagtt tgtcaggca tcaacctctt ggtgtaact
1801  ctctttaata aatatttccg aagggcttctc tctaatatt tgggtgctgga tttataaggca
1861  gagaaaaagc ctacgcttgct cxagattctt aggottgctg cgactgtgtt gttgggggagq
1921  gacgcattq ttaacattta tggccataacc aatgaacgtq tggctaggaa agcataatgac
1981  acctgaccctg gcataagagat gcagttggag aacttagagc tcaccatcaa cccctctaat
2041  gttgctcagcg agaggattag tagttgttaa gcgaagagca gcgcagactt ctacagggaa
2101  agttcctgta ggaagtcct ccccaccccc cgtgattttc ctgtagatatca taactaatgt
2161  aatatttctgt gtgtgacaag acagtgtttt ttaaatagc ttgcaacccc tgtaccttac
2221  atcatgcggttt aatagtgagca ttgcgg

FIGURE 36 - CONTINUED
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C07K 14/705; C12N 15/10, 15/12  
US CL :435/69.1, 172.3; 530/350, 536/23.5  
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
U.S. : 435/69.1, 172.3; 530/350, 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Kao et al. Site-directed mutagenesis of a single residue changes the binding properties of the serotonin 5-HT2 receptor from a human to a rat pharmacology. FEBS Letts. August 1992, Vol. 307, No. 3. pages 324-328, see in particular Figures 1 and 2.</td>
<td>1-13, 16-24</td>
</tr>
</tbody>
</table>

[X] Further documents are listed in the continuation of Box C.  
[ ] See patent family annex.

* Special categories of cited documents:  
"A" document defining the general state of the art which is not considered to be of particular relevance  
"E" earlier document published on or after the international filing date  
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
"O" document referring to an oral disclosure, use, exhibition or other means  
"P" document published prior to the international filing date but later than the priority date claimed  
"R" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
"T" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
"X" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  
"Y" document member of the same patent family

Date of the actual completion of the international search  
14 MAY 1998

Date of mailing of the international search report  
2 3 JUN 1998

Name and mailing address of the ISA/US  
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<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Boone et al. Mutations that alter the third cytoplasmic loop of the a-factor receptor lead to a constitutive and hypersensitive phenotype. Proc. Natl. Acad. Sci. November 1993. Vol. 90. pages 9921-9925. see especially Figure 2.</td>
<td>1-13, 16-24</td>
</tr>
<tr>
<td>Y</td>
<td>Ren et al. Constitutively Active Mutans of the alpha2-Adrenergic Receptor. Journal of Biological Chemistry. 05 August 1993. Vol. 268. No. 22. pages 16483-16487. especially Figure 1.</td>
<td>1-13, 16-24</td>
</tr>
<tr>
<td>Y</td>
<td>Bluml et al. Functional Role of a Cytoplasmic Aromatic Amino Acid in Muscarinic Receptor-mediated Activation of Phospholipase C. Journal of Biological Chemistry. 15 April 1994. Vol. 269. No.15. pages 11537-11541. especially Figure 1.</td>
<td>1-13, 16-24</td>
</tr>
</tbody>
</table>
B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN/MEDLINE
search terms: polymerase chain reaction, site directed mutagenesis, cleavage site#, constitutiv?, activ?, receptor#, G protein#

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-13, 16-22, drawn to a nucleic acid encoding a constitutively active receptor and the protein encoded thereby.
Group II, claims 14-15, drawn to a method of site directed mutagenesis.
Group III, claims 23-24, drawn to a transgenic animal.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Inventions I and III are materially different inventions which lack a common utility based upon a common special technical feature that is disclosed as responsible for that utility and which is lacking from the prior art. Invention II is unrelated to inventions I and III because it is a method which performs a different function, operates upon a different principal and achieves a different result. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.