HUMAN 5-HT_{2C} RECEPTOR VARIANTS: FUNCTIONAL PROPERTIES AND GENETIC ASSOCIATIONS IN MAJOR DEPRESSIVE DISORDER

By

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To my grandfather Sam and late grandmother Lessie, the wisest two I've ever known, to my parents, Vera and Cliff, forever caring, to my loving wife Tamara, constantly supportive, and God, my source of strength and hope, my everything.

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5-HT	Serotonin, 5-hyroxytryptamine
cGMP	Guanosine-3′,5′-cyclic monophosphate
CNS	Central nervous system
DAG	Diacyl glycerol
DIC	Differential interference contrast
DMEM	Dulbecco's modified Eagle's medium
DOI	(±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane
EC_{50}	Concentration of drug that gives 50% of the maximal response
FRET	Fluorescence resonance energy transfer
GDP	Guanosine 5′-diphosphate
G-protein	Guanine nucleotide binding protein
GPCR	G-protein coupled receptor
GppNHp	5'-(β,γ-imido)triphosphate
GTP	Guanosine 5′-triphosphate
HBSS	Hank's balanced salt solution
IP_3	Inositol 1,4,5-triphosphate
LSD	Lysergic acid diethylamide
mCPP	m-chlorophenylpiperazine
PI hydrolysis	Phosphatidylinositol hydrolysis
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PKC	Protein kinase C
SNP	Single nucleotide polymorphism
TM	Transmembrane domain

CHAPTER I

INTRODUCTION

Serotonin Function and History

Serotonin (5-hydroxytryptamine, 5-HT) is an indolamine neurotransmitter that is involved in many psychophysiological responses such as mood, appetite, aggression, arousal, sleep, learning, and motor control (Lentes et al., 1997; Breier, 1995). It was initially identified as a vasoconstricting substance in the blood stream that enhanced platelet aggregation in the gut (Rapport et al., 1948). The highest concentrations of 5-HT are found in the gastrointestinal tract where it is made in enterochromaffin cells, followed by platelets and the central nervous system (CNS) (Erspamer 1966). Since serotonin is involved in a diverse array of behavioral and physiological processes, defects in serotonergic function have been implicated in a number of neuropsychiatric disorders including depression, anxiety, eating disorders, suicide, obsessive-compulsive disorder, schizophrenia, and migraines (Heisler, 2000). However, the exact role of 5-HT in these diseases is not very well understood.

5-HT appeared early in evolution as its distribution can be found throughout both the plant and animal kingdoms. It can be found in the most primitive organisms that possess a nervous system such as coelenterates, *Platyhelminths* (flatworms), *Caenorhabditis elegans* (nematodes), *Aplysia californica* (mollusks), annelids, echinoderms, crustaceans, and *Drosophila melanogaster* (Weiger, 1997). In these invertebrates, 5-HT modulates many behaviors such as feeding, egg laying, biting, and

escape swimming. The study of these lower organisms have led to great insights into the actions of 5-HT as a neurotransmitter, neuromodulator, and neurohormone in both invertebrates and vertebrates. Nonetheless, the mechanisms by which 5-HT affects behavior and lead to disease are yet to be clearly understood.

Serotonin Receptor Subtypes

The physiological effects of 5-HT are elicited through binding to one of fourteen receptor subtypes (Barnes and Sharp, 1999). These receptors are classified into seven families based on three criteria: amino acid sequence homology, gene structure, and intracellular signaling cascades (Figure 1; Hoyer et al., 1994). All but one of the 14 receptor subtypes belong to class A of the superfamily of G protein coupled receptors (GPCRs); the 5-HT_3 receptor subtype is a ligand gated ion channel. The 5-HT receptor subtypes are the largest of all known neurotransmitter receptor families to date, showing the importance and the complexity of the serotonergic system. Each 5-HT receptor subtype has varying affinities for ligands along with different expression profiles throughout the body, including the brain. Therefore, 5-HT can modulate many physiological processes depending on the receptor subtype and tissue distribution.

The 5-HT_1 receptor subfamily contains five members all of which are coupled to G_i/G_o to inhibit the production of adenylyl cyclase to increase K^+ conductance and to inhibit voltage gated calcium channels (Hoyer et al., 1994). In mesenchymal cells, 5-HT_1 receptors also mediate stimulatory pathways that include activation of phospholipase Cβ (PLCβ) via Gβγ subunits and mitogen-activated protein kinase (MAPK), resulting in cell proliferation and transformation (Albert and Tiberi, 2001).

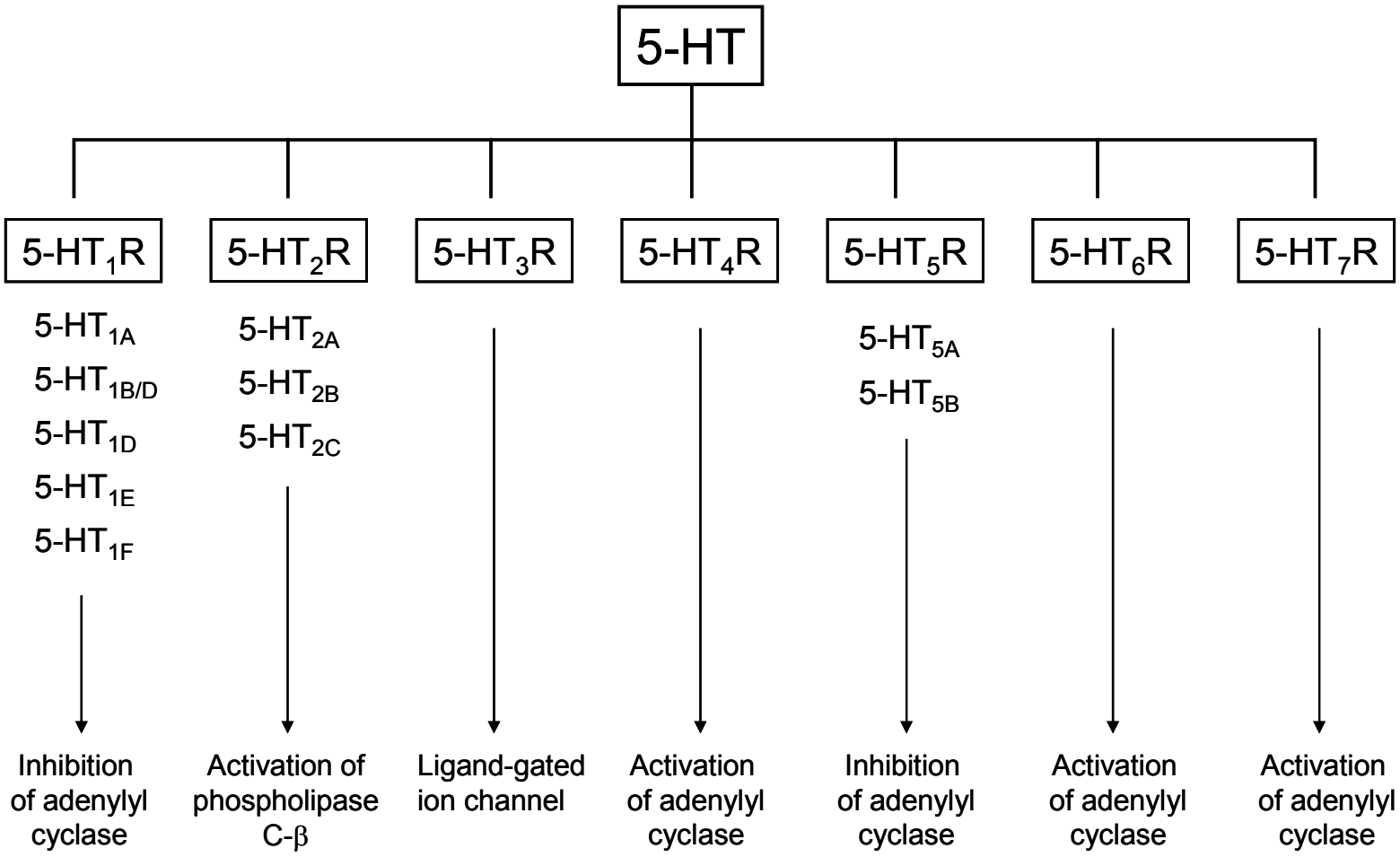

Figure 1: Serotonin receptor family

The 5-HT_{1A} receptor is a somatodendritic autoreceptor on cell bodies of serotonergic neurons in the raphe nuclei of the brainstem, where it also activates a receptor-operated K^+ channel and inhibits a voltage-gated Ca^{2+} channel via Gβγ subunit interaction with the Ca^{2+} channel's α1 subunit (Chen and Patterson, 1997). It is also found in target neurons in the hippocampus where its function is less understood. The 5-HT_{1D} receptor (homolog to the rat 5-HT_{1B} receptor) is highly expressed in the substantia nigra and basal ganglia where it functions as an autoreceptor on axon terminals, inhibiting the release of 5-HT.

As mentioned earlier, the 5-HT_3 receptor is unique in that it is the only member of the family and also the only monoamine neurotransmitter receptor known to function as a ligand-gated ion channel. It is found in the CNS in regions such as the cerebral cortex, hippocampus, amygdala and medulla and is also found in the peripheral nervous system (PNS). Upon activation, postsynaptic 5-HT_3 receptors induce a rapidly desensitizing depolarization mediated by the gating of cation influx. These receptors are also found presynaptically where they are thought to modulate neurotransmitter release (Hooft and Yakel, 2003).

The 5-HT_4, 5-HT_6, and 5-HT_7 receptors all couple to G_s and positively activate adenylate cyclase but are divided into different subfamilies based upon lack of sequence homology. The 5-HT_4 receptor was first identified in cultured mouse colliculi neurons and the guinea pig brain using a functional assay, stimulation of adenylyl cyclase (Dumuis et al., 1988; Bockaert et al., 1990). These receptors are found consistently in the nigrostriatal and mesolimbic systems in the brain of many different species (Grossman et al., 1993; Mengod et al., 1996). Four different 5-HT_4 receptors variants have been

identified as a result of alternative splicing (Gerald et al., 1995; Blondel et al., 1998; Claeysen et al., 1998). Upon activation of 5-HT_4 receptors, there is increased neuronal excitability and a slowing of repolarization, suggesting that these receptors enhance neurotransmitter release (Chaput et al. 1990; Roychowdhury et al., 1994). The 5-HT_6 receptor was initially identified by two groups after finding a cDNA sequence that encoded for a 5-HT sensitive-receptor with distinctive pharmacology (Monsma et al., 1993; Ruat et al., 1993). Abundant levels of 5-HT_6 mRNA have been detected in the caudate nucleus, nucleus accumbens, and hippocampus of human, rat, and guinea pig where they are thought to be mostly postsynaptic receptors (Barnes and Sharp, 1999). The 5-HT_7 receptor is the most recently identified 5-HT receptor. Although the 5-HT_7 receptor has four splice variants, only three have been found in rat and human tissue, in regions such as the thalamus, hypothalamus, and hippocampus (Heidmann et al., 1997).

The 5-HT_5 receptor is the least understood of all of the receptors in the family. The 5-HT_5 receptor consists of two members, the 5-HT_{5A} and 5-HT_{5B} receptors. The 5-HT_{5A} receptor has been identified in mouse, rat and human (Plassat et al., 1992; Hen, 1992; Erlander et al., 1993; Wisden et al., 1993; Rees et al., 1994). However, the 5-HT_{5B} receptor is only expressed in the mouse and rat because in the human, the coding sequence is interrupted by a stop codon (Grailhe et al., 2001). For the most part, both receptors have their distribution limited to the CNS; 5-HT_{5A} receptors have been found on neuronal and neuronal-like cells of the carotid body (Nelson, 2004). The human 5-HT_{5A} receptor has been shown to couple to $G_i/_o$ proteins to lead to inhibition of adenylate cyclase in HEK293 cells (Francken et al., 1998; Hurley et al., 1998). However, the physiological function of 5-HT_5 receptors is still not clear.

5-HT_2 Receptors

The 5-HT_2 receptor family consists of three subtypes, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors which share 46-50% sequence identity. Through the years, these receptors have been renamed 5-HT_{2A}, formerly 5-HT_2; 5-HT_{2B}, formerly 5-HT_{2F}; and 5-HT_{2C}, formerly 5-HT_{1C}. Most of the homology is within the seven transmembrane domains but they are structurally distinct from other 5-HT receptors. This family is also characteristic of coupling positively to the activation of PLC and mobilization of intracellular calcium.

The 5-HT_{2A} receptor was originally classified as the 5-HT D receptor that mediated contractions in the guinea pig ileum (Gaddum and Picarelli, 1957). Brain 5-HT_{2A} receptors were identified in the rat much later as a binding site with high affinity for [^{3}H]-spiperone and low affinity for 5-HT (Leysen et al., 1978; Peroutka and Snyder, 1979). The 5-HT_{2A} receptor was cloned from rat brain (Julius et al., 1990) and later from humans (Saltzman et al., 1991). The amino acid sequence of the 5-HT_{2A} receptor has 5 potential glycosylation sites, 11 phosphorylation sites, and 1 palmitoylation site (Saltzman et al., 1991). Within the PNS, 5-HT_{2A} receptors are found on platelets and in the gastrointestinal tract. In the CNS, 5-HT_{2A} receptors are highly expressed in the prefrontal cortex, claustrum, and the caudate nucleus where they function as post-synaptic receptors (Pazos et al., 1985; Lopez-Gimenez et al., 1997; Aghajanian and Marek, 1999). 5-HT_{2A} receptors have been recently characterized electrophyisiologically in the raphe nucleus where they are believed to have a pre-synaptic role in regulating serotonergic function (Boothman et al., 2003).

Although the 5-HT_{2A} receptor has low affinity for 5-HT, it has high affinity for the hallucinogenic agonists (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (DOI) and lysergic acid diethylamide (LSD). Until recently, it was difficult to differentiate between the 5-HT_2 receptors. However, with the development of selective 5-HT_{2A} receptor antagonists such as MDL 100907, we are now able to differentiate these receptors in vivo and in vitro (Sorensen et al., 1993). In addition to the 5-HT_{2A} receptor activating PLC, it also activates a biochemical cascade that leads to the altered expression of genes such as brain-derived neurotrophic factor (BDNF) (Vaidya et al., 1997). Excitatory responses to 5-HT_{2A} stimulation in rat brain slices lead to a reduction in potassium conductance (Marek and Aghajanian, 1995).

The 5-HT_{2B} receptor was the original receptor found to mediate 5-HT-induced contraction of the rat stomach fundus with similar pharmacological properties of what is now known as the 5-HT_{2C} receptor (Vane, 1959). The presence of 5-HT_{2B} receptors in the brain has been controversial, but they are thought to be present in limited amounts in the mouse and human (Loric et al., 1992; Bonhaus et al., 1995). These receptors are restricted to the cerebellum, lateral septum, dorsal hypothalamus, and medial amygdala (Barnes and Sharp, 1999). 5-HT_{2B} receptors are thought to mediate in the mitogenic effects of 5-HT during neural development.

The 5-HT_{2C} receptor was identified in the choroid plexus as a [^{3}H]-5-HT binding site that could also be labeled with [^{3}H]-mesulergine and [^{3}H]-LSD, but not [^{3}H]-ketanserin (Pazos et al., 1984). Because of its high affinity for 5-HT, it was originally thought to be a member of the 5-HT_1 family and named 5-HT_{1C}. However, upon cloning and further characterization of the receptor, it was reclassified and moved to the 5-HT_2

family because of its close sequence homology and gene structure and renamed the 5-HT_{2C} receptor. The mouse 5-HT_{2C} receptor was first partially cloned by Lubbert et al. (1987) followed by sequencing of the full length clone in rat (Julius et al., 1988), the mouse (Yu et al., 1991) and then the human (Saltzman et al., 1991; Xie et al., 1996).

Structure and Function of 5-HT_2 Receptors

GPCRs share many structural features, including seven transmembrane α-helices connected by six loops (3 extracellular and 3 intracellular) of varying lengths with an extracellular amino terminus and an intracellular carboxyl terminus. More specifically, class A GPCRs are characterized by their sequence homology, which includes shared cysteine residues in extracellular loops 1 and 2 (e1 and e2), a DRY motif in intracellular loop 2 (i2), and a NPXXY motif in transmembrane 7 (TM 7) (Fig. 2; Bockaert and Pin, 1999). Ligand binding takes place on the extracellular or transmembrane domains and causes conformational changes that act as a switch to relay the signal to G-proteins that in turn induce an intracellular response. Both mutagenesis and biochemical studies of a variety of class A GPCRs suggest that receptor activation by ligand binding causes changes in the relative orientation of TM helices 3 and 6 (Wess, 1997; Shapiro et al., 2002). To date, the rhodopsin receptor is the only GPCR to be resolved by X-ray crystallography at high resolution. This receptor was crystallized in its inactive form, showing the orientation of the seven transmembrane α helices, but the structural characteristics of its intracellular loop regions are still lacking (Palczewski et al., 2000). Upon receptor activation and interaction with the G-protein, this interaction causes GDP to be released by the G-protein. The receptor contact site on the G-protein is thought to

be distant from the GDP-binding pocket, so the receptor must work "at a distance" to change the conformation of the protein (Bourne, 1997).

Many of the ligands that bind 5-HT_{2C} receptors have polar side chains that need to be buried within the membrane in a binding pocket. In TM 3 of 5-HT_2 receptors, a conserved aspartic residue (D155) is thought to bind and anchor at least one amine moiety in 5-HT and other agonists, thus hiding these polar residues within the membrane (Choudhary et al., 1995; Weinstein, 1995). Site-directed mutagenesis of D155 (D155N) revealed that this residue is needed for optimal ligand binding for many agonists and antagonists (Wang et al., 1993). If this aspartic acid is mutated into a glutamic acid (D155E), there is a marked decrease in targeting of the receptor to the membrane (Kristiansen et al., 2000). Additional evidence for conserved side chains acting in concert to mediate activation comes from mutational studies of aspartic acid residue (D120) in TM 2 of the 5-HT_{2A} receptor. Mutation of the conserved aspartic acid (D120N) eliminated coupling while an additional mutation in TM 7 (N376D) restores function (Sealfon et al., 1995), suggesting that these residues are adjacent and interact via a hydrogen-bonding network.

5-HT_{2C} Receptors

5-HT_{2C} Receptor Distribution

In contrast to the 5-HT_{2A} and 5-HT_{2B} receptors, there is very little evidence for expression of the 5-HT_{2C} receptor outside of the CNS. Studies using radioligands such as [^{3}H]-5-HT, [^{3}H]-mesulergine, and [^{3}H]-LSD to perform autoradiography have provided

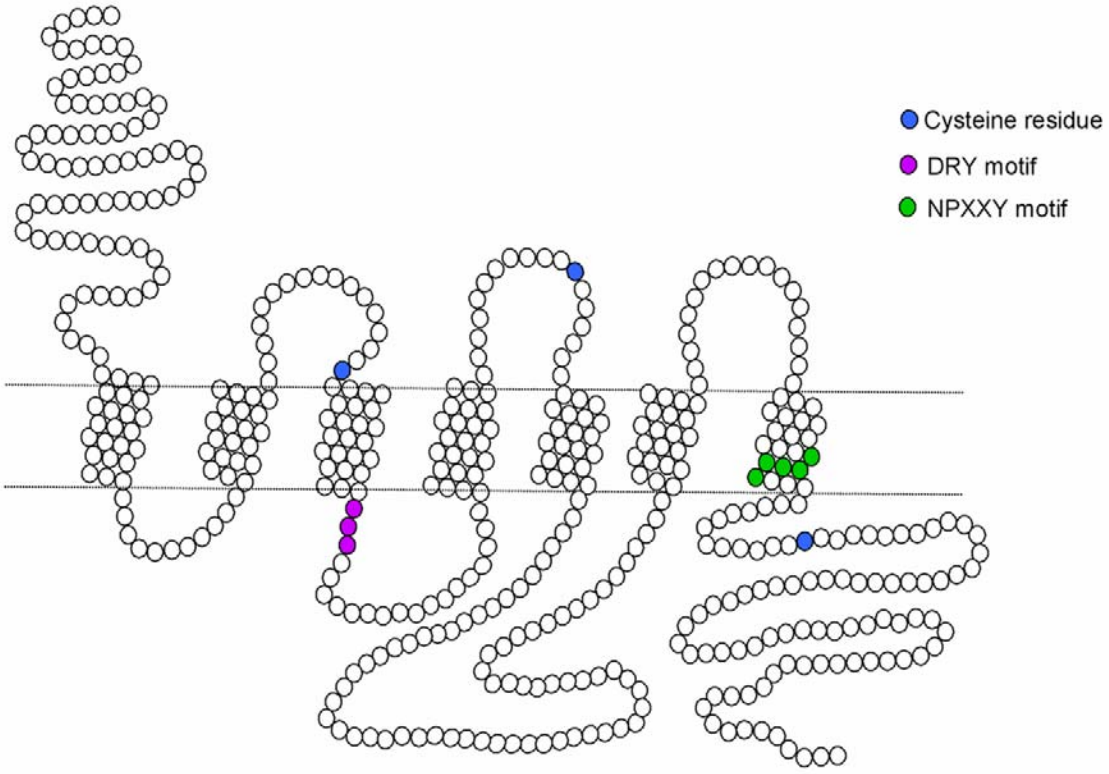

Figure 2: Class A GPCR conserved domains and residues

detailed maps of the distribution of 5-HT_{2C} binding sites in the rat and many other species (Pazos et al., 1984). 5-HT_{2C} receptor binding is highest in the choroid plexus where its function is still not clear, but is also present in areas of the cortex (olfactory nucleus, pyriform, cingulate, and retrosplenial), limbic system (amygdala, nucleus accumbens, and hippocampus), and the basal ganglia (caudate nucleus and the substantia nigra). The existence of 5-HT_{2C} receptor binding in the pyriform cortex and the substantia nigra, support the findings of 5-HT_{2C} receptor-mediated electrophysiological responses in these regions (Sheldon and Aghajanian, 1991; Rick et al., 1995).

For the most part, there is good correlation between the distribution of 5-HT_{2C} receptor mRNA and 5-HT_{2C} receptor binding sites (Mengod, 1990). However, one exception is the high levels of 5-HT_{2C} receptor mRNA in the lateral habenular nucleus where 5-HT_{2C} receptor binding sites are very low. Therefore, 5-HT_{2C} receptors may be presynaptically located based upon the projections of the habenula. It has also been reported that the distribution of the 5-HT_{2C} receptor-like immunoreactivity also follows the binding data (Abramowski et al., 1995). 5-HT_{2C} receptor mRNA has been reported in the midbrain raphe nuclei in two studies (Hoffman and Mezey, 1989; Molineaux et al., 1989), however, these results were not confirmed in another study (Mengod et al., 1990b). A recent study used double in situ hybridization to examine the cellular localization of 5-HT_{2C} receptor mRNA in relation to serotonergic and GABAergic neurons in the anterior raphe nuclei of the rat (Serrats et al., 2005). In the dorsal and median raphe nuclei, 5-HT_{2C} receptor mRNA was not detected in serotonergic neurons, however, it was found in most GABAergic cells. Together, these data provide evidence that the 5-HT_{2C} receptor is located both on the presynaptic and postsynaptic terminal.

5-HT_{2C} Receptor Pharmacology

The pharmacological profile of the 5-HT_{2C} receptor is similar to but distinguishable from other members of the 5-HT_2 receptor family (Baxter et al., 1995). 5-HT has a higher affinity for the cloned human 5-HT_{2C} receptor (K_d = 2-56 nM) than for the 5-HT_{2A} receptor (K_d = 63-250 nM). The antagonists ritanserin, LY 53857, mesulergine, mianserin and the agonists m-chlorophenylpiperazine (mCPP) and DOI do not discriminate well between the different 5-HT_2 receptors. On the other hand, the 5-$HT_{2B/2C}$ receptors can be distinguished from the 5-HT_{2A} receptor by their high affinity for SB 200646 and SB 206553, and for their low affinity for the antagonists MDL 100907, ketanserin, and spiperone (Table 1). Additionally, the novel compound SB 204741 is approximately 20-60 fold more selective for the 5-HT_{2B} receptor over the 5-HT_{2C} receptor. The development of the recent selective antagonists SB 242084 and RS-102221 have been profound in the study of 5-HT_{2C} receptors because these compounds are at least two orders of magnitude more selective for the 5-HT_{2C} receptor versus the 5-HT_{2A}, 5-HT_{2B}, and other binding sites (Bonhaus et al., 1997; Kennett et al., 1997). The typical and atypical antipsychotics chlorpromazine, clozapine, loxapine all have relative high affinity for both 5-HT_{2C} and 5-HT_{2A} binding sites as well as some antidepressants (e.g. tricyclics, doxepin, mianserin, and trazadone) (Canton et al., 1990; Roth et al., 1992; Jenck et al., 1993).

Not only does 5-HT_{2C} receptor have a high affinity for 5-HT, it is also able to exhibit complex binding consistent with multiple affinity states. In stable cell lines expressing the 5-HT_{2C} receptor, competition binding experiments using 5-HT to compete off [^{3}H]-mesulergine exhibited shallow curves. In order to determine if the shallow

Table 1: **Preference for various 5-HT2 antagonists at human 5-HT2 receptors**
K_i values are in nM; pA_2 values were determined in rat fundus preparations.

	5-HT$_{2A}$	**5-HT$_{2B}$**	**5-HT$_{2C}$**	**Reference**
5-HT$_{2A}$ preferring				
Risperidone	0.4	29	64	Wainscott et al., 1996
Pirenperone	1.1	61	77	Wainscott et al., 1996
Spiperone	1.4	590	3,830	Ismaiel et al., 1996
Ketanserin	2.0	395	160	Wainscott et al., 1996
MDL 11,939	6.1	3,020	1,020	Wainscott et al., 1996
AMI-193	7.7	710	8,495	Ismaiel et al., 1996
MDL 100907	0.85	261	136	Kehne et al., 1996
5-HT$_{2B}$ preferring				
LY23728	66	1	123	Audia et al., 1996; Bonhaus et al., 1999
LY266097	19	pA_2 = 9.80	25	Audia et al., 1996
SB 204741	>10,000	79	2,000	Bonhaus et al., 1995
5-HT$_{2B/2C}$ preferring				
SB 206553	1,600	pA_2 = 8.48	10	Forbes et al., 1995
SB 200646	>10,000	630	400	Bonhaus et al., 1995
SDZ SER-082	630	pA_2 = 7.34	16	Nozulak et al., 1995
SB 242084	300	100	1	Kennett et al., 1997
RS-102221	1,122	813	3.8	Bonhaus et al., 1997

curves for agonists were due to multiple affinity states or multiple binding sites, a non-hydrolysable form of GTP, (GppNHp), was used, which eliminates high affinity agonist binding. This was found to be the case for the unedited form of the 5-HT_{2C} receptor but only with 5-HT (Niswender et al., 1999). More recent data suggests the unedited (INI) and edited (VSV) isoforms of the 5-HT_{2C} receptor are able to bind 5-HT, DOI, and mCPP with multiple affinity states (Fentress et al., 2005). Although LSD has a high affinity for the 5-HT_{2C} receptor, it has not been demonstrated to bind with multiple affinity states.

Intracellular Signaling of 5-HT_{2C} Receptors

Originally, it was thought that receptor activation resulted in a single intracellular signaling pathway initiating a cellular response. However, the 5-HT_{2C} receptor, like many other GPCRs, is able to activate numerous signaling pathways by interacting with multiple G-proteins. Traditional activation of the 5-HT_{2C} receptor activates phospholipase C (PLC) in the choroids plexus of various species (Sanders-Bush et al., 1988) and stably transfected cells (Julius et al., 1988) by coupling to the G-protein, G_q (Fig. 3; Chang et al., 2000). PLC then promotes the hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG then goes on to activate protein kinase C (PKC) while IP_3 activates intracellular calcium stores (Figure 4; Sanders-Bush et al., 1990). The activation of intracellular calcium stores activate calcium-dependent calmodulin kinases leading to the inactivation of potassium channels. Calcium release may also lead to the activation of chloride currents. The second messenger DAG may also release arachidonic acid (AA) that can result in elevated cGMP levels (Fig.4).

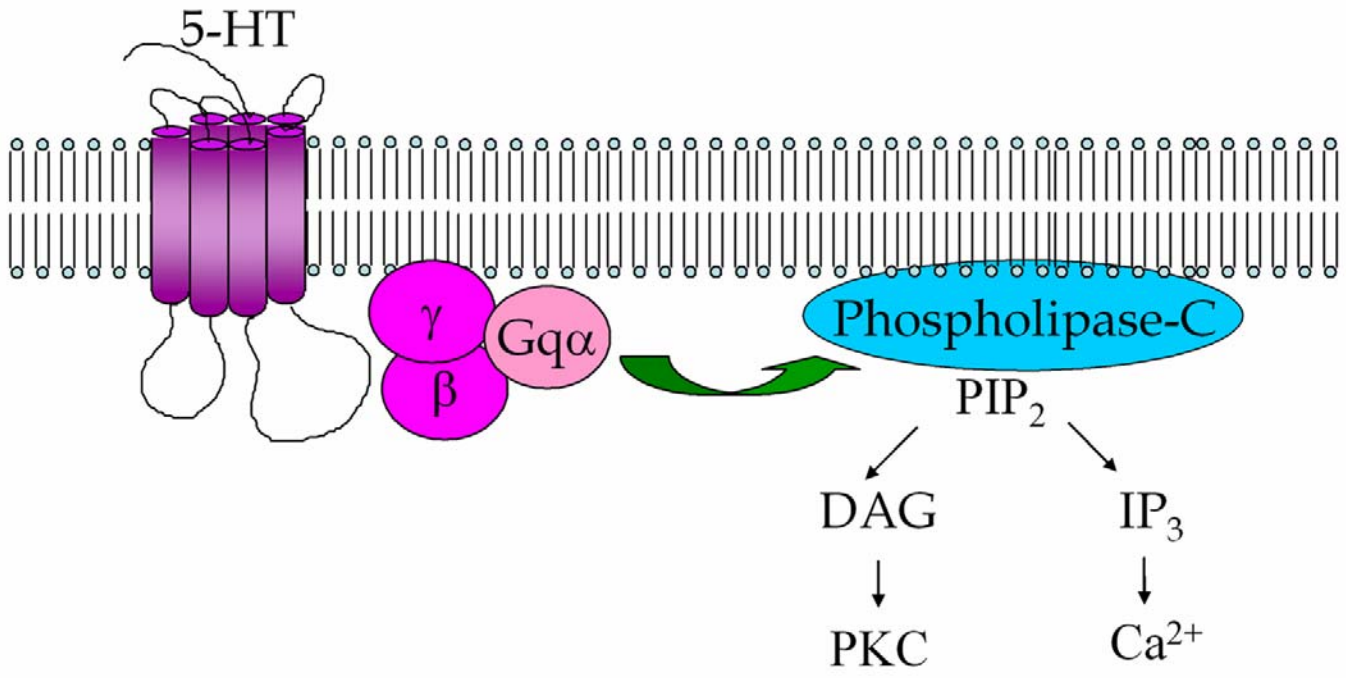

Figure 3: Intracellular signaling cascade of the 5-HT$_{2C}$ receptor.
Gq, heterotrimeric G protein subunit; PIP2, phosphatidylinositol-1,4-bisphosphate; IP3, inositol-1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C

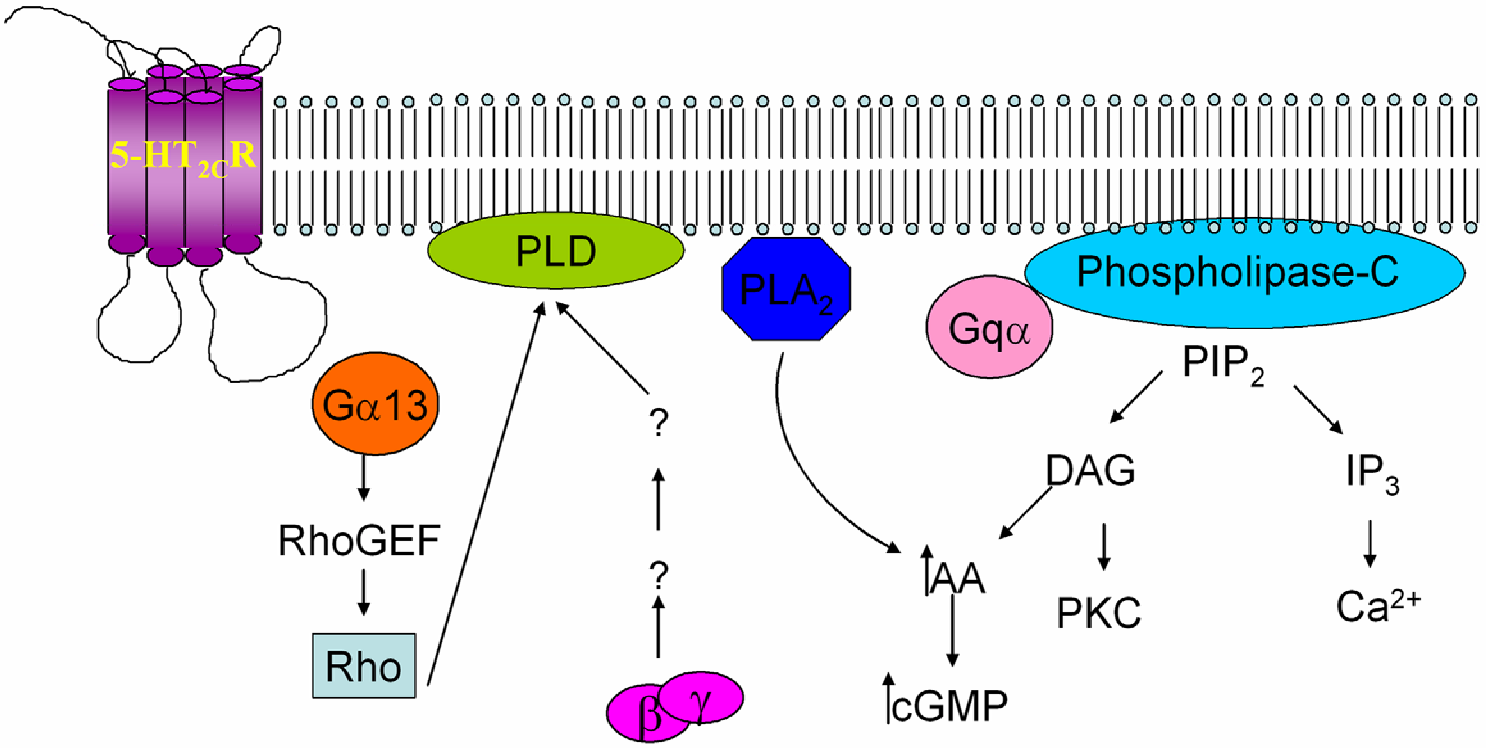

Figure 4: Mutiple signaling cascades of the 5-HT_{2C} receptor.
The 5-HT_{2C} receptor can activate multiple G-proteins, stimulating multiple pathways. PIP2, phosphatidylinositol-1,4-bisphosphate; IP3, inositol-1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C; PLD, phospholipase D; PLA_2, Phospholipase A_2; cGMP, guanosine-3',5'-cyclic monophosphate; AA, arachidonic acid

In addition to activating PLC, 5-HT_{2C} receptors also activate phospholipase A_2 (PLA_2) (Fig.4). Activation of PLA_2 via a pertussis toxin-insensitive G-protein(s), results in the release of arachidonic acid from various membrane phospholipids. Arachidonic acid has many cellular functions of its own and is also metabolized to a myriad of bioactive compounds (eicosanoids) such as prostaglandins, leukotrienes, and thromboxanes. In transfected CHO cells, 5-HT_{1B} receptor-mediated inhibition of forskolin-stimulated cAMP accumulation is inhibited by 5-HT_{2C} receptors by increasing arachidonic acid via a PLA_2 mechanism. Although the 5-HT_{2A} has also been found to activate PLA_2, it was unable to inhibit 5-HT_{1B} receptor activation in CHO cells (Berg et al., 1996; Berg et al., 1998).

The 5-HT_{2C} receptor has also been shown to increase cGMP (Fig. 4) in pig choroid plexus tissue slices. This activation was insensitive to the treatment with pertussis toxin but was sensitive to calcium. 5-HT_{2C} receptor cGMP activation is thought to be dependent upon PLA_2 and lipoxygenase since inhibitors of either protein significantly decreased cGMP production (Kaufman et al., 1995). However, it is still not clear whether or not cGMP formation is secondary to PI turnover although the reverse has been ruled out (Conn and Sanders-Bush, 1986). In the choroid plexus, the non-selective 5-HT_2 receptor agonists TFMPP, quipazine, DOM, mCPP, and MK 212 all behave as agonists. However, only MK 212 had an equal efficacy to 5-HT (Conn and Sanders-Bush, 1987; Sanders-Bush et al., 1988). It is thought that 5-HT_{2C} receptors in the choroid plexus may regulate CSF formation as a result of their ability to mediate cGMP formation (Kaufman et al., 1995).

Activation of endogenous 5-HT_{2C} receptors in choroid plexus epithelial (CPE) cells leads to the stimulation of phospholipase D (PLD) (Fig. 4). This effect is blocked by the 5-HT_{2C} specific antagonist SB206553 but not by the 5-HT_{2A} specific antagonist MDL100907. 5-HT_{2C} receptor activation of PLD is mediated through coupling to the G_{13} protein and activation of the small G-protein Rho. These interactions were further characterized and found to be a result of both Gα and Gβγ subunits from G_{13} (McGrew et al., 2002). PLD activation has been suggested to be a mediator of stress fiber formation (Gohla et al., 1999), and has also been linked to vesicle trafficking (Brown et al., 1993; Malcolm et al., 1994), the formation of oxygen radicals (Grewal et al., 1999), and cell cycle control (for review see Exton, 1999).

Constitutive Activity of 5-HT_{2C} Receptors

Some antagonists have been found to possess negative intrinsic activity, meaning its affinity for receptors is increased following uncoupling from G-proteins. The phenomenon of negative intrinsic activity was first recognized in the actions of β-carbolines at the ionotropic $GABA_A$ receptor (Braestrup et al., 1982). The ability to detect negative intrinsic activity depends upon a measurable amount of constitutive or agonist-independent activity. Constitutively active receptors are receptors that are able to undergo a conformation change in the absence of agonist that allows coupling to G-proteins, thereby activating a signaling cascade. This phenomenon has been found to occur at many GPCRs including the 5-HT_{2C} receptor (Barker et al., 1994).

Importantly, studies of constitutively activating mutations have provided insight into the role of specific side chains in receptor activation. As one may not expect, most

activating mutations do not occur in conserved regions. Mutation of the non-conserved Ala293 in the α_{1B} adrenergic receptor to any amino acid yields a constitutively active receptor (Kjelsberg et al., 1992). Other studies have shown that mutations of the conserved Asp-Arg domain at the cytoplasmic boundary of TM 3 cause constitutive activity in some GPCRs (Scheer et al., 1997; Fanelli et al., 1999). In addition, the Tyr368 in the conserved Asn-Pro-X-X-Tyr motif in TM 7 has been studied in many GPCRs, and found to affect agonist affinity, signaling, and sequestration in different receptors (Barak et al., 1995; Gabilondo et al., 1996). Constitutively active GPCRs have also been created in vitro by site-directed mutagenesis in several transmembrane domains, extracellular and intracellular loops. Specifically, constitutive receptor activation has been achieved by mutating amino acid residues in i2, i3, TM 3, and TM 5 of adrenergic receptors (Kjelsburg et al., 1992; Scheer et al., 1996; Hwa et al., 1997), TM 6 of the M5 muscarinic receptor (Spalding et al., 1995), and e1, e2, i3, TM 2, TM 3, TM 6, and TM 7 of the TSH receptor (Tonacchera et al., 1996), and e2 of the thrombin receptor (Nanevicz et al., 1996). As for the 5-HT_{2C} receptor, mutation of the Tyr368 to a cysteine or alanine in this conserved domain in TM 7 resulted in a marked increase in basal PI hydrolysis which was abolished by the addition of the inverse agonist SB 206553. Introduction of a phenylalanine to this locus eliminated both basal and agonist-stimulated signaling. All three mutations caused an increase in binding affinity for the structurally different agonists 5-HT, DOI, and quipazine, suggesting that both the activating and inactivating mutations stabilize a high affinity state (Rosendorff et al., 2000). These data suggest that this conserved Tyr368 has both a structural and functional role in 5-HT_{2C} receptor.

5-HT_{2C} receptor antagonists such as mianserin and mesulergine have been shown to have negative intrinsic activity, as evidenced by a decrease in agonist-independent, receptor mediated PI hydrolysis in transfected cells. The antagonists ketanserin and spiperone also caused dose-dependent decreases in basal PI hydrolysis. On the other hand, BOL did not reduce basal activity; although it did block 5-HT's actions completely. BOL was also able to prevent the decreased in basal activity produced by mianserin and mesulergine. These findings combined with the fact that the antagonists had no effect in untransfected cells indicate that the decrease in basal was receptor-mediated. Therefore, it was concluded that mianserin, ketanserin, mesulergine, and spiperone act as inverse agonists at 5-HT_{2C} receptors, with varying degrees of negative intrinsic activity, while BOL acts as a neutral antagonist (Barker et al., 1994). Moreover, in the revised ternary complex model of G-protein coupled receptors, inverse agonists are preferred to bind and stabilize a coupling-inconcomitant conformation.

In order to mimic the active conformation of the 5-HT_{2C} receptor, serine 312 in i3 was mutated to a phenylalanine (S312F) or lysine (S312K). Upon expression of the mutant receptors into cells, the K_i values of 5-HT for [^{3}H]-mesulergine-labeled 5-HT_{2C} receptors decreased from 203 nM (native) to 76 nM for S312F and 6.6 nM for S312K mutant receptors. The potency of 5-HT for stimulation of PI hydrolysis increased (EC_{50}= 70 nM, 28 nM, and 2.7 nM respectively). These mutant receptors were also constitutively active, stimulating PI hydrolysis in the absence of agonist. The S312F and the S312K mutants resulted in two-fold and five-fold increases, respectively, in basal levels of PI hydrolysis. Mianserin and mesulergine both displayed inverse agonist activity by decreasing basal levels of PI hydrolysis stimulated by the S312K mutants.

[^{3}H]5-HT and [^{3}H]-mesulergine labeled the same number of S312K mutant receptors and 5′-guanylylimidodiphosphate (Gpp(NH)p) had no effect on [^{3}H]5-HT binding. These results indicate that the S312K mutation produces an agonist high affinity state of the 5-HT_{2C} receptor that spontaneously couples to G-proteins and stimulates PI hydrolysis in the absence of agonist to a greater level than normal (Herrick-Davis et al., 1997).

Homodimerization of the 5-HT_{2C} Receptor

GPCR dimerization is an emerging area of research on the molecular mechanisms of receptor activation. The first studies examining GPCR dimerization used membrane solubilization and Western blotting and were criticized because of the potential for non-specific protein aggregation during membrane preparation and solubilization. Another issue was the fact that the higher molecular weight bands observed on Western blots, presumed to be receptor dimers/oligomers, were insensitive to detergents and reducing agents. However, subsequent studies using techniques such as co-immunoprecipitation (Hebert et al., 1996; Romano et al., 1996; Zeng et al., 1999) and biophysical techniques (Angers et al., 2000; Rocheville et al., 2000; Kroeger et al., 2001) have provided stronger evidence of GPCR formation of dimers/oligomers.

The first evidence that 5-HT receptors may form homodimers was provided by Western blots of solubilized membrane protein from Sf9 insect cells expressing 5-HT_{1B} or 5-HT_{1D} receptors (Ng et al., 1993; Xie et al., 1999). In a later study, co-immunoprecipitation of differentially tagged 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors suggested that 5-HT_1 receptors may form heterodimers (Salim et al., 2002). There have been no reports demonstrating the presence of 5-HT receptor dimers/oligomers on the

plasma membrane of live cells until the recent report by Herrick-Davis et al. (2004). In their study, using biochemical and biophysical techniques, they examined the ability of the 5-HT_{2C} receptor to form homodimers on living cells. Immunoprecipitation followed by Western blotting revealed the presence of immunoreactive bands the predicted size of 5-HT_{2C} receptor monomers and homodimers that were detergent and cross-linker sensitive. In HEK293 cells expressing 5-HT_{2C} receptors labeled with Renilla luciferase and yellow fluorescent protein, bioluminescence resonance energy transfer (BRET) was accessed. BRET levels were not altered by pretreatment with 5-HT. With confocal microscopy, they were able to directly visualize fluorescent resonance energy transfer (FRET) on the plasma membrane of living cells expressing 5-HT_{2C} receptors labeled with cyan (donor) and yellow (acceptor) fluorescent proteins. FRET, accessed by acceptor photobleaching, was dependent on the donor/acceptor ratio and independent of acceptor expression levels. This indicated that FRET resulted from receptor clustering and not from overexpression of randomly distributed receptors, providing evidence for GPCR dimers/oligomers in a clustered distribution on the plasma membrane. Together, these results suggest that 5-HT_{2C} receptors exist as constitutive homodimers on the plasma membrane of living cells.

Electrophysiological Responses

There is also evidence for the 5-HT_{2C} receptor mediated excitation of neurons in certain brain regions. Particularly, neurons in the rat substania nigra reticulata in vitro are excited by 5-HT and this response can be blocked by ketanserin and methysergide but not spiperone or selective antagonists of 5-HT_1, 5-HT_3, and 5-HT_4 receptors (Rick et al.,

1995). In the rat pyriform cortex, activation of 5-HT_{2C} receptors leads to depolarization of pyramidal neurons. The response of these neurons to 5-HT was blocked by spiperone, ritanserin, and LY 53857 but at concentrations that appeared to be somewhat higher than those need to block the 5-HT_{2A} receptor mediated responses in the same preparation (Sheldon and Aghajanian, 1991).

Motor neurons of the facial nucleus in vitro and in vivo are activated by the local application of 5-HT and 5-HT_2 receptor agonists. This effect may be mediated by the 5-HT_{2C} receptor (for review see Aghajanian, 1995). In earlier in vitro studies in the response of these neurons to 5-HT was block by methysergide, but not ketanserin or spiperone (Larkman and Kelly, 1991). On the other hand, other studies have shown that excitation of facial motor neurons by 5-HT and other 5-HT agonists were blocked by ritanserin and spiperone (Aghajanian, 1995). However, the lack of 5-HT_{2A} receptor-like immunoreactivity in the rat facial nucleus suggests that the physiological factors are non-5-HT_{2A} mediated; presumably 5-HT_{2C} receptors (Morilak et al., 1993). The development of more 5-HT_2 subtype specific agonists will aid in solving these problems.

Behavioral and other Physiological Repsones

Several behavioral responses have been shown to be associated with activation of central 5-HT_{2C} receptors. These include hypolocomotion, hyperphagia, anxiety, penile erections, and hyperthermia (for review see Koek et al., 1992). Most of these associations are based upon behavioral effects seen in rats with non-selective 5-HT_2 receptor agonists such as mCPP, TFMPP, and MK 212 along with antagonism by non-selective 5-HT_2 antagonists such as mianserin and ritanserin. However, the evidence for

involvement for the 5-HT_{2C} receptor in these behaviors is becoming convincing as we continue to study these behaviors with more specific ligands and the use of transgenic animal models.

Although the agonists mCPP and TFMPP are partial agonists at the 5-HT_{2C} receptor, these compounds normally have antagonistic properties at the 5-HT_{2A} receptor (Conn and Sanders-Bush, 1987; Baxter et al., 1995). Futhermore, 5-HT_{2A} receptor antagonists such as ketanserin are generally inactive against mCPP responses (Koek et al., 1992). The 5-HT_{2C} receptor's role in mCPP-induced hypophagia is further supported by the fact that the 5-HT_{2C} receptor knockout mice are obese (Tecott et al., 1995). Additionally, mCPP-induced hyperphagia, hypolocomotion, and anxiety are all antagonized by the 5-$HT_{2C/2B}$ antagonists SB 200646 or SB 206553 (Kennett et al., 1994, 1996). The 5-HT_{2C} receptor selective antagonist SB 242084 also potently blocks mCPP-induced hypolocomotion and hypophagia (Kennett et al., 1997). When 5-HT_{2C} receptor antagonists are given alone, they are anxiolytic in various animal models (Kennett et al., 1996, 1997). However, evidence suggests that normal animals treated with these antagonists do not over-eat or have a high propensity for epileptic seizures, even though these are hallmark features of the 5-HT_{2C} receptor knock mice (Tecott et al., 1995). Therefore, the phenotypes in the knock out mice may be a result of developmental abnormalities versus a loss of the receptor in adult mice (Kennett et al., 1997; Bonhaus et al., 1997).

There is also evidence that 5-HT_{2C} receptors are able to regulate the release of other neurotransmitters. Antagonists of these receptors have been reported to increase the release of noradrenaline and dopamine in microdialysis experiments (Millan et al.,

1998; Di Matteo et al., 1998) and via in vivo extracellular single cell recording (Blackburn et al., 2002). These data suggest that 5-HT_{2C} receptors have a tonic inhibitory effect on mesocortical/mesolimibic dopaminergic and noradrenergic projections. Corticosterone and ACTH responses to mCPP in rats may be mediated by the 5-HT_{2C} receptor (Fuller, 1996). In humans, mCPP-induced prolactin secretion involves the 5-HT_{2C} receptor (Cowen et al., 1996). Blockade of 5-HT_{2C} receptors in human, causes increase in slow wave sleep (Sharpley et al., 1994).

Molecular Diversity: Regulation at the Level of RNA processing

Proteins are able to generate molecular diversity through processing mechanisms at various levels, including at the RNA level. This enables many protein isoforms to be generated with different functions from a single gene product. Two RNA processing events that alter the 5-HT_{2C} receptor are RNA splicing and RNA editing. These processes together generate diverse 5-HT_{2C} receptor isoforms with altered functions.

RNA Splicing

The genes for both the 5-HT_{2A} and 5-HT_{2C} receptors undergo alternative splicing of their pre-mRNAs. This process entails removal of introns in more than one way before RNA translation. Most of the nucleotide sequences around the intron begin at the 5′-end with a GU dinucleotide sequence and terminate at the 3′-end with an AG dinucleotide sequence. These, as well as other consensus sequences attract the spliceosome, a molecular complex essential to splicing of the introns and ligation of the exons. As these introns are spliced out of the nascent RNA at alternative splice sites,

multiple protein isoforms result from a single gene locus. To date, all of the protein isoforms generated by alternative RNA splicing of the 5-HT_{2A} and 5-HT_{2C} receptors encode pharmacologically inactive proteins. For example, the 5-HT_{2A} receptor is alternatively spliced producing a variant RNA with a 112-base insert which results in a frame shift and a premature stop codon (Guest et al., 2000). Consequently, this transcript encodes a nonfunctional, truncated protein that terminates in the region of TM 4. While it is possible that the truncated protein could interact with the full-length 5-HT_{2A} receptor protein in a dominant-negative manner to regulate its function, careful evaluation of this phenomenon indicated that this is not the case (Guest et al., 2000).

The 5-HT_{2C} receptor has two alternative splice variants. The first one discovered has a 96-bp deletion that occurs at the junction of exon II and III, resulting in a protein that is truncated near the putative junction of the second intracellular loop and TM 4 (Canton et al., 1996; Xie et al., 1996). The full-length and the truncated mRNAs are expressed in parallel throughout the brain. Studies examining the function of the truncated protein expressed alone or in combination with the full-length protein suggest that the splice variant is inactive. The second splice variant was identified by Wang et al (2000). This splice variant had a truncated C-terminus that displayed no ligand binding capability or G-protein coupling activity. Upon further examination of the truncated splice variant, the data suggested that RNA editing of this variant occurred after completion of splicing, resulting in complete editing at all five sites (Wang et al., 2000).

RNA Editing: Definition and Historical Overview

A second less common mechanism for creating protein diversity at the level of RNA processing is the relatively recently discovered process, RNA editing. RNA editing was initially described as a phenomenon in which uridine residues were inserted or deleted from mitochondrial RNAs of kinetoplastid protozoa (Benne et al., 1986). Now this process is broadly defined as any event that changes the coding potential of primary RNA transcripts by mechanisms other than RNA splicing. The most common form of editing involves substitution of nucleotides and this phenomenon has been observed in the mouse and a viral pathogen that affects humans (Samuel, 2003). The nucleotide substitution consists of either cytidine-to-uridine (C-to-U) or adenosine-to-inosine (A-to-I) alterations which may result in altered coding potential of the mRNA and altered function of the encoded protein.

Transcripts in the mouse intestine encoding apolipoprotein B (apoB) were the first example of RNA editing on a nucleus encoded mRNA. In apoB, cytidine at the C4 position is converted to uridine by a specific cytidine deaminase. This deamination converts a glutamine codon (CAA) into a stop codon (UAA), thereby producing a novel apoB protein isoform with distinct physiological properties in comparison to the full length protein (Davidson, 1993). A tripartite regulatory sequence surrounding the edited cytidine residue along with a multi protein complex called an editosome is required for this modification to take place (Gott and Emeson, 2000). This C-to-U change can be easily detected. In contrast, A-to-I editing is more difficult to detect.

RNA Editing of Glutamate Receptor Subunits

The AMPA subtype of the glutamate receptor is composed of four subunits, GluR-A, -B, -C, and –D which can form homomeric and heteromeric ligand-gated ion channels (Hollmann et al., 1989; Keinanen et al., 1990; Dingledine et al., 1999). Comparisons between genomic and cDNA sequences of transcripts encoding subunits of the GluR-B receptor revealed adenosine-to-guanosine changes. It was later found that these discrepancies actually resulted from the enzymatic deamination of adenosine residues to inosines. Inosines have base pairing properties similar to guanosines; therefore they are translated as guanosines by the decoding ribosomes (Higuchi et al., 1993; Melcher et al., 1995). This modification involves the hydrolytic deamination of adenosine at the C6 position of the purine ring (Melcher et al., 1995; Polson et al.1994). A-to-I editing requires short complementary RNA sequences embedded in an adjacent intron to form an imperfect RNA duplex with the exonic target sequence around the adenosine to be deaminated (Seeburg, 2002). Given that A-to-I editing may occur in introns of pre-mRNAs and intron-exon base pairing interactions are required, it is clear that RNA editing is a nuclear event that precedes splicing (Higuchi et al., 1993).

The presence or absence of the GluR-B subunit defines the divalent cation permeability of the AMPA receptor (Hollmann et al., 1991). AMPA receptors with the GluR-B subunit are impermeant to calcium ions while those without the subunit increase their permeability to calcium. RNA editing of the GluR-B determines its calcium permeability, suggesting that RNA editing process is a significant regulatory mechanism for neuronal activation and signaling (Verdoorn et al., 1991). Editing occurs at a specific arginine residue within the second transmembrane (TM2) region of the protein.

Nucleotide sequence analysis revealed an arginine (CGG) codon in GluR-B cDNAs, while a glutamine (CAG) codon was found in the GluR-B genomic DNA (Sommer et al., 1991). Southern analyses of genomic DNA ruled out multiple genes or alternative splicing of the arginine codon, leaving the hypothesis that RNA editing caused the base change (Sommer et al., 1991). Later it was found that this discrepancy resulted from conversion of an adenosine to inosine (Rueter et al., 1995). The GluR-B receptor is edited nearly 100% of the time, thus dictating the calcium impermeability of this ion channel. The subunits of heteromeric kainate receptors (GluR-5 and GluR-6) have also been shown to be regulated by RNA editing (Sommer et al., 1991; Kohler et al., 1993).

Adenosine Deaminases that act on RNA (ADAR1)

Even before the discovery of the editing in the GluR-B subunit of the glutamate receptor, an activity that unwinds double-stranded RNA (dsRNA) was identified and described in *Xenopus laevis* (Bass and Weintraub, 1987). Later studies discovered that instead of unwinding duplex RNA, this activity catalyzed the conversion of adenosine to inosine by way of hydrolytic deamination (Polson et al., 1991). This enzymatic activity causes the RNA to become more single stranded by converting the stable A-U base pairs to less stable I-U pairs, ultimately leading to destabilization of the RNA duplex (Bass and Weintraub, 1988). For several years, this enzyme became known as dsRNA-specific adenosine deaminase (dsRAD or DRADA), and is now referred to as adenosine deaminases that act on RNA (ADAR1) (Reuter and Emeson, 1998). Two other ADARs (ADAR2 and ADAR3) have been clone with unique substrate specificity and regional localization.

RNA Editing of the 5-HT_{2C} Receptor

Editing of the 5-HT_{2C} receptor was discovered by comparing sequences from rat genomic DNA and cDNAs from the striatum; four A-to-G discrepancies were identified. The cDNA library predicted the presence of valine (GTG), serine (AGT), and valine (GTT) (5-$HT_{2C\text{-}VSV}$) at positions 157, 159, and 161 respectively. Conversely, the genomic DNA was found to be encoded by isoleucine (ATA), asparagine (AAT), and isoleucine (ATT) (5-$HT_{2C\text{-}INI}$) at these three positions (Burns et al. 1997). As a result, it was proposed that the four adenosine residues in the 5-HT_{2C} receptor RNA (Fig. 5; sites termed A, B, C, and D) were converted into inosines in the mature mRNA by a process analogous to that seen for editing of the subunits of the AMPA and kainate glutamate receptors. Further analysis of cDNA sequences isolated from rat brain revealed the tissue specific expression of seven major 5-HT_{2C} receptor isoforms, encoded by eleven distinct RNA species. The most prominent variant found in whole rat brain extracts was encoded by editing at the A, B, and D sites and expressed the 5-$HT_{2C\text{-}VNV}$ receptor protein (Fig. 6). On the other hand, editing at the A and B sites was reduced in choroid plexus, thus generating high levels of the 5-$HT_{2C\text{-}INI}$, 5-$HT_{2C\text{-}INV}$, and 5-$HT_{2C\text{-}ISV}$ receptor variants (Burns et al., 1997). In the human brain, a novel editing site was found and termed E (previously named C') (Fig. 5). This modification occurs at codon 158, converting the asparagine into a glycine resulting in the fully edited 5-$HT_{2C\text{-}VGV}$ protein (Niswender et al., 1999). These editing events take place in the putative second intracellular loop of the receptor (Burns et al., 1997); thereby altering the coding potential in a region implicated in receptor:G-protein coupling (Arora et al., 1995; Blin et al., 1995; Arora et al., 1997).

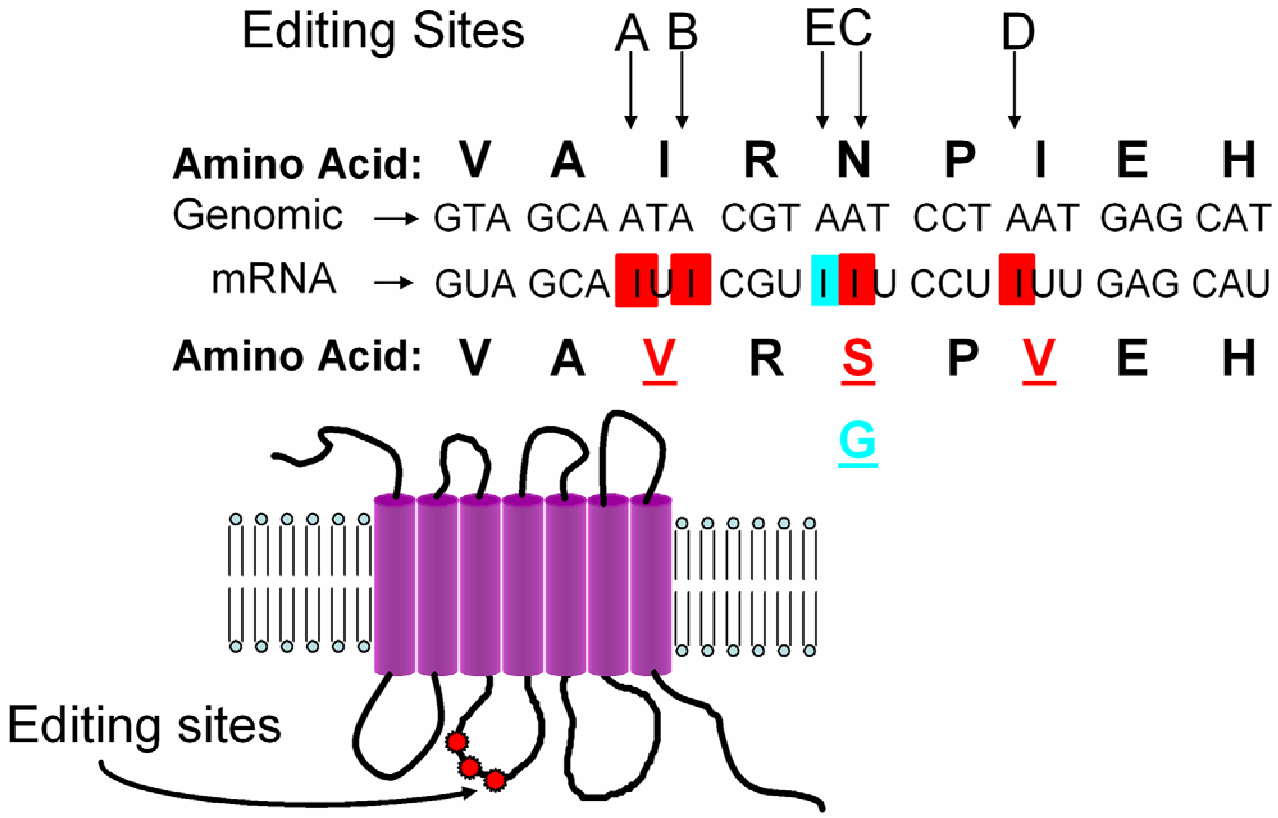

Figure 5: 5-HT$_{2C}$ receptor editing sites

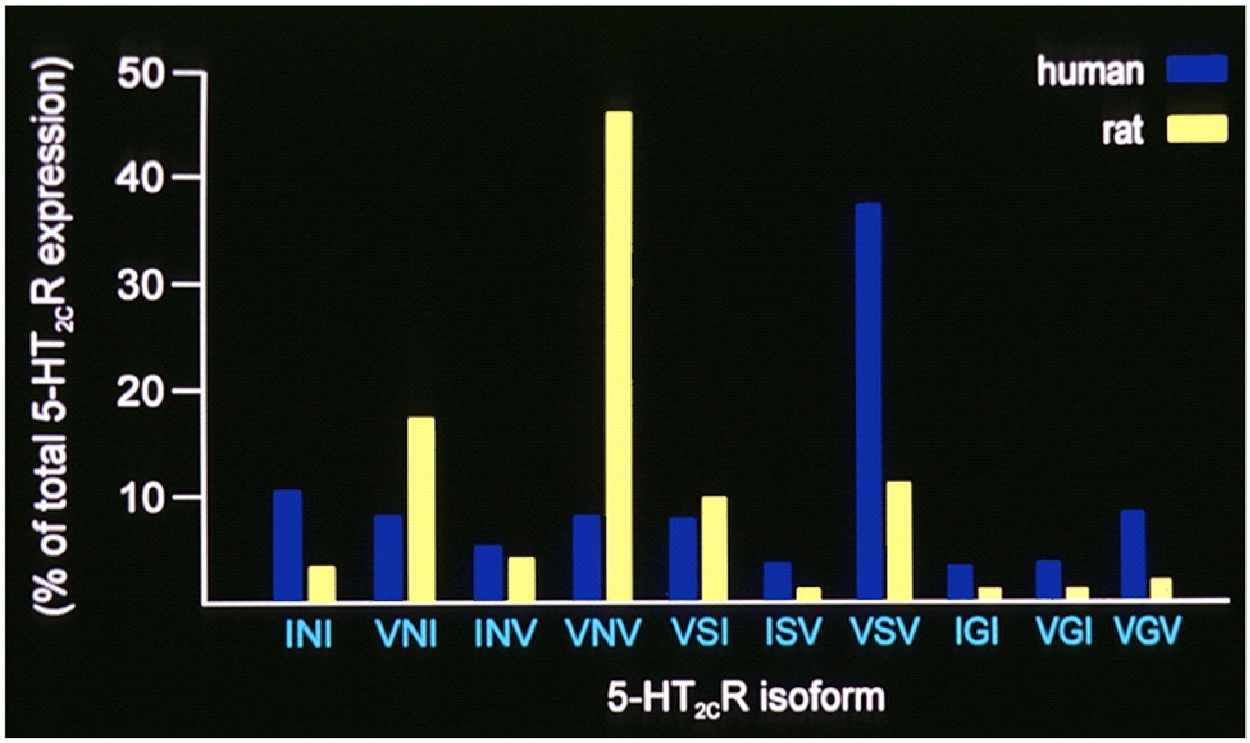

Figure 6: 5-HT_{2C} receptor expression in rat and human brain
(Niswender et al., 1999).

Molecular Mechanisms of 5-HT_{2C} Receptor RNA Editing

The family of adenosine deaminases that act on RNA (ADAR) consists of three members; ADAR 1 and 2, which are ubiquitously expressed, and ADAR3, which is highly enriched in brain. These enzymes bind specifically to double-stranded RNA and catalyze the conversion of adenosine to inosine by hydrolytic deamination. Analysis of DNA from the rat 5-HT_{2C} receptor gene, in the region surrounding the potential editing sites, suggested the existence of an imperfect inverted repeat forming a putative RNA duplex between the 3' end of exon 3 and the proximal region of intron 3 (Fig. 7; Rueter and Emeson, 1998). This region is conserved between human and rodents. In order to study the molecular mechanisms of the post-transcriptional modification of 5-HT_{2C} receptor transcripts, Burns et al. (1997) developed an *in vitro* editing system using rat brain nuclear extracts fractionated by cation-exchange chromatography and a 5-HT_{2C} receptor RNA substrate labeled with [α-^{32}P] adenosine 5'-triphosphate. These experiments revealed two peaks of inosine that co-eluted with two distinct peaks of editing activity. Activity of the first peak was responsible for editing 5-HT_{2C} receptor transcripts at sites A, B, and C and co-eluted with the activity of ADAR1, which has also been shown to modify the B subunit of AMPA glutamate receptors (Yang et al., 1995). The second peak of activity specifically modified the D site of the transcripts and co-eluted with the activity of ADAR2, the enzyme that has been shown to modify the Q/R site of GluR-B (Maas et al., 1996).

Functional Consequences of 5-HT_{2C} Receptor Editing

Since editing of the 5-HT_{2C} receptor occurs in a region involved in G-protein

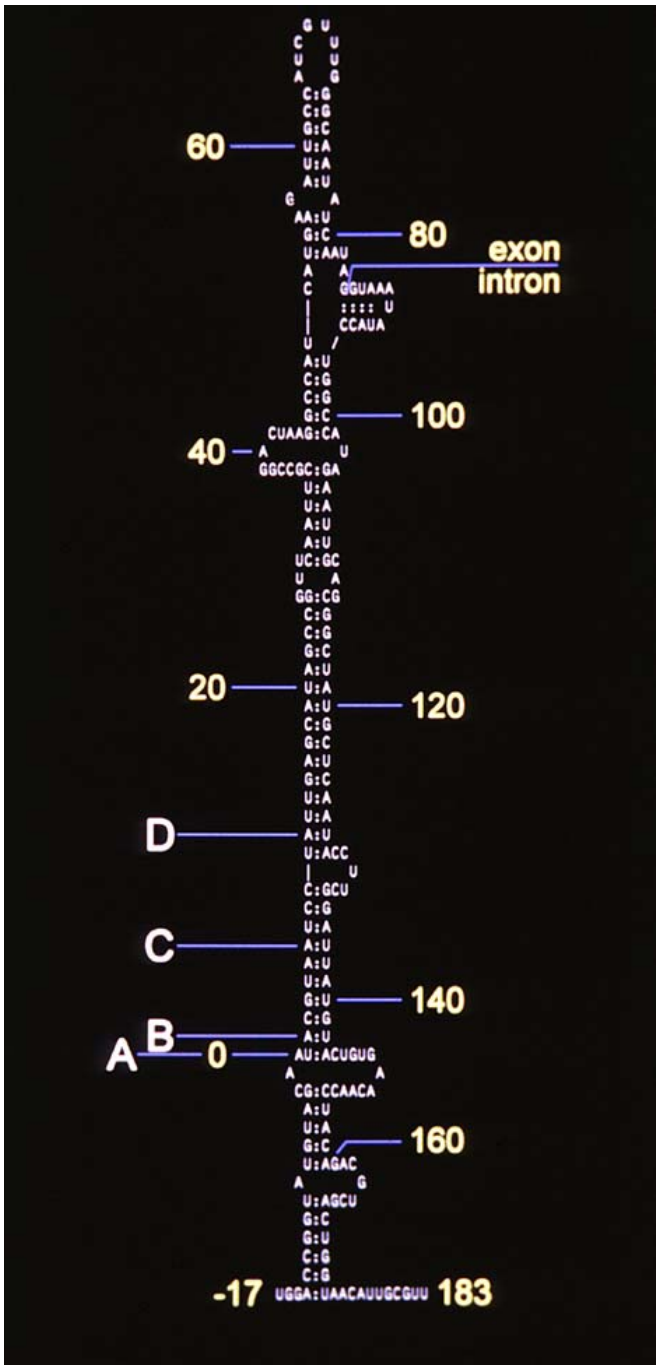

Figure 7: Rat 5-HT$_{2C}$ receptor RNA duplex structure
(Rueter and Emeson, 1998).

coupling, it was hypothesized that this post-transcriptional modification may alter receptor function. In order to test the functional consequences of RNA editing, NIH-3T3 fibroblasts were transfected with different 5-HT_{2C} receptor isoforms and their ability to activate phospholipase C (PLC) was assayed. The rat 5-$HT_{2C\text{-}VSV}$ isoform displayed 10-15 fold lower potency upon 5-HT stimulation compared to the unedited 5-$HT_{2C\text{-}INI}$ isoform. Affinity changes, desensitization, and spare receptors were all ruled out as possible explanations for these differences, leaving reduced receptor G-protein coupling as the mechanism for the alteration in potency (Burns et al., 1997). The pattern of editing in human brain differs from that found in rat, principally due to increased editing at the C and E positions. This leads to the production of the 5-$HT_{2C\text{-}VSV}$ isoform as the most prominent variant in human brain as opposed to 5-$HT_{2C\text{-}VNV}$ in the rat (Fig. 7; Niswender et al., 1999; Fitzgerald et al., 1999). The human 5-$HT_{2C\text{-}VSV}$ receptor exhibited a 5-fold shift in potency for 5-HT when compared to the 5-$HT_{2C\text{-}INI}$ receptor (Niswender et al., 1999), while the human 5-$HT_{2C\text{-}VGV}$ variant showed an even greater shift (29-fold) (Fig. 8). It was also found that the 5-$HT_{2C\text{-}INI}$ receptor existed in a high and low affinity state for 5-HT, while the 5-$HT_{2C\text{-}VGV}$ receptor lost the agonist high affinity state (Niswender et al., 1999). The 5-$HT_{2C\text{-}VGV}$ receptor was also unable to stimulate inositol phosphate formation in the absence of agonist, a property referred to as constitutive activity that has been well established to occur at both the rat and human 5-$HT_{2C\text{-}INI}$ receptors (Barker et al., 1994). Price and Sanders-Bush (2000) later showed that editing also delayed agonist-stimulated calcium release. Taken together, these functional data in cell lines show that unedited 5-HT_{2C} receptors couple more efficiently to G proteins even when no agonist is presence, suggesting that editing may be a way to decrease tone and signaling in certain

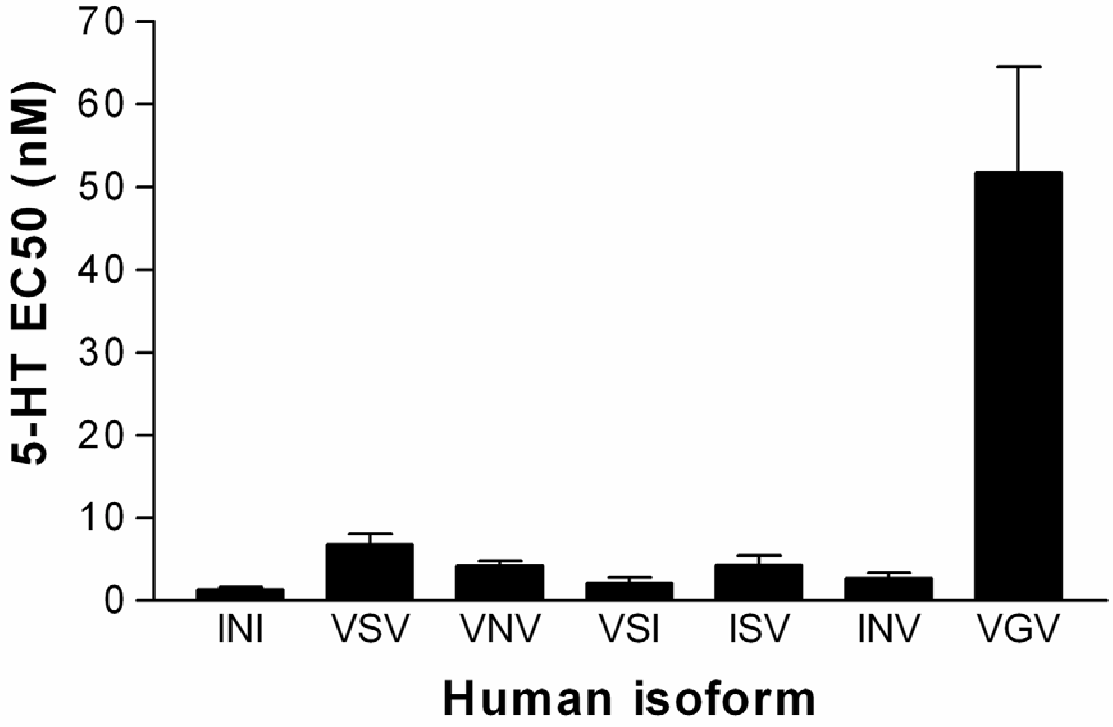

Figure 8: RNA editing changes EC_{50} values for PI hydrolysis
EC_{50}, effective concentration at 50% response; PI, Phosphoinositide (Niswender et al., 1999).

brain regions. More recently, studies of the profile of G protein coupling utilizing a high throughput functional assay have demonstrated that not only is coupling efficiency reduced, but also, the pattern of G protein coupling is altered (Price et al., 2001). The 5-$HT_{2C\text{-}INI}$ receptor isoform couples to Gq/11, as well as, to G13. The 5-$HT_{2C\text{-}VGV}$ and 5-$HT_{2C\text{-}VSV}$ receptor isoforms have loss the ability to couple to G13 and this in turn leads to altered intracellular signaling (Fig. 9).

In order to determine the role of RNA editing in drug response, cell lines expressing variant 5-HT_{2C} receptor isoforms were tested with the hallucinogenic drug lysergic acid diethylamide (LSD) and antipsychotic drugs. A marked reduction was found at 5-$HT_{2C\text{-}VGV}$ receptors in the efficacy of LSD and antipsychotics, suggesting a possible role for editing in the etiology and treatment of schizophrenia. Initial studies of antipsychotic drugs suggested that a reduction in the constitutive activity of the non-edited INI isoform differentiated atypical from typical antipsychotics (Herrick-Davis et al., 1999; Niswender et al., 1999); however, a more recent study failed to confirm this difference (Rauser et al., 2001). Niswender et al. (2001) examined the isoform distribution in human post-mortem brain tissue of normal controls and found the VSV variant to be the most abundant isoform. They also examined total RNA from the prefrontal cortex (PFC) (Brodmann areas 8 and 9) of 13 controls, 13 major depressed, and 13 schizophrenic subjects and found no significant differences in editing between the groups. However, upon analyzing suicide versus non-suicide, the suicide population exhibited significantly higher levels of editing at the A-site, independent of diagnosis. On the other hand, a study by Sodhi et al. (2001) found that RNA editing frequencies in the PFC (Brodmann area 46) of schizophrenics was reduced at all editing sites, but only

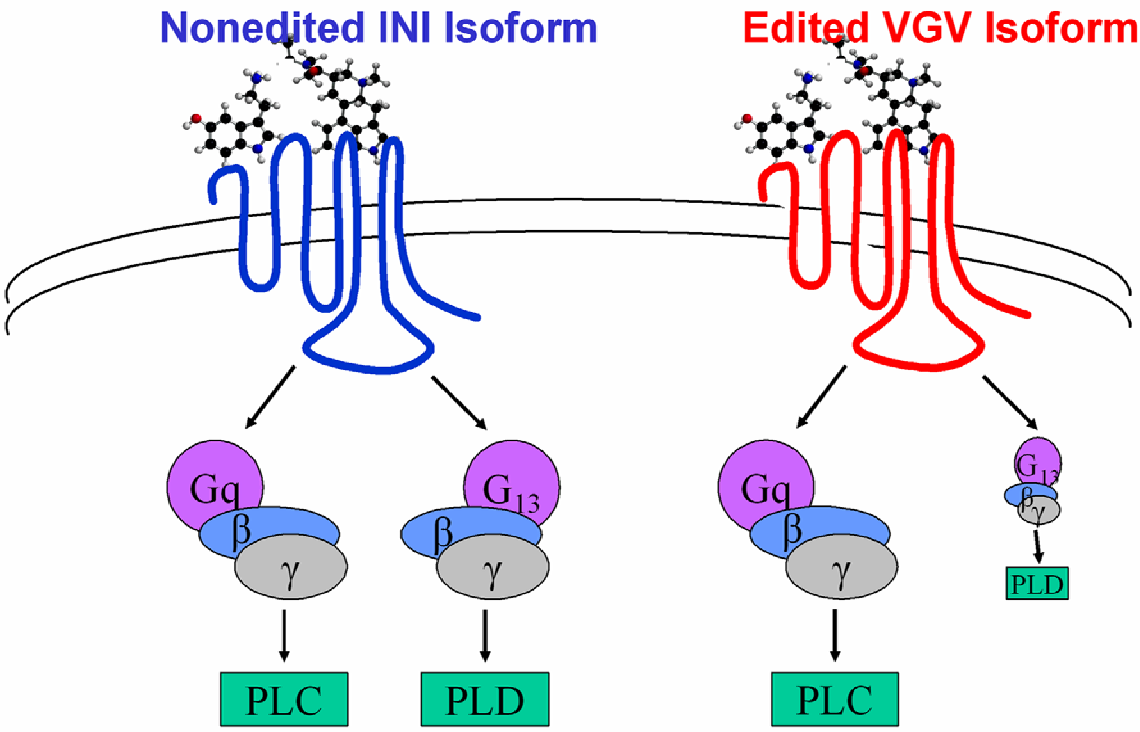

Figure 9: Functional consequences of RNA editing
RNA editing alters the G-protein coupling profile of the 5-HT_{2C} receptor with subsequent fine-tuning of downstream signals. INI and VGV refer to the amino acids at positions 156, 158, and 160; PLC-phospholipase C; PLD-phospholipase D. (Sanders-Bush et al., 2003).

editing at the B-site reached statistical significance. Consequently, this association was found with only five schizophrenic patients and was largely due to the INI isoform which was found at 20% in the schizophrenics and not at all in the controls.

A more recent study has shown that depressed suicide victims have alterations in RNA editing (Gurevich et al., 2002a). Gurevich et al. examined the dorsal PFC (Brodmann area 9) of 5 controls and 6 matched suicide victims (5 of which were diagnosed with a depressive illness). Only one of the suicide victims had a history of antidepressant and neuroleptic drug treatment prior to death. Upon comparison of the two groups, there was a significant increase in editing of the C′-site (also known as the E-site) and a significant decrease in editing of the D-site in the depressed suicide victims. There was also a trend for increased editing at the C-site. Furthermore, the suicide victim with a history of neuroleptic and antidepressant treatment prior to death differed from both controls and the other five suicide victims, mainly due to the high percentage of the non-edited INI isoform. Iwamoto and Kato (2003) examined the RNA editing efficiencies of the A and D-sites of the 5-HT_{2C} receptor mRNA in the PFC (Brodmann area 10) of patients with bipolar disorder (n=12), schizophrenia (n=13), major depressive disorder (n=11) and control subjects (n=15). The authors did not find significant alterations in the editing efficiencies at these sites, but there was a trend for increased editing at the D-site in depressed patients and increased editing at the A-site in suicide victims. Although not significant, this increased in editing efficiency of the A-site supports the results of Niswender et al. (2001). Shortly after Iwamoto and Kato's report came a study which investigated alternative splicing and RNA editing in the PFC (Brodmann area 46) of 15 schizophrenic patients and 15 controls. No significant

differences in RNA editing efficiencies or alternative splicing were found confirming Niswender et al. (2001) results that RNA editing is not altered in schizophrenia. Recent evidence suggests that 5-HT_{2C} receptor editing is modulated by changes in 5-HT neurotransmission such as 5-HT depletion and treatment with agonists and antagonists (Gurevich et al., 2002b).

Clearly, there is still a debate as to whether on not RNA editing of the 5-HT_{2C} receptor is altered in psychiatric disorders. Additional studies are needed with larger numbers of patient samples to give the groups more power to find a significant difference between the patient and control groups. The inability of these groups to replicate each other's findings could also be due to the different regions of the PFC analyzed or differences in the way brains were stored. Although preliminary, Gurevich et al.'s (2002a) findings in the one suicide victim with a history of drug treatment lends support to the hypothesis that drug treatment may alter RNA editing. This serves as another potential problem when trying to replicate these findings since various drugs may affect RNA editing of the 5-HT_{2C} receptor differently. These findings lend further support to the hypothesis that RNA editing is an important process that regulates synaptic input in certain brain areas by fine tuning optimal signaling. This in turn may lead to novel drug treatments for psychotic patients.

Genetics of 5-HT_{2C} Receptors

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are substitutions of the wild-type nucleotide for a novel nucleotide within the genomic DNA. SNPs are the most common type of genetic variation and occur at a frequency greater than 1% (Wang et al., 1998). Within the coding region of a gene, SNPs that cause a change in the encoded amino acid (non-synonymous SNP) may have a deleterious consequence on protein folding. Many such polymorphisms produce an unstable conformation of the protein, resulting in retention in the endoplasmic reticulum and decreased plasma membrane expression (Wenkert et al., 1996). Non-synonymous SNPs can also interfere with plasma membrane retention mechanisms, such as palmitoylation and glycosylation. Finally, alterations in the amino acid structure can have dramatic consequences on the function of a protein. SNPs that alter the primary protein structure of a G-protein coupled receptor could modify the binding pocket of the receptor, disrupting receptor-ligand interactions, and thus changing the binding properties of the drug or the ability of the receptor to isomerize to the active form. Alternatively, non-synonymous SNPs may interfere with the G-protein coupling region of the receptor, changing the kinetics of receptor-G-protein interaction in either the ligand-activated or basal state of the receptor. SNPs in this region could also alter phosphorylation of the receptor or the binding of accessory scaffolding proteins necessary for internalization and desensitization of the receptor. Each of these problems would result in a diminished signal downstream of the receptor leading to altered, and possibly inappropriate, cellular response to stimuli.

Other forms of variation include coding polymorphisms that do not alter the amino acid sequence (silent or synonymous), promoter polymorphisms and variable number of tandem repeat (VNTRs) in 5' and 3' untranslated regions (UTRs). However, the consequences of these polymorphisms have been more difficult to deduce. Each of these types of SNPs may have an indirect impact on receptor expression by altering the efficiency of transcription or translation. Diminished or enhanced receptor expression will impact the signaling cascade downstream of neurotransmitter, thus altering the overall cellular response to the stimulus.

Most polymorphisms are synonymous or non-coding in nature. This is believed to occur because there is selection against non-synonymous polymorphisms in most genes due to the high likelihood of deleterious effect on protein structure and function. SNP frequency is also known to vary by race (Jorde, 2001; Tishkoff, 2002) and by gender. The frequency of SNPs can vary tremendously between populations, and there are many reports of enriched frequencies of SNPs in African populations (Alonso, 2001; Jorde, 2001). This ethnic variability – diminished diversity in non-African populations – is consistent with a genetic bottleneck during migration out of Africa and should be carefully considered when choosing a population for association analysis. Gender is a factor for polymorphisms that occur on genes of the X- and Y-chromosomes. SNPs that occur in this region may have a dose-dependent effect in both males and females. Clearly, the single copy of the X- or Y-chromosome in men can impact the frequency at which these SNPs can occur among males. Additionally, the X-chromosome also undergoes the phenomena of X-chromosome inactivation (XCI) – the silencing of one copy of the X-chromosome. XCI will not change the genotypic frequency of a

polymorphism in females, but it can alter the phenotypic consequence of variability of the silenced gene. By means similar to XCI, several autosomal genes are imprinted – an event wherein one or several genes on a chromosome are selectively inactivated in a tissue- or developmental-specific manner. Polymorphisms that occur only in an imprinted gene may have no phenotypic or functional consequence upon the individual, yielding a greater degree of complexity to association studies. Lastly, some genes are reported to be imprinted in a polymorphic manner, including the 5-HT_{2A} receptor gene (Bunzel et al., 1998). This indicates that the gene is silenced in some individuals but not in others, and selective inactivation may result from a SNP within the gene or the imprinting machinery.

HTR2C Gene

The HTR2C gene that encodes 5-HT_{2C} receptor is X-linked (human chromosome Xq24; mouse chromosome X D-F4). The HTR2C gene contains six exons and five introns spanning at least 230 kb of DNA. However, the coding region of the 5-HT_{2C} receptor contains three introns as opposed to two introns in the 5-HT_{2A} and 5-HT_{2B} receptors. The complete cDNA consists of 4775 nucleotides of which 728 are in the 5′-untranslated region, 1377 in the coding region, and 2670 in the 3′-untranslated region encoding a protein of 458 amino acids with an estimated mass of 53 kDa (Xie et al., 1996). The 5′ leader region comprises exon I, II, and a small portion of exon III. The 5-HT_{2C} receptor cDNA coding region extends from the rest of exon III to exon VI (Fig. 10). The 5′-untranslated region of the human receptor is 80% homologous to the rat sequence except for a sequence of 80 bases 5′ of the translation start site (Saltzman et al., 1991),

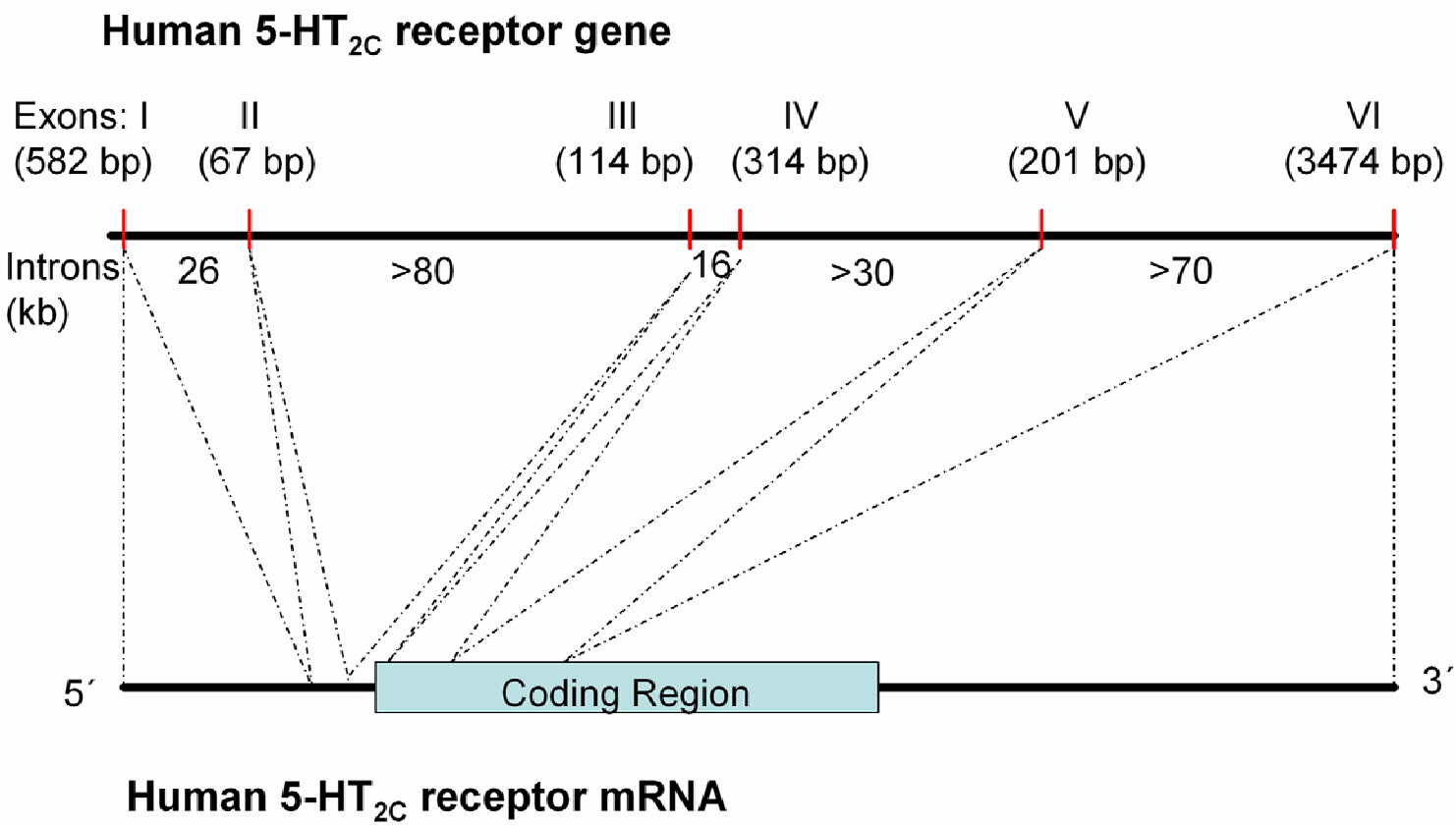

Figure 10: 5-HT$_{2C}$ receptor gene and mRNA structure

however there is controversy as to whether this region actually exists in the human receptor (Xie et al., 1996). Overall, the human 5-HT_{2C} receptor sequence is 90% homologous to the rat receptor.

The human and rat 5-HT_{2C} receptors are most divergent at the N-terminal extracellular domain (78% homology) and their third cytoplasmic loop (71% homology). However, there is a single conserved potential N-linked glycosylation site in the N-terminus of both species (Fig. 11), which may play a role in trafficking of the receptors (Saltzman et al., 1991). There are a total of six potential glycosylation sites in the mouse and rat 5-HT_{2C} receptor (Yu et al., 1991) while there are four in the human (Saltzman et al., 1991). The mouse, rat, and human 5-HT_{2C} receptors have greater than 80% sequence homology in their transmembrane domains. The 5-HT_{2C} receptor possesses an extra hydrophobic domain at its N-terminus that could serve as an extra transmembrane domain, but recent evidence suggests that this may not be the case (Hurley et al., 1999). There are eight serine/threonine residues in the rat 5-HT_{2C} receptor which represent potential phosphorylation sites, and are conserved in the human receptor (Barnes and Sharp, 1999). Two different RNA processing events, RNA splicing and RNA editing take place in the 5-HT_{2C} RNA generating diversity through expression of different isoforms of the protein.

Polymorphisms within the 5-HT_{2C} Receptor Gene

Overall sequence identity between the 5-HT_{2A} and 5-HT_{2C} receptors is ~50% across the entire receptor and approximately 80% in the transmembrane regions. The serotonin 5-HT_{2C} receptor gene lies on the X-chromosome at Xq24 and it contains three

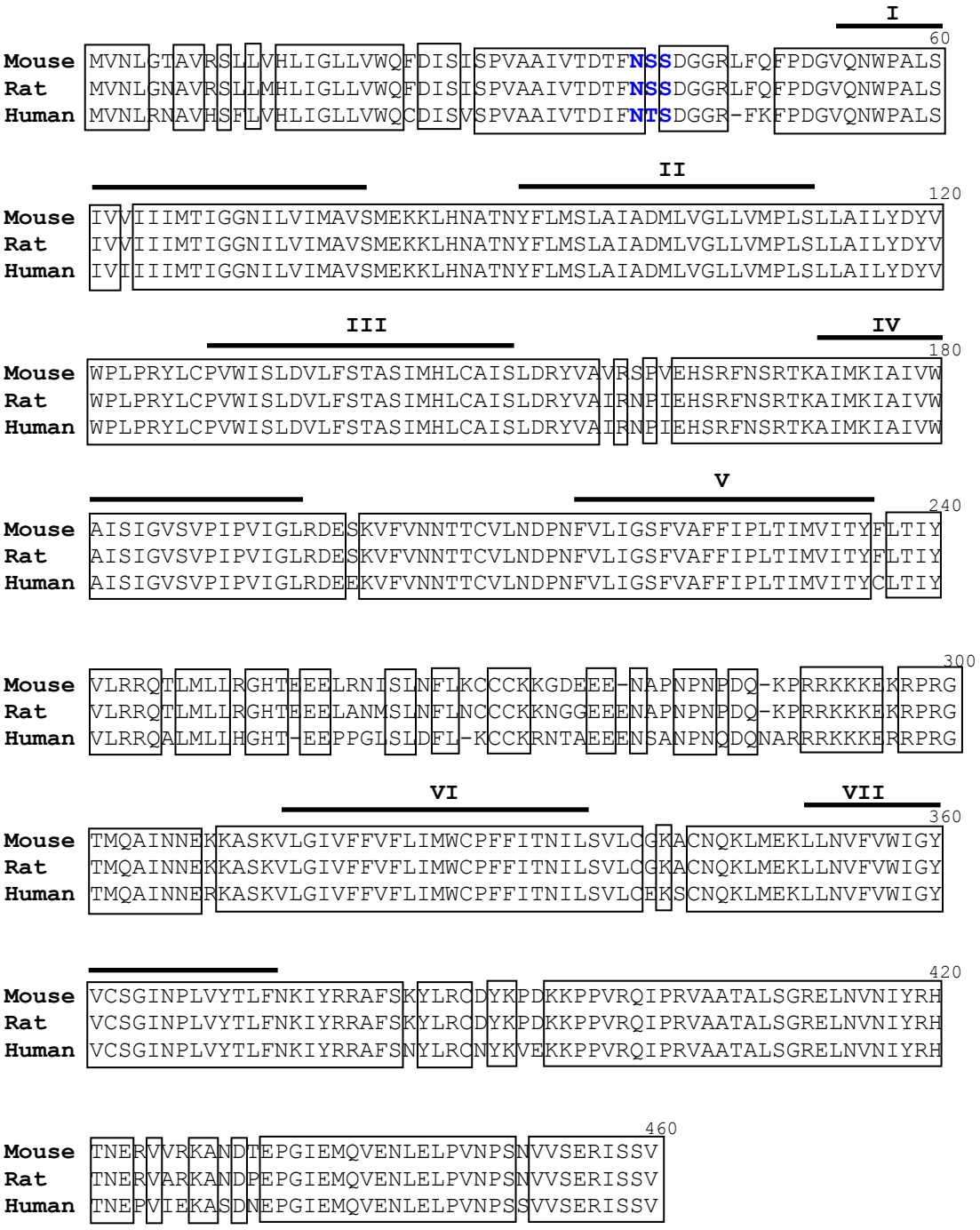

Figure 11: 5-HT$_{2C}$ receptor species alignment.
Alignment of the mouse, rat, and human 5-HT$_{2C}$ receptors. Amino acids in blue represent a putative conserved glycosylation sequence. Squared regions are regions of homology. Lines represent putative transmembrane domains.

introns within the coding region which spans approximately 1.4 Kb. Not only does the 5-HT_{2C} receptor achieve its diversity through molecular mechanisms such as RNA splicing and RNA editing, it also creates genetic diversity with the incorporation of SNPs. To date, there are three reported SNPs and two dinucleotide repeats in the promoter region of this receptor and one SNP in the 3' untranslated region (Fig. 12; Table 2). There have been two different reports of dinucleotide repeats in the promoter region of the 5-HT_{2C} receptor (Yuan et al., 2000; Meyer et al., 1999). Based upon the numbered locations of the repeats the two groups reported, it seems that there was three different dinucleotide repeats; two GT repeats and one CT repeat. However, careful examination of the promoter sequence (Xie et al., 1996) reveals that the two GT repeats were one and the same. The differences in sequence number resulted from one group numbering from the translation initiation codon (exon 3) and the other from the first exon. The functional characteristics of the promoter SNPs in the 5-HT_{2C} receptor have been examined in only two reports, both of which evaluated a series of alleles found at linked loci on a single chromosome know as haplotypes using luciferase reporter gene constructs (Yuan et al., 2000; Meyer et al., 2002). Yuan's group found that promoter activity of haplotype 2 (-1,027(GT)11, -697C) and haplotype 3 (-1,027(GT)11, -995A, -759T, -697C) was 1.44 and 2.58 fold higher than the wild type haplotype (-1,027(GT)17, -995G, -759C, -697C). Although there is not much functional data on these SNPs in the promoter of the 5-HT_{2C} receptor, many groups have begun to examine them in disease association studies as summarized in Table 3. Of these, the most promising seems to be the -759C/T polymorphism. These haplotypes containing the nucleotide substitutions in the promoter have been associated with higher transcription levels and resistance to obesity and Type

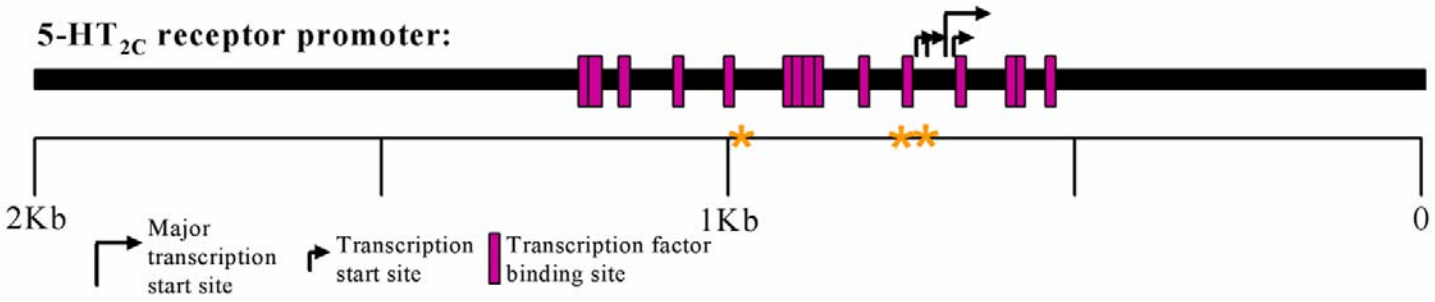

Figure 12: 5-HT$_{2C}$ receptor promoter map and polymorphisms. Yellow asterisks represent SNPs (Sanders-Bush et al., 2003)

Table 2: Frequency and distribution of 5-HT_{2C} receptor SNPs.

Nucleotide Substitution	*Nucleotide Position*	*Amino Acid Substitution*	*Domain*	*Minor Allele Frequency*	*Reference*
5-HT_{2C} Receptor					
(CT)4-5	-963	-	Proximal to NF-IL6 binding site	-	Myer et al., 1999
$(GT)_n$	-1,027	-	Between two NF-IL6 binding sites	Major: (GT)17	Yuan et al., 2000
[G/A]	-995	-	Between two NF-IL6 binding sites	11%	Yuan et al., 2000
[C/T]	-759	-	Proximal to LF-A1 binding site	11%	Yuan et al., 2000
[G/C]	-697	-	Between -703 and -692 transcription initiation sites	15%	Yuan et al., 2000
[G/C]	68	C23S	NT	13%	Lappalainen et al., 1995
[T/G]	2831	-	3′ UTR	10%	Song et al., 1999
[A/G]	1255	T419A	CT	<1%	Gibson et al., 2004
[C/G]	10	L4V	NT	1%	dbSNP

II diabetes (Yuan et al., 2000). All together, these studies support the hypothesis that SNPs in the promoter region of the 5-HT_{2C} receptor may alter transcription levels of the protein, and could potentially change the neuronal regulation of food intake in the hypothalamus. Therefore, continued identification and characterization of polymorphisms in the promoter region of this receptor are important. Two SNPs has been identified in the coding region of the 5-HT_{2C} receptor, one converting a cysteine (Cys) to a serine (Ser) at amino acid codon 23 (Lappalainen et al., 1995) and the other converting a threonine (Thr) to an alanine (Ala) at amino acid codon 419 (Fig. 13; Gibson et al., 2004). The latter SNP was only found in one early onset obese patient and has not been confirmed. Still another SNP found in the dbSNP database, converts a leucine (Leu) to a valine (Val) at amino acid condon 4. This SNP has not been reported elsewhere. Besides the present work, there have been only two functional studies performed on the Cys23Ser polymorphism. The first was conducted by the group that originally discovered it. Recombinant human 5-$HT_{2C\text{-}Cys}$ and 5-$HT_{2C\text{-}Ser}$ receptors were expressed in frog oocytes and 5-HT's ability to activate Ca^{2+} activated chloride channels evaluated (Lappalainen et al., 1995). Although no significant differences were found in this study, more detailed studies are needed to functionally characterize this variant in mammalian cell lines since so many association studies have found position correlations with the 5-$HT_{2C\text{-}Ser}$ allele (Table 3). Therefore, our laboratory has embarked upon a thorough pharmacological characterization of the 5-$HT_{2C\text{-}Ser}$ receptor variant in both an edited and non-edited backbone.

Numerous human studies have examined the association of the Cys23Ser SNP with disease states and drug responses (Table 3). One of the most frequently examined

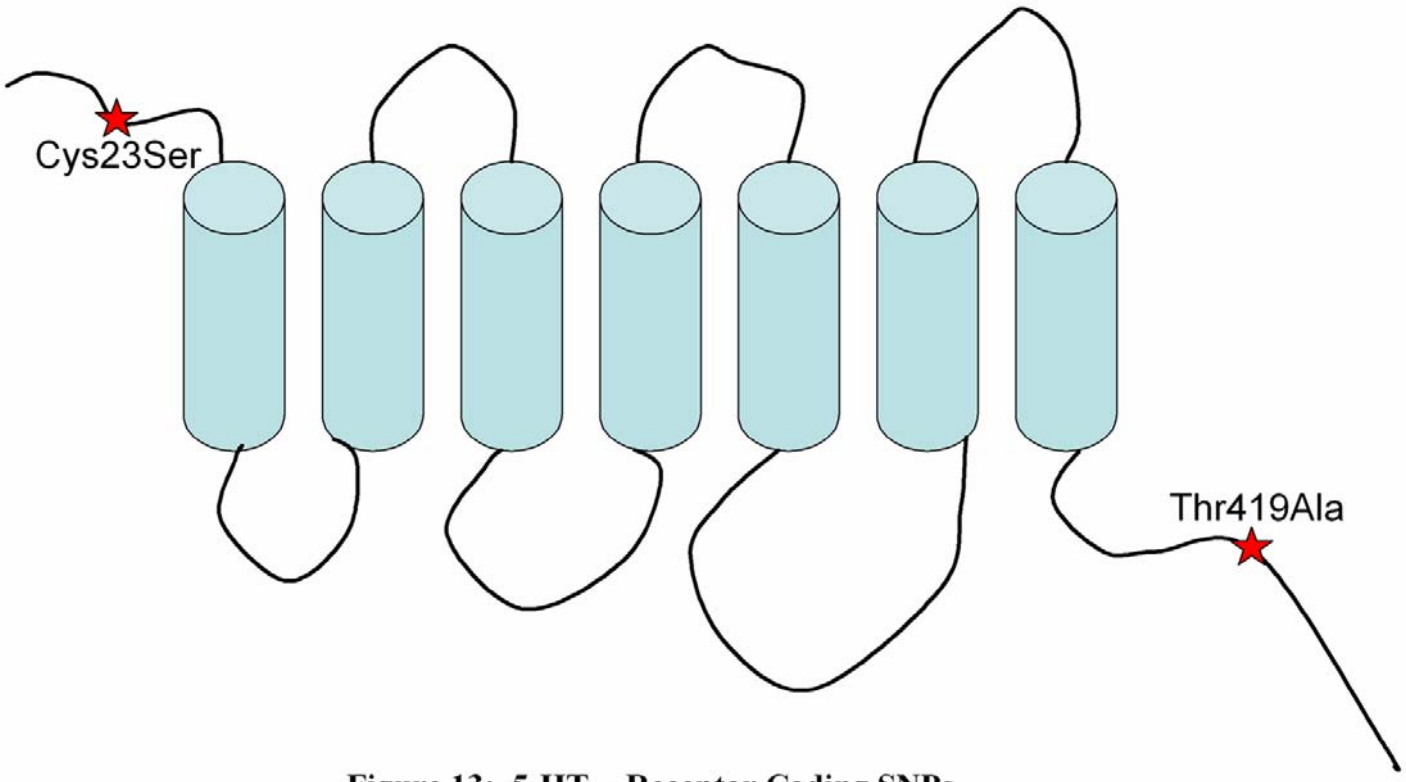

Figure 13: 5-HT_{2C} Receptor Coding SNPs

Table 3: Association studies of SNPs in the human 5-HT_{2C} receptor

MDD: Major Depressive Disorder; SAD: Seasonal Affective Disorder; Sp.-Clz response: Schizophrenia-Clozapine response; Sp./TD: Schizophrenia and/or Tardive Dyskinesia; BD: Bipolar Disorder; WG/ED: Weight Gain/Eating Disorder; AD: Alzheimer's Disease; Alc: Alcoholism; Neg: negative association; Pos: positive association; Mix: reports of positive and negative association; N/A: not studied.

SNP	**MDD**	**Suicide**	**SAD**	**Sp.-Clz response**	**Sp./TD**	**BD**	**WG/ED**	**AD**	**Alc**
5-HT_{2C} Receptor									
(GT)12-18/ (CT)4-5	N/A	N/A	N/A	Pos.[23]	N/A	Neg.[22]	Pos.[12]	N/A	N/A
-759C/T	N/A	N/A	N/A	Neg.[27]	Mix.[9,26,32]	N/A	Mix.[12,13,29]	N/A	N/A
-697G/C	N/A	N/A	N/A	N/A	Pos.[9,32]	N/A	Pos.[12]	N/A	N/A
C23S	Mix.[1,2,21]	Neg.[24,25]	Neg.[4]	Mix.[5,6,7]	Pos.[8]	Mix.[2,10,11,21]	Mix.[14,15,16,28]	Mix.[17,19,30]	Mix.[18,20,31]

References (superscript) **1:** Frisch et al., 1999; **2:** Lerer et al., 2001; **4:** Johansson et al., 2001; **5:** Malhotra et al., 1996; **6:** Sodhi et al., 1995; **7:** Masellis et al., 1998; **8:** Segman et al., 2000; **9:** Zhang et al., 2002; **10:** Gutierrez et al., 1996; **11:** Oruc et al., 1997; **12:** Yuan et al., 2000; **13:** Reynolds et al., 2002; **14:** Westberg et al., 2002; **15:** Burnet et al., 1999; **16:** Lentes et al., 1997; **17:** Holmes et al., 1998; **18:** Himei et al., 2000; **19:** Holmes et al., 2003; **20:** Hill et al., 2002; **21:** Serretti et al., 2000 ; **22:** Gutierrez et al., 2001; **23** Arranz et al., 2000; **24:** Stefulj et al., 2004; **25:** Pooley et al., 2003; **26:** Ellingrod et al., 2004; **27:** Theisen et al., 2004; **28:** Hu et al., 2003; **29:** Pooley et al., 2004; **30:** Assal et al 2004; **31:** Johann et al., 2003; **32:** Reynolds et al., 2003

phenotypes is clozapine response in schizophrenic patients; both positive and negative results have been reported with more evidence leaning towards a lack of association. The Cys23Ser polymorphism has also been shown to be associated with visual hallucinations and hyperphagia in Alzheimer's patients (Holmes et al., 1998); however, this finding has not been confirmed. Moreover, association studies of 5-HT_{2C} receptor polymorphisms require extra care since the gene is on the X chromosome. Some groups fail to address this issue although many have begun to analyze males and females separately.

Consequences of Genetic Variation

It has recently been hypothesized that common genetic variation may significantly contribute to risk for common diseases – the common disease-common variant (CD-CV) hypothesis (Cargill, 1999). If this hypothesis is accurate, then normal genetic variation within serotonin receptors could increase the likelihood of developing a psychiatric disorder linked to serotonergic pathways, such as depression or schizophrenia. Thus far, much of the association data that have been generated for SNPs in the 5-$HT_{2A/2C}$ receptor genes have been inconclusive. Often times a larger sample size would address many of the problems with these association studies. However, many of the disease states that could be impacted by the serotonergic system are polygenic in nature and are further influenced by environmental factors. This necessitates a large, well-defined disease population with several sub-phenotypes that can each be specifically evaluated with respect to the disease of interest. It is only by thorough investigation of the intricacies of these diseases that we will uncover the role that 5-$HT_{2A/2C}$ receptor polymorphisms may play in complex polygenic disorders. Finally, although functional investigations of the

promoter and non-synonymous SNPs of the 5-$HT_{2A/2C}$ receptors have been initiated, there is still a need for a deeper investigation into the consequence of these polymorphisms. Studies are currently underway to further clarify the impact of these polymorphisms on the structure and function of the human 5-$HT_{2A/2C}$ receptors. Additional understanding of the consequence of these SNPs will provide greater insight into the role of the serotonin 5-$HT_{2A/2C}$ receptors in complex human diseases, and may help define unique populations and sub-phenotypes for future association studies.

Specific Aims

Since the original discovery of the Cys23Ser polymorphism in the human 5-HT_{2C} receptor, little work has been done to look at the structural and functional consequences of this amino acid substitution. On the other hand, there have been numerous association studies in human subjects trying to link the polymorphism to various disease states, including major depressive disorder. The Cys23Ser polymorphism has been shown to associate with individuals who have late onset Alzheimer's disease with visual hallucinations and hyperphagia (Holmes et al., 1998), in addition to schizophrenia patients with tardive diskinesia (Segman et al., 2000). Recently, a genetic component of unipolar depression was identified (Sullivan et al., 2000). There have been only two association analyses of the Cys23Ser SNP with unipolar depression. One of these studies, performed on a population of European subjects found a positive association between the occurrence of the Ser23 allele with unipolar depression and bipolar disorder (Lerer et al., 2001). Such studies are driven by the emerging pharmacological evidence that the 5-HT_{2C} receptor may be involved in depression. For example, in studies of the

rat forced swim test, three novel selective 5-HT_{2C} receptor agonists, WAY 161503, RO 60-0175, and RO 60-0332, all decreased immobility and increased swimming, a pattern of behavior similar to that produced by the selective serotonin reuptake inhibitor (SSRI) fluoxetine. Furthermore, the selective 5-HT_{2C} receptor antagonist SB 206553 blocked the antidepressant-like effects of both WAY 161503 as well as fluoxetine (Cryan and Lucki, 2000), suggesting a role for 5-HT_{2C} receptors in the behavioral effects of antidepressant drugs.

Depression is a complex disease, making it highly unlikely that one SNP could cause all forms of unipolar depression. On the other hand, it is plausible that the Cys23Ser polymorphism in the 5-HT_{2C} receptor could cause an endophenotype of depression such as anxiety. The fact that this polymorphism occurs at a relatively high percentage (13%), suggests that this variation could have a functional consequence and thereby be of physiological relevance. The goal of this project is to determine the functional impact of the Cys23Ser SNP in mammalian cells and to examine its occurrence in selected patient populations.

In order to reach these goals, I have developed several specific aims. My first aim was to generate and express edited and non-edited polymorphic human 5-HT_{2C} receptors. Polymorphic receptors were created using PCR site-directed mutagenesis and were then transfected into cells to create cell lines that express the variant receptors. My next aim was to examine the functional impact of the Cys23Ser SNP on the human 5-HT_{2C} receptor. This involved performing radioligand binding using various ligands to determine receptor densities and to examine changes in affinity and conducting phosphoinositide hydrolysis to determine the EC_{50} and maximal effect after agonist

stimulation. In order to investigate alterations in the expression patterns of these variant receptors, imunocytochemistry and surface biotinylation in combination with Western blot analysis was used. The third and final aim was to elucidate the association between the Cys23Ser polymorphism and subpopulations of depressed patients. First I identified subjects homozygous or heterozygous for the polymorphism within populations of depressed patients and control patients by genotyping DNA samples. Then I analyzed the associations of the polymorphism with an endophenotype of depression by using statistical analyses with STATA software.

CHAPTER II

PHARMACOLOGICAL PROPERTIES OF THE CYS23SER SINGLE NUCLEOTIDE POLYMORPHISM IN HUMAN 5-HT_{2C} RECEPTOR ISOFORMS

Introduction

The serotonin 2C (5-HT_{2C}) receptor is a seven transmembrane G-protein-coupled receptor (GPCR) that has only been found in the brain, where it is widely expressed (Mengod et al., 1990). Upon activation through interactions with the heterotrimeric G-protein, G_q (Chang et al., 2000), the 5-HT_{2C} receptor leads to activation of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Several mechanisms exist for the generation of molecular diversity in the 5-HT_{2C} receptor, one of which is RNA editing. RNA editing is a post-transcriptional event in which the coding potential of primary RNA transcripts is changed by mechanisms other than splicing. The human 5-HT_{2C} receptor mRNA undergoes A-to-I editing at five positions, generating multiple protein isoforms with different distributions and signaling capabilities (Niswender et al., 1999). The human 5-HT_{2C} receptor edited isoforms, which have VSV or VGV at positions 156, 158, and 160 have been shown to cause a decrease in agonist potency (Burns et al., 1997; Niswender et al., 1999; Fitzgerald et al., 1999) and constitutive activity (Niswender et al., 1999; Herrick-Davis et al., 1999), as well as altered patterns of G-protein coupling (Price et al., 2001), suggesting that this editing process may be used to regulate signaling tone in the brain.

Another mechanism for creating diversity is at the genetic level. A growing hypothesis is that common genetic variations may contribute significantly to the genetic risk for common disease (Lander, 1996). The most common type of genetic variation is the single nucleotide polymorphism (SNP) (frequency > 1%; Wang et al., 1998), which occurs in the coding or non-coding region of the gene. SNPs may change the amino acid coding potential of a GPCR which could interfere with protein folding, ligand binding, interactions with G-proteins and post-translational modifications of the protein (for review see Rana et al., 2001). Since serotonin has been implicated in a variety of behavioral and neurochemical responses, genetic variations within the 5-HT_{2C} receptor may increase the likelihood of the development of psychiatric disorders such as depression, anxiety, and schizophrenia. In the coding region of the 5-HT_{2C} receptor, a cysteine (C) to serine (S) change has been identified at the 23rd amino acid (C23S) (Lappalainen et al., 1995). The 5-HT_{2C} receptor C23S polymorphism has been shown to associate with many disease states. One such disease is schizophrenia, in which the C23S SNP has been shown to associate with a positive response to the atypical antipsychotic drug clozapine (Sodhi et al., 1995) and tardive diskinesia (Segman et al., 2001). Bipolar disorder, major depression (Lerer et al., 2001), and hallucinations in Alzheimer's disease (Holmes et al., 1998) have also been positively associated with this polymorphism. Given that this genetic variation has been associated with numerous diseases implies that this SNP may have an actual functional consequence on the protein which may increase the likelihood of a disease. It is therefore important to examine the function of the polymorphic variant. Furthermore, since the edited VSV isoform is the principal 5-HT_{2C} receptor isoform found in the human brain, it is necessary to determine the properties of

the C23S SNP in this edited background. Therefore, the purpose of this study was to examine the functional impact of the C23S polymorphism on the 5-HT_{2C} receptor in non-edited and edited VSV receptors expressed in three different mammalian cell lines.

Materials and Methods

Site-directed mutagenesis

Human 5-HT_{2C} receptor variants were prepared by PCR site-directed mutagenesis. PCR amplification was executed with two primer sets in two rounds. The first round consisted of the following primers: 1) 5′-CACCCCAGGCTTTACACTTTAT-3′ and 2) 3′-AACCAAACCGTTAGACTATAGAGACACTCGGGT-5′. Round two consisted of the following primers: 1) 5′-CACCCCAGGCTTTACACTTTAT-3′ and 2) 5′-ACAGGCCTTCCCACAAAGAACAGACAGAATATTGG-3′. Each set consisted of a primer containing the SNP and a flanking primer. The expression vector Bluescript (Stratagene, La Jolla, CA) containing wild-type human 5-HT_{2C} receptor (INI or VSV) served as the template. Once amplified by recombinant Pfu polymerase (Promega, Madison, WI), the mutated region was cut with the restriction enzymes EcoR I and Avr II and ligated into wild-type cDNA to generate the full-length expression construct. S23 receptors were verified by restriction digests and ABI 310 automated DNA sequencing. The entire receptor sequence containing the S23 SNP was cut out with EcoR I and Xba I and ligated into the mammalian expression vector pCMV2 (a gift of Dr. David Russell).

Expression and Cell Culture

In order to make stable cell lines, NIH-3T3 fibroblasts were transfected by co-electroporation of pCMV2 containing C23 or S23 5-$HT_{2C\text{-}INI}$ or 5-$HT_{2C\text{-}VSV}$ receptor cDNA and empty pcDNA3 plasmid (0.3 μg; Invitrogen, Carlsbad, CA) using the Bio-Rad Gene Pulser II. Cells with the receptor were initially selected in Dulbecco's modified eagle medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT), antibiotics (5 units/ml penicillin and 5 μg/ml streptomycin; Life Technologies, Inc.), and 2 mg/ml G418 (Geneticin, Life Technologies, Inc.). The concentration of G418 was then gradually decreased to 0.5 mg/ml and stable cell lines were maintained in this medium at 37°C with 5% CO_2. For transient expression in NIH-3T3 fibroblasts and COS-7 cells, cells were plated in 12 or 24 well, or 100 mm plates 24 hours prior to transfection. Both cells types were transfected by combining Fugene 6 reagent (Roche Diagnostics Corporation, Indianapolis, IN) and (0.25-1 μg) plasmid in serum free DMEM according to manufacturer's instructions. HEK293 cells ($4x10^6$ cells/100 mm dish) were transfected with 1 μg 5-$HT_{2C\text{-}INI}$ using Lipofectamine Reagent (Life Technologies, Inc.) and replated in 24 well plates at $2x10^5$ cells/well.

Human 5-HT_{2C} Receptor Antibody

In order to generate antibodies to the human 5-HT_{2C} receptor, peptides were directed against the C-terminus of the receptor as previously described (Backstrom et al., 1997). Rabbits were immunized with constructs containing the human 2C-CT peptide and purified using two columns. Serum was passed through a column containing the rat 2C-CT peptide and the flow-through applied to a second column containing the human

2C-CT peptide. Thus, the anti-human antibodies have considerably higher reactivity against human receptors relative to rat receptors.

Fluorescence microscopy

HEK293 cells ($2x10^6$ cells) were transfected with 0.1 μg of C23 or S23 5-HT_{2C-INI}/YFP cDNA. Twenty-four hours post transfection, the cells were replated on poly-D-lysine-coated coverslips in MEM (with serum) and incubated overnight prior to imaging. The cells were washed in PBS and viewed live using a Zeiss LSM 510 Meta Confocal Imaging System.

Surface Biotinylation and western blotting

For each receptor variant, one 100 mm plate of NIH-3T3 stable cell line was serum starved 16 hours prior to assay. After aspiration of medium, the plates were washed 3 times with cold Hank's balanced salt solution (HBSS). Sulfo-NHS-SS-Biotin in HBSS (1mg/mL; Pierce, Rockford, IL) was added (total volume=2 ml) and incubated at 4°C for 20 minutes while rocking. The biotin solution was aspirated and 2 ml of fresh biotin solution (1 mg/ml) was added for an additional 20 minutes at 4°C. After aspiration of the biotin solution, cells were washed three times with cold HBSS and scraped into phosphate-EDTA buffer (PE; 0.04 M NaH_2PO_4, 0.01 M Na_2HPO_4, 0.01 M disodium EDTA, 0.002 M EGTA, pH 7.2) with 0.5 μg/ml leupeptin and 100 μM PMSF. Cell were then sonicated for 5 seconds and centrifuged at 16000xg for 10 minutes. Pellets were resuspended in 1mL PE-CHAPS (PE plus 0.01 M CHAPS) with 0.5 μg/ml leupeptin and 100 μM PMSF, sonicated for 5 seconds, and incubated on ice for 30 minutes. Samples

were then centrifuged at 16000xg for 10 minutes at 4°C and the BCA protein assay (Pierce) performed on the supernatants. After dilution to equal protein concentrations, 30 µl aliquots of each sample were set aside for future western analyses. The remainder of the sample was added to 100 µl of immobilized streptavidin beads (Pierce) and rocked at 4ºC for 1 hour. Streptavidin-biotin complexes were separated by centrifugation at 16000xg for 2 minutes and washed 3 times with 1 ml of PE-CHAPs buffer. Biotinylated proteins were eluted from the complex by adding sample buffer containing 50 mM DTT and incubating at 50ºC for 40 minutes. Samples were resolved on a 10% SDS-PAGE precast minigels (Cambrex Bio Science, Rockland, ME) and proteins transferred to nitrocellulose. Nitrocellulose membranes were washed and blocked by incubation in 3% BSA for 1 hour and subsequently incubated with a primary antibody directed against the C-terminus of the human 5-HT_{2C} receptor (2 µg/ml) for 1.5 hours. After washing 3 times, the membranes were incubated with alkaline-phosphatase conjugated goat-anti-rabbit secondary antibody (1:1000; Dako, Carpinteria, CA) for 1 hour. Receptor protein was detected with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyphosphate (BCIP) (Pierce) in buffer (100 mM Tris, 100mM NaCl, 5 mM $MgCl_2$, pH 9.5).

Quantification of immunoblots

Protein band densities were scanned on an Epson Expression 636 scanner and quantified using the public domain Image J program developed at the National Institutes of Health. Graphs represent the mean optical density from three different experiments. Student's *t* test (two-tailed) was used to compare the means of the band densities.

Radioligand Binding

Membranes were prepared after washing cells with Hank's balanced salt solution (HBSS) (+Ca^{2+}/Mg^{2+}). Ice-cold binding buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4,) was added and cells were scraped off plates and placed in ultracentrifuge tubes. After brief homogenization (2 seconds with a Polytron), the membranes were spun at 20,000g for 20 minutes at 4°C. The pellet was resuspended in binding buffer and protein concentrations were determined with the BioRad protein assay. For saturation experiments, 500 µl (50 µg) of membrane preparation, 50 µl of 50 mM Tris buffer, and 50 µl of varied concentrations [N^6-methyl-^{3}H]-mesulergine (Amersham Pharmacia Biotech, UK) were mixed together and incubated at 37°C for 30 minutes. Competition binding was carried out on membranes prepared as described above and incubated with 1nM [^{3}H]-mesulergine and varying concentrations of competitor. Nonspecific binding was determined with 10 µM methysergide. Following incubation, free drug was separated from bound drug by vacuum filtration onto Whatman GF/C glass filters (Brandel, Gaithersburg, MD). The filters were placed in scintillation vials and counted on a Packard Tri-Carb scintillation counter (Packard Instrument Company, Downers Grove, IL). B_{max} and K_D values were determined from saturation isotherms using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). In some competition binding experiments, 100 µM guanosine 5'-(β,γ-imido)triphosphate [Gpp(NH)p] was added to shift all receptors to the low affinity state. IC_{50} values were determined by fitting data to a sigmoidal curve with variable slope using GraphPad Prism; one-site and two-site binding curves were compared using the *F* ratio. IC_{50} values were converted to K_i values using the transformation of Cheng-Prusoff (1973).

Phosphoinositide (PI) Hydrolysis

For transients, 24 hours post transfection cells were washed and then incubated for 16-20 hours in serum-free, inositol-free DMEM with 1 μCi *myo*-[^{3}H]inositol/ml (20-25 Ci/mmol, NEN Life Science Products). The experiment was initiated after adding 10 mM lithium chloride and 10 μM pargyline and incubating for 15 minutes at 37ºC. Agonist (either 5-HT or DOI) was added and plates were incubated for an additional 30 minutes at 37°C. The reaction was stopped by aspirating the medium and fixing the cells with 50 μl of methanol per well. [^{3}H]-inositol monophosphates were isolated as previously described (Barker et al., 1994). The data were analyzed with GraphPad Prism 3.0 software to determine maximum responses and EC_{50} values.

Fluorescence Resonance Energy Transfer (FRET)

HEK293 cells (4 x 10^6 cells/100mm dish) were co-transfected with 0.2ug of 5-$HT_{2C\text{-}INI}$/CFP (donor) and 0.4 μg of 5-$HT_{2C\text{-}INI}$/YFP (acceptor) using 20 μl of lipofectamine. Twenty-four hours post transfection, cells were plated in serum-free media on poly-lysine coated glass cover slips for 16 hours prior to the FRET assay. The cells were viewed live in phosphate buffered saline using a Zeiss LSM-510 META confocal imaging system with a 30 mW argon laser and 63x 1.4 NA oil immersion objective. FRET was measured by acceptor photobleaching (Bastiaens et al., 1996) using linear unmixing of CFP and YFP emission spectra as previously described.[20] Briefly, confocal microscopy was used to isolate a 2μm thick optical section through the middle of a live cell expressing both 5-$HT_{2C\text{-}INI}$/CFP and 5-$HT_{2C\text{-}INI}$/YFP. A prebleach image was captured using an argon laser with a 458 nm/514 nm dual dichroic. A region of the

plasma membrane was scanned with the 514 nm laser (100% intensity) for 30 seconds to photobleach YFP, and postbleach images were captured. FRET was measured as an increase in CFP fluorescence intensity (donor de-quenching) following YFP photobleaching. FRET efficiency was calculated as follows: 100 x (CFP postbleach - CFP prebleach) / CFP postbleach. Donor/acceptor ratios were calculated as donor fluorescence divided by acceptor fluorescence, measured prior to photobleaching. FRET efficiencies measured in 10 cells transfected with 5-$HT_{2C\text{-}INI}$/CFP alone ranged from -3.6% to +2.9%, with an average of 0.3%. Similar results were obtained from non-bleached regions of the plasma membrane.

Results

Cellular distribution of C23 and S23 5-HT_{2C} receptors are similar

Many GPCR SNPs cause improper folding of proteins, leading to retention in the ER or Golgi and reduced receptor at the cell surface (for recent review, see Bernier et al., 2004). In order to examine the impact of the C23S polymorphism on protein localization, HEK293 cells were transiently transfected with C23 or S23 5-$HT_{2C\text{-}INI}$/YFP cDNA and subjected to confocal imaging. C23 and S23 5-$HT_{2C\text{-}INI}$ receptors showed similar fluorescence patterns with intense plasma membrane labeling and some intracellular fluorescence (Fig. 14). A more quantitative estimate of receptor expression on the plasma membrane was obtained using surface biotinylation of NIH-3T3 fibroblasts stably expressing C23 or S23 5-$HT_{2C\text{-}INI}$ or 5-$HT_{2C\text{-}VSV}$ receptors. The amount of surface biotinylated 5-HT_{2C} receptor was not significantly different between cells expressing C23

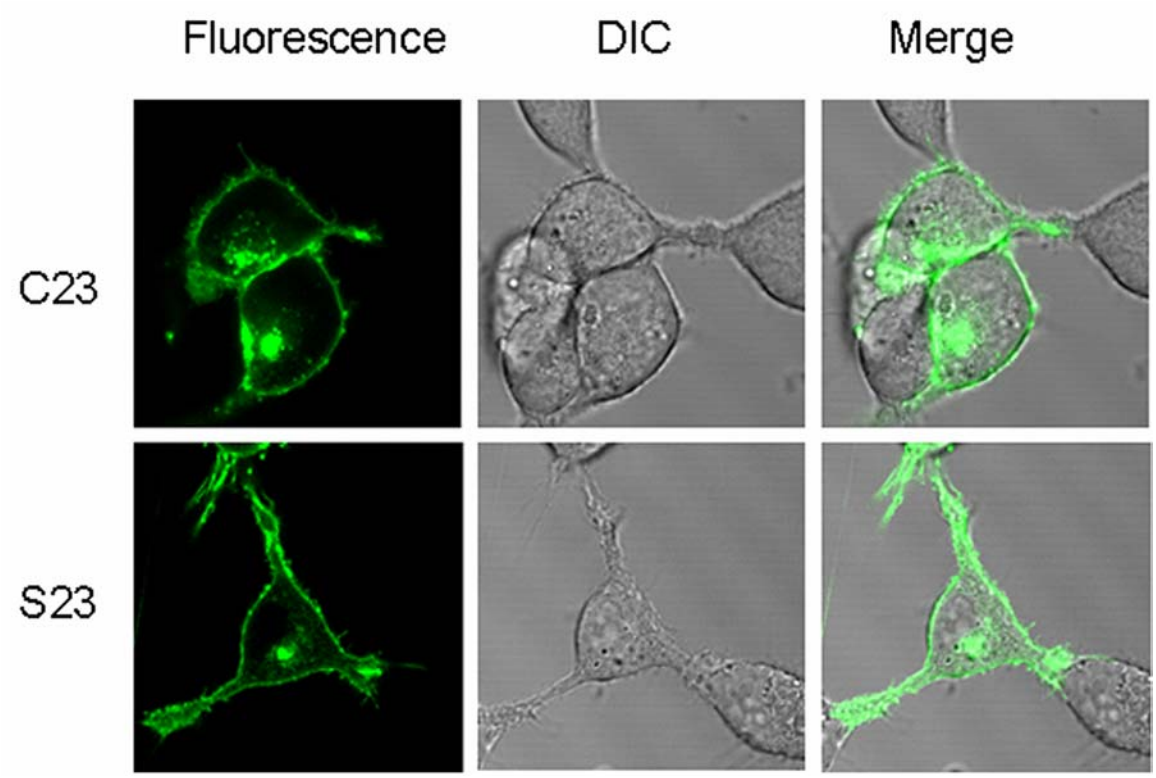

Figure 14: Cellular distribution of C23 5-HT_{2C-INI} and S23 5-HT_{2C-INI} receptors
HEK293 cells were transfected with 0.1μg of C23 or S23 5-HT_{2C-INI}/YFP cDNA. Twenty-fours hours post transfection, the cells were re-plated on poly-D-lysine-coated coverslips in MEM (with serum) overnight prior to imaging. The cells were washed in PBS and viewed live using a Zeiss LSM 510 Meta Confocal Imaging System. DIC: differential interference contrast.

or S23 5-HT_{2C-INI} or 5-HT_{2C-VSV} receptor isoforms (Fig. 15 and 16), suggesting no difference in protein targeting to the cell surface. All receptor isoforms migrated as broad bands with similar patterns between 40-68 kDa (Figure 15), agreeing with previous studies that the 5-HT_{2C} receptor is glycosylated (Backstrom et al., 1995) and suggesting that the CS23S SNP does not alter the degree of glycosylation.

High affinity binding is retained at S23 receptors

NIH-3T3 fibroblasts were stably transfected with 5-HT_{2C-INI} or 5-HT_{2C-VSV} receptor cDNAs with or without the C23S polymorphism. Competition binding was performed with [^{3}H]-mesulergine to determine the affinity of agonists and antagonists at the variant 5-HT_{2C} receptors. All of the ligands tested had similar affinity for C23 and S23 receptor variants (Table 4). However, the competition binding curves were shallow both at C23 and S23 5-HT_{2C-VSV} receptors, as illustrated in Figures 17 and 18, for the hallucinogenic agonist (±)-1-(2,5-dimethoxy-4-phenyl)-2-aminopropane (DOI), suggesting multiple affinity states of the receptors. The C23 5-HT_{2C-VSV} receptor curve was best fit by a two site model with the high affinity state having a K_i of 0.73 ± 0.43 nM. The addition of the GTP analog Gpp(NH)p shifted the competition curve to a single, low affinity state with a K_i of 40.3 ± 7.3 nM (Fig. 17). The S23 5-HT_{2C-VSV} receptor curve was also best fit by a two site model with the high affinity state having a K_i of 1.17 ± 0.63 nM. Addition of Gpp(NH)p shifted the competition curve to a single, low affinity state with a K_i of 42.3 ± 2.6 nM (Fig. 18). The portion of high affinity agonist binding was equal for the C23 and S23 receptors (Table 5). Similar results were observed with the agonists 5-HT and m-chlorophenylpiperazine (m-CPP), but not lysergic acid diethylamide (LSD) (Table 5).

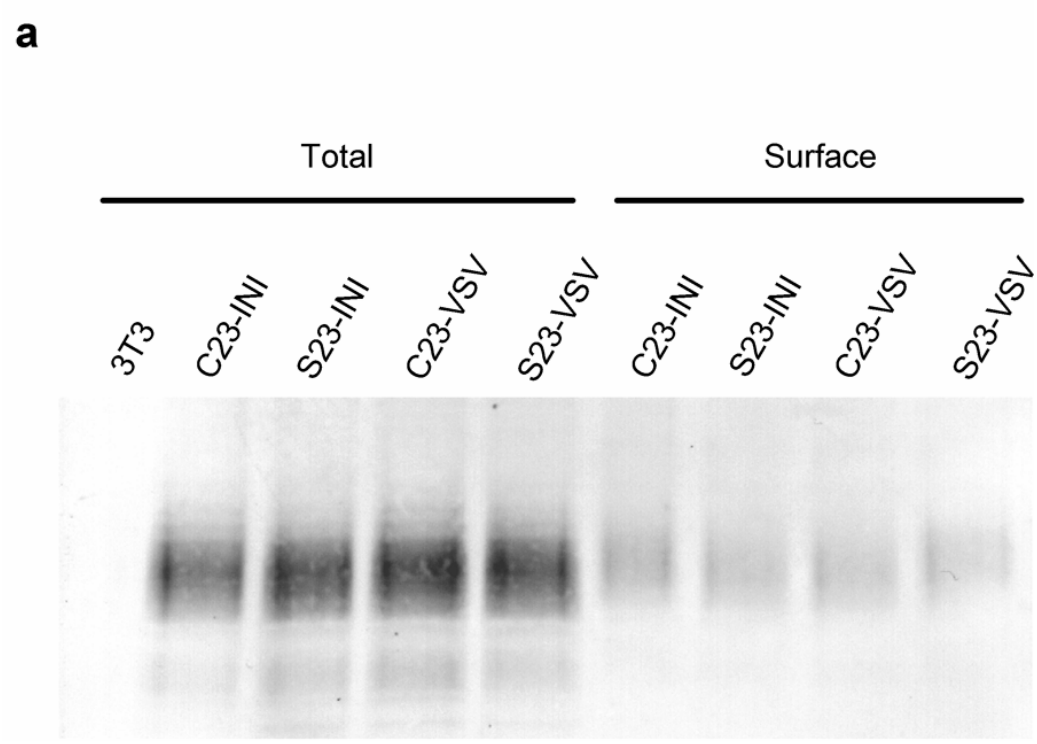

Figure 15: Surface biotinylation of C23 and S23 5-HT_{2C} receptors and Western blotting

NIH-3T3 fibroblasts stably expressing C23 5-$HT_{2C\text{-}INI}$ (B_{max} 2.1 ± 0.2 pmol/mg), C23 5-$HT_{2C\text{-}VSV}$ (B_{max} 2.5 ± 0.3 pmol/mg), S23 5-$HT_{2C\text{-}INI}$ (B_{max} 3.0 ± 0.3 pmol/mg), or S23 5-$HT_{2C\text{-}VSV}$ (B_{max} 2.2 ± 0.2 pmol/mg) receptors were labeled with biotin, extracted with streptavidin beads, and blotted with a polyclonal 5-HT_{2C} receptor antibody as described in Materials and Methods. Blot shown is a representative experiment that was replicated in three independent experiments.

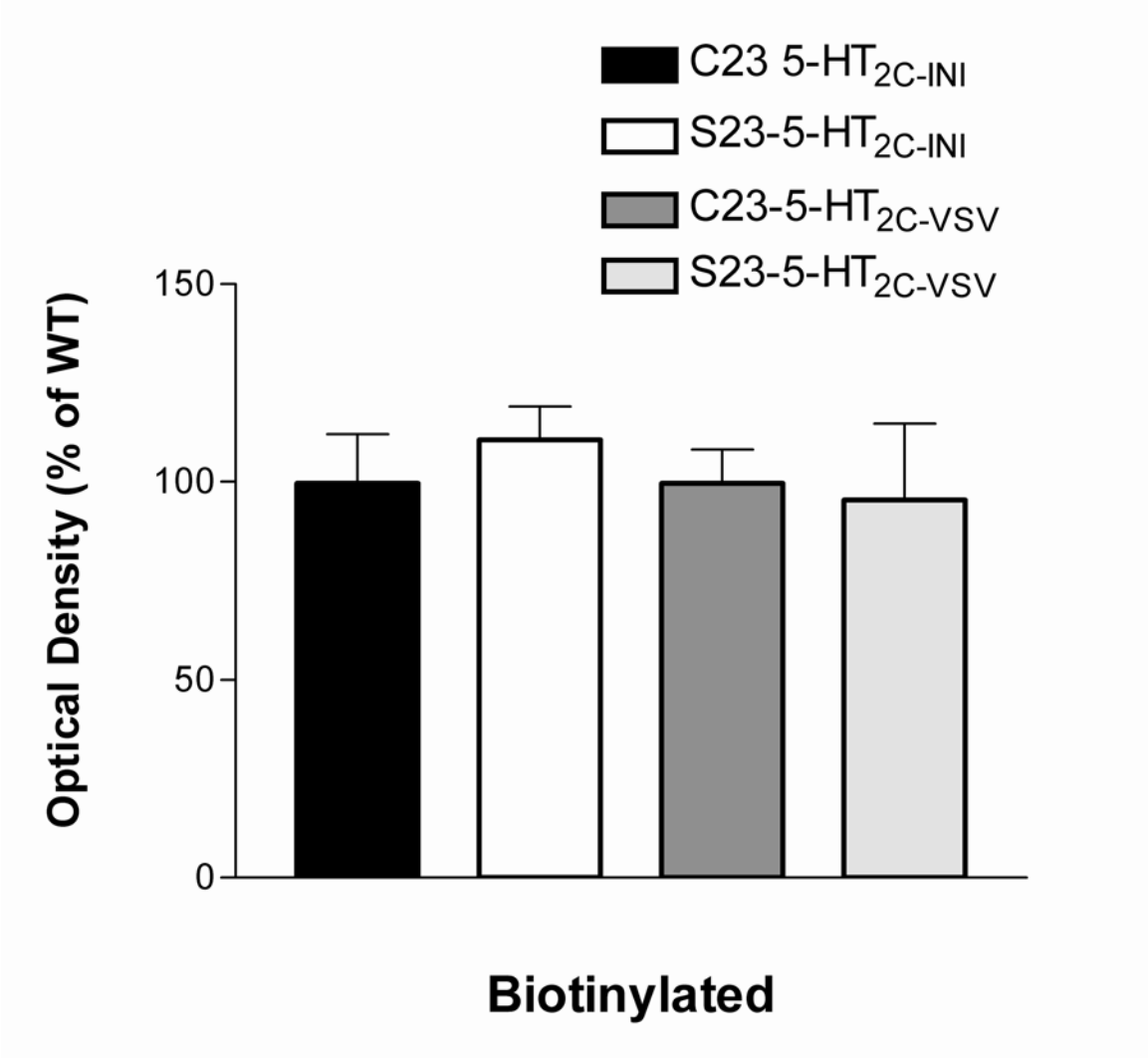

Figure 16: Quantification of biotinylated 5-HT_{2C} receptors
Protein bands from three independent cell surface biotinylation experiments were quantified. Results are expressed as percentages ± S.E.M. of WT (either C23 5-$HT_{2C\text{-}INI}$ or C23 5-$HT_{2C\text{-}VSV}$) receptor band density for each protein species. There was no significant difference in band densities from total cell lysates. Optical density was measured for each band using Image J software as described in Materials and Methods. 3T3: untransfected parental NIH-3T3 fibroblasts.

Table 4: Relative affinities for agonists and antagonists for C23 5-HT_{2C}-VSV and S23 5-HT_{2C-VSV} receptors

K_i values for the ligands were determined by competition for 1 nM [^{3}H]-mesulergine in NIH-3T3 fibroblasts stably expressing C23 or S23 5-HT_{2C-VSV} receptors. K_i values are in nM and were determined using the method of Cheng and Prusoff. Agonist competition curves were determined in the presence of 100 μM Gpp(NH)p. Data represent the mean ± S.E.M. of 3-8 independent experiments performed in duplicate.

Agonist	**C23**	**S23**
5-HT	89 ± 9	97 ± 18
DOI	41 ± 17	40 ± 3
LSD	5.3 ± 0.96	6.5 ± 1.3
mCPP	138 ± 60	150 ± 65
Antagonist		
Clozapine	13.4 ± 4	18.2 ± 2
Mianserin	2.87 ± 1.4	2.38 ± 1.2
Fluoxetine	228 ± 98	190 ± 56

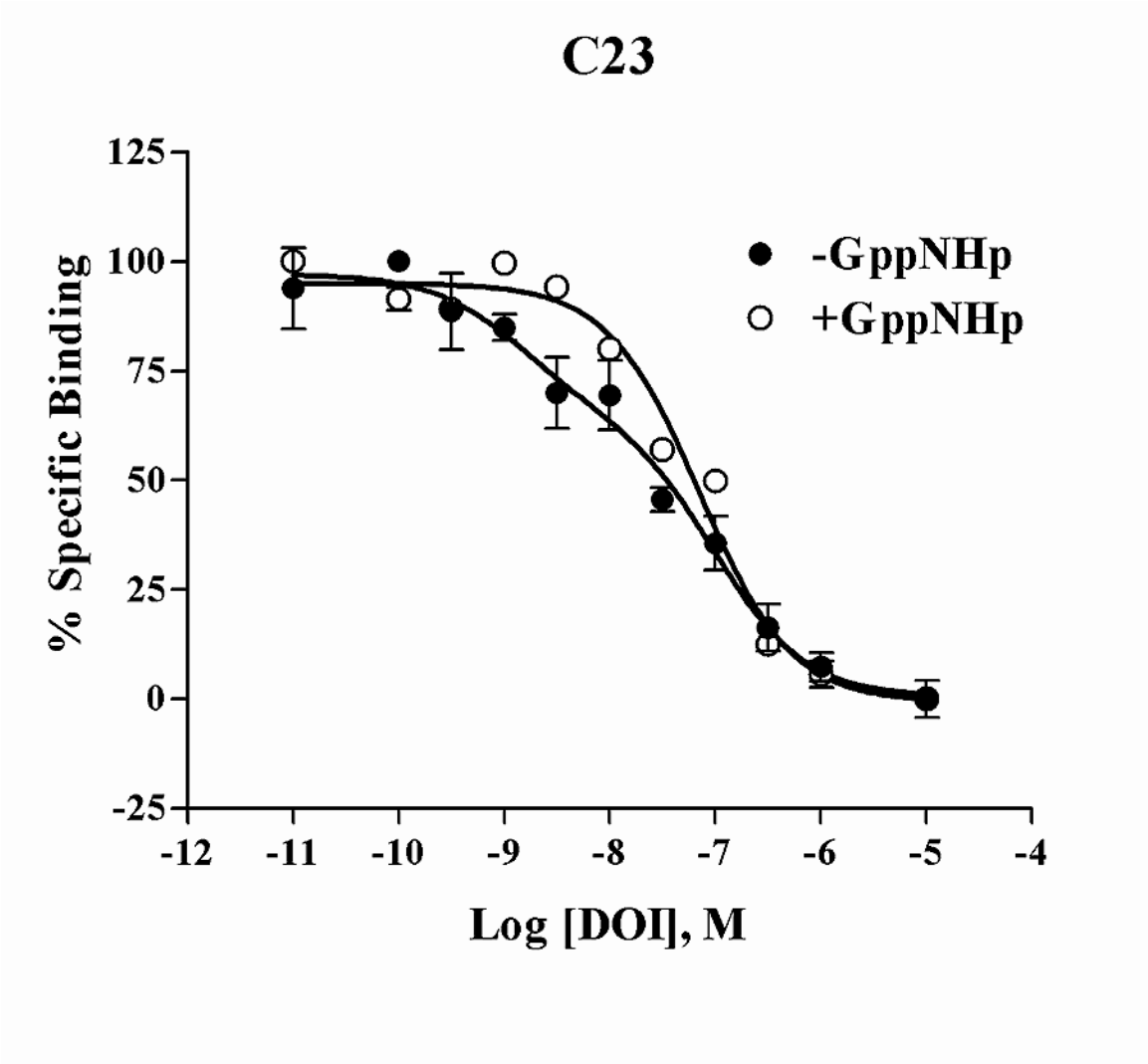

Figure 17: Competition binding of DOI for [^{3}H]-mesulergine labeled C23 5-HT$_{2C\text{-}VSV}$ receptors

DOI competition for 1 nM [^{3}H]-mesulergine binding was measured in membranes prepared from NIH-3T3 fibroblasts stably transfected with C23 5-HT$_{2C\text{-}VSV}$ receptors in the presence (open circles) and absence (closed circles) of Gpp(NH)p. The receptor density was1.5 ± 0.09 pmol/mg protein.

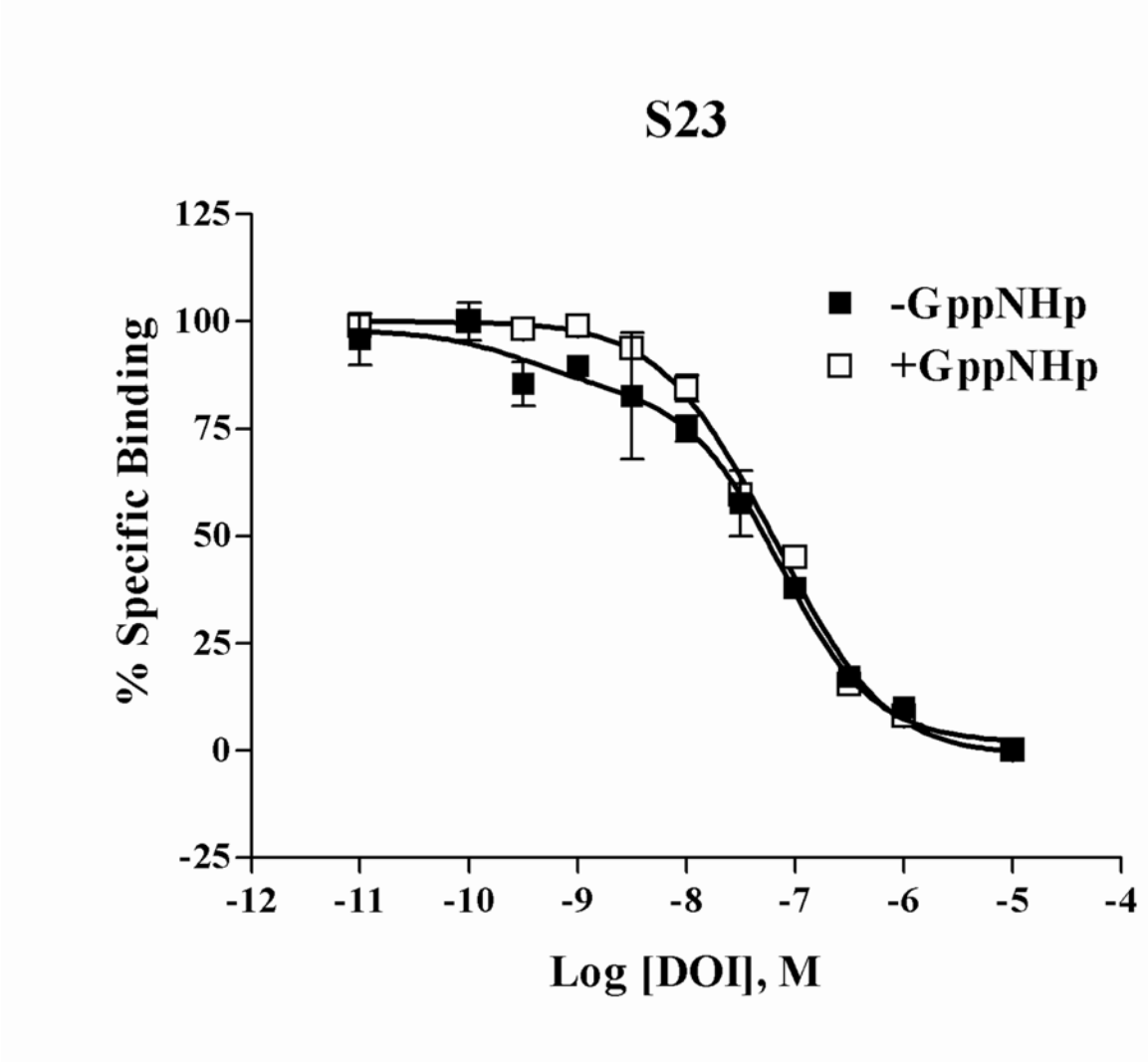

Figure 18: Competition binding of DOI for [^{3}H]-mesulergine labeled S23 5-HT$_{2C\text{-}VSV}$ receptors

DOI competition for 1 nM [^{3}H]-mesulergine binding was measured in membranes prepared from NIH-3T3 fibroblasts stably transfected with S23 5-HT$_{2C\text{-}VSV}$ receptors in the presence (open squares) and absence (closed squares) of Gpp(NH)p. The receptor density was 1.5 ± 0.08 pmol/mg protein.

Table 5: High and low affinities for agonists at C23 and S23 5-$HT_{2C\text{-}VSV}$ receptors
K_i values for agonists were determined by competition for 1 nM [^{3}H]-mesulergine in NIH-3T3 fibroblasts stably expressing C23 or S23 5-$HT_{2C\text{-}VSV}$ receptors. K_i values are in nM and were determined using the method of Cheng and Prusoff. Competition curves were in the absence of Gpp(NH)p and were best fit by a two site model. Percentage of agonist in the high affinity state was calculated by GraphPad Prism. Data represent the mean ± S.E.M. of four independent experiments.

Agonist	High Affinity (K_{iH})		% High Affinity		Low Affinity (K_{iL})	
	C23	S23	C23	S23	C23	S23
5-HT	1.1 ± 0.3	1.7 ± 0.7	38 ± 4	42 ± 3	107 ± 14	154 ± 32
DOI	0.6 ± 0.4	5.3 ± 4.2	19 ± 1	17 ± 1	68 ± 17	120 ± 55
m-CPP	0.8 ± 0.3	0.8 ± 0.2	24 ± 2	23 ± 3	138 ± 25	150 ± 27

G-protein coupling and receptor signaling are comparable between C23 and S23 receptor variants

The 5-HT_{2C} receptor has been shown to interact with G-proteins and activate intracellular signaling in the absence of agonist, an action termed constitutive activity (Barker et al., 1994). RNA editing dramatically reduces constitutive activity of the 5-HT_{2C} receptor (Niswender et al., 1999; Herrick-Davis et al., 1999), therefore, we determined if the C23S polymorphism altered the constitutive activity of the 5-HT_{2C} receptor. COS-7 cells were transiently transfected with increasing concentrations of C23 or S23 5-$HT_{2C\text{-}INI}$ or 5-$HT_{2C\text{-}VSV}$ receptors and ^{3}H-IP formation was measured in the absence of agonist for 30 minutes. For the 5-$HT_{2C\text{-}INI}$ expressing cells, ^{3}H-IP formation increased as the DNA concentration increased. However, there was no significant difference between cells expressing C23 or S23 5-$HT_{2C\text{-}INI}$ receptors (Fig. 19). Similar results were observed in cell lines expressing 5-$HT_{2C\text{-}VSV}$ receptors except constitutive activity was lower (Fig. 20), which agrees with previous studies (Herrick-Davis et al., 1999).

Because cell types may differ in their signaling machinery, it was important to test an additional cell type. Therefore, we measured basal [^{3}H]-IP formation in HEK293 cells expressing C23 or S23 5-$HT_{2C\text{-}INI}$ receptors. Under basal conditions, expression of C23 or S23 5-$HT_{2C\text{-}INI}$ receptors (at 4.7±0.6 pmol/mg) significantly increased [^{3}H]-IP production compared to vector alone. However, there was no difference in basal activity between C23 and S23 5-$HT_{2C\text{-}INI}$ receptors (Fig. 21). Clozapine, a 5-HT_{2C} receptor inverse agonist (Herrick-Davis et al., 2000), reversed the constitutive activity of C23 and S23 receptors to the same extent (Fig. 21). HEK293 cells expressing C23 or S23 5-HT_{2C}-

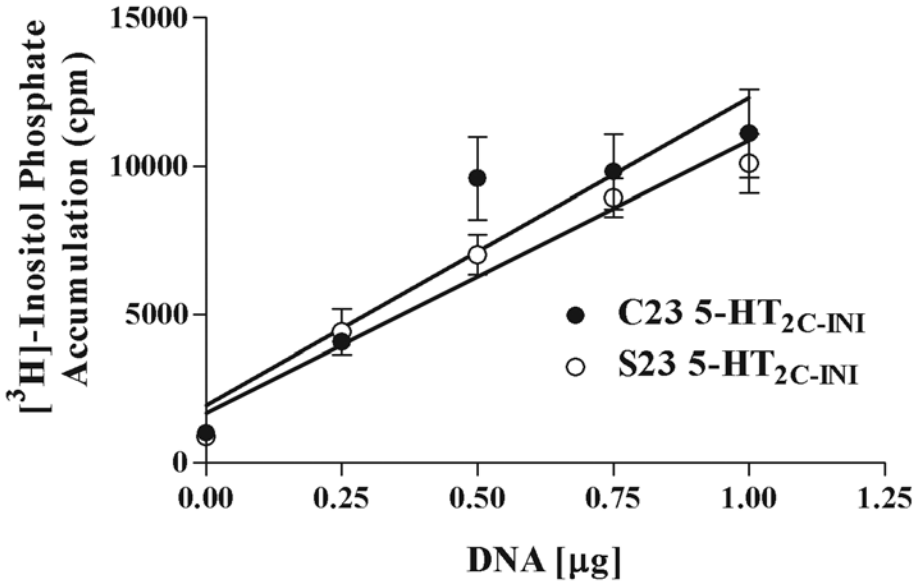

Figure 19: Constitutive activity of C23 and S23 5-HT$_{2C-INI}$ receptors
Basal [^{3}H]-IP formation was measured after 30 minutes according to the Materials and Methods section. COS-7 cells were transiently transfected with increasing concentrations of C23 5-HT$_{2C-INI}$ or S23 5-HT$_{2C-INI}$ cDNAs (0.25-1.0 µg). Overall, the slopes of the two lines were not different: F=0.48. Data represent three independent experiments performed in duplicate.

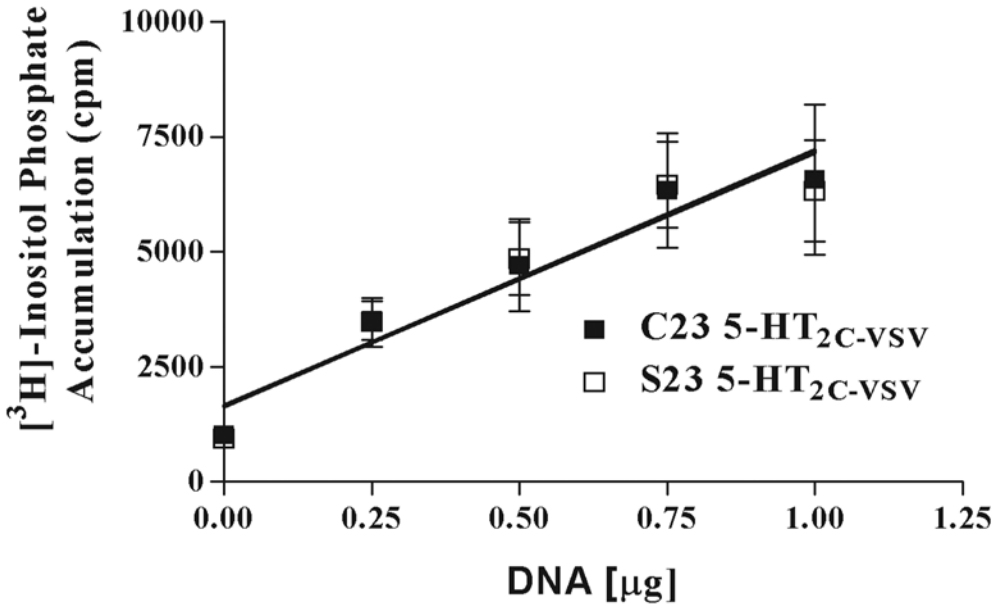

Figure 20: Constitutive activity of C23 and S23 5-$HT_{2C\text{-}VSV}$ receptors
COS-7 cells were transiently transfected with increasing concentrations of C23 5-$HT_{2C\text{-}VSV}$ or S23 5-$HT_{2C\text{-}VSV}$ cDNAs (0.25-1.0 μg). Overall the slopes were not significantly different: F=0.00. Data represent three independent experiments performed in duplicate.

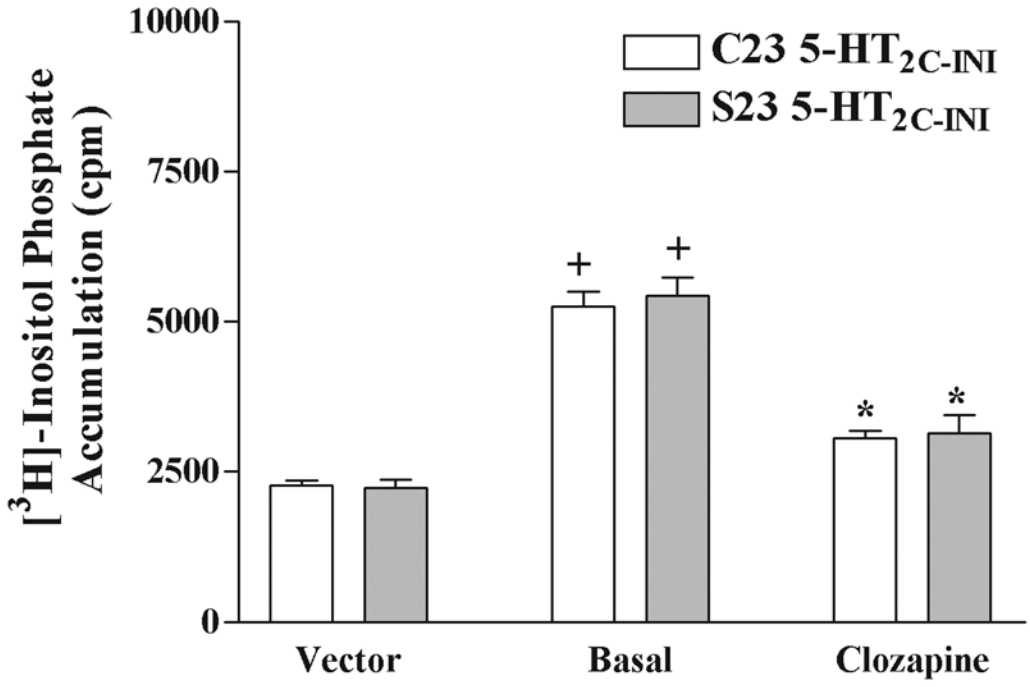

Figure 21: Constitutive activity of C23 and S23 5-HT$_{2C\text{-}INI}$ receptors in HEK293 cells
HEK293 cells were transfected with 0.5 μg of 5-HT$_{2C\text{-}INI}$ cDNA. Average receptor expression was 4.7 ± 0.6 pmol/mg protein. Data represent the mean ± S.D. from six experiments each performed in triplicate. +$p<0.01$ vs Vector; *$p<0.01$ vs Basal. There was no significant difference between C23 and S23.

$_{INI}$ receptors at 10.4 ± 0.4 pm/mg protein also showed no significant differences in constitutive activity (data not shown).

Agonist-promoted ^{3}H-IP formation was evaluated in cells transiently expressing C23 or S23 5-HT$_{2C-INI}$ receptors to determine if there are differences in agonist potencies to activate PLC. EC_{50} values from three independent experiments for C23 and S23 were 1.1 ± 0.2 nM and 1.4 ± 0.5 nM, respectively, showing no significant differences in potency (Fig. 22). The hallucinogen DOI also yielded similar results between C23 and S23 receptors with EC_{50} values of 3.1 ± 0.8 nM and 6.3 ± 0.9 nM, respectively (Fig. 23). We also evaluated 5-HT in HEK293 cells expressing C23 or S23 5-HT$_{2C-INI}$ receptors. EC_{50} values from three independent experiments were 2.4 ± 0.5 nM for C23 and 1.9 ± 0.6 nM for S23 5-HT$_{2C-INI}$ receptors, again showing no significant difference in 5-HT potency (Fig. 24).

Dimerization of the 5-HT$_{2C-INI}$ receptor is not altered by the C23S polymorphism

Fluorescence resonance energy transfer (FRET) experiments were performed to determine if the C23S polymorphism alters 5-HT$_{2C}$ receptor homodimerization. Acceptor photobleaching was used to measure FRET in selected regions of plasma membrane from 12 cells expressing C23 5-HT$_{2C-INI}$/CFP (donor) and C23 5-HT$_{2C-INI}$/YFP (acceptor) and 12 cells expressing S23 5-HT$_{2C-INI}$/CFP and S23 5-HT$_{2C-INI}$/YFP. FRET efficiency was significantly correlated with the donor/acceptor ratio for cells expressing C23 and S23 5-HT$_{2C-INI}$ receptors (Fig. 25). Since FRET efficiency is dependent on the donor/acceptor ratio, the cells were divided into three groups based on these ratios and

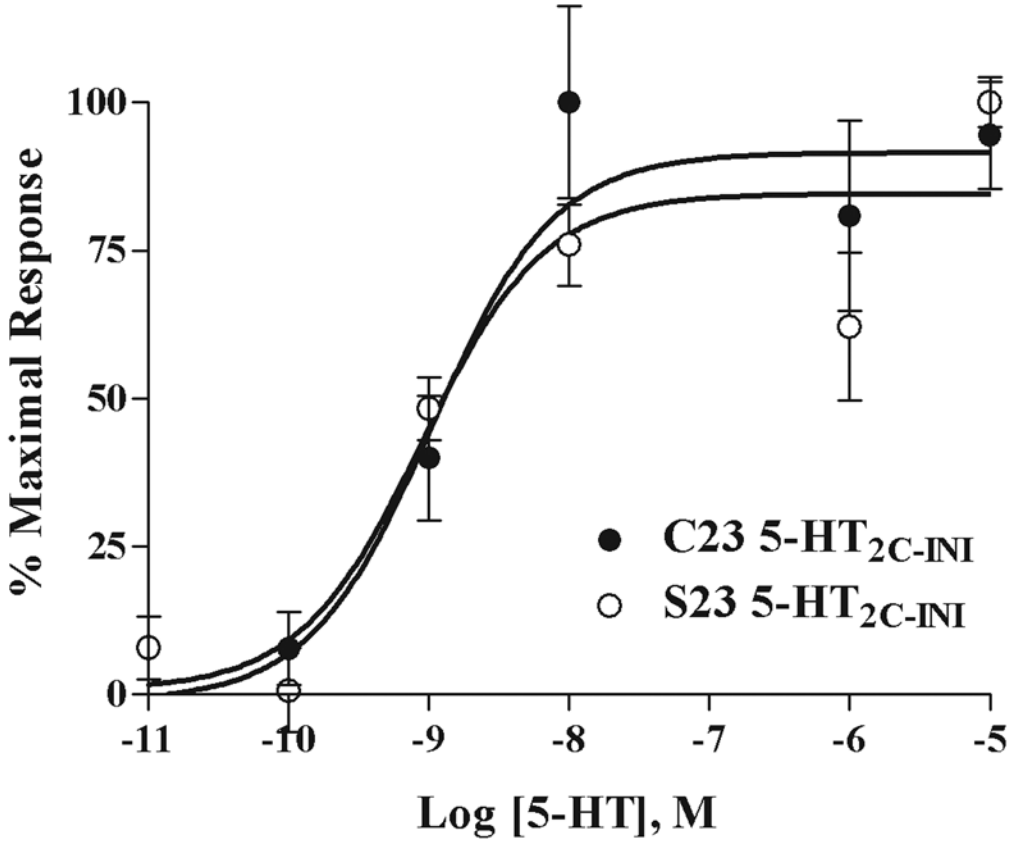

Figure 22: 5-HT stimulation of phospholipase C at C23 and S23 5-HT$_{2C\text{-}INI}$ receptors
NIH-3T3 fibroblasts transiently transfected with C23 5-HT$_{2C\text{-}INI}$ or S23 5-HT$_{2C\text{-}INI}$ cDNA were stimulated with 5-HT for 30 minutes. Receptor densities were 456 ± 137 fmol/mg and 492 ± 172 fmol/mg for C23 and S23, respectively.

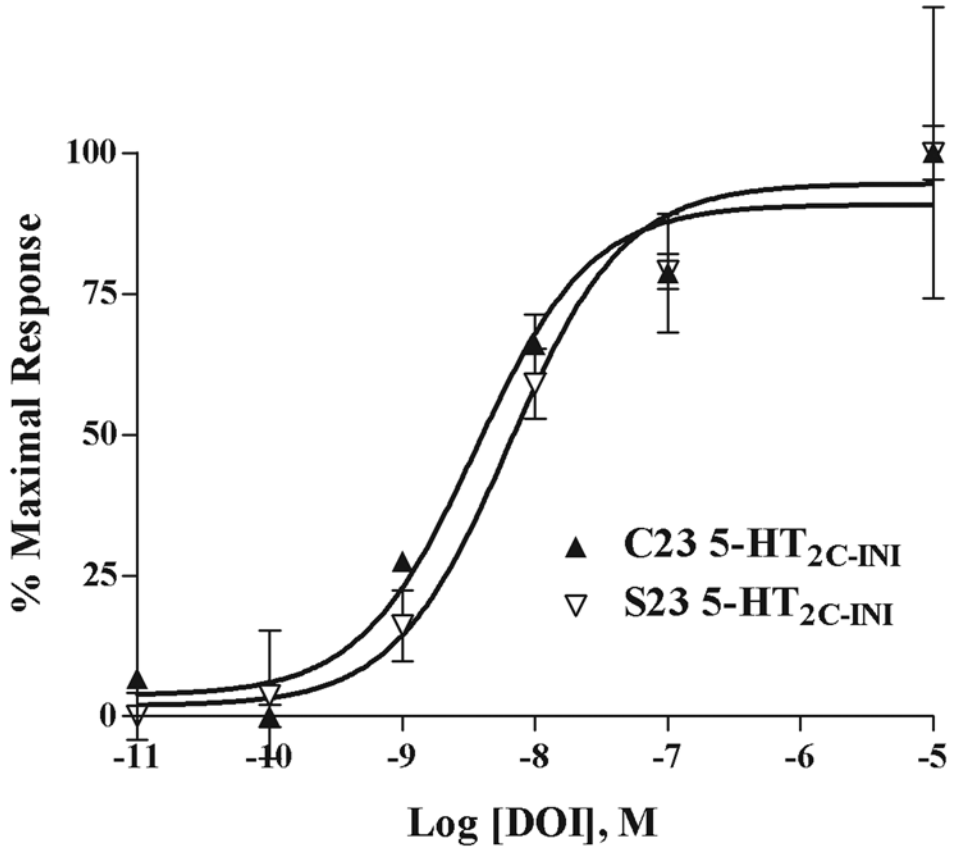

Figure 23: DOI stimulation of phospholipase C at C23 and S23 5-HT$_{2C\text{-}INI}$ receptors
NIH-3T3 fibroblasts were transiently transfected with C23 5-HT$_{2C\text{-}INI}$ or S23 5-HT$_{2C\text{-}INI}$ cDNA and stimulated with DOI for 30 minutes. Receptor densities were 594 ± 78 fmol/mg for C23 and 413 ± 91 fmol/mg for S23.

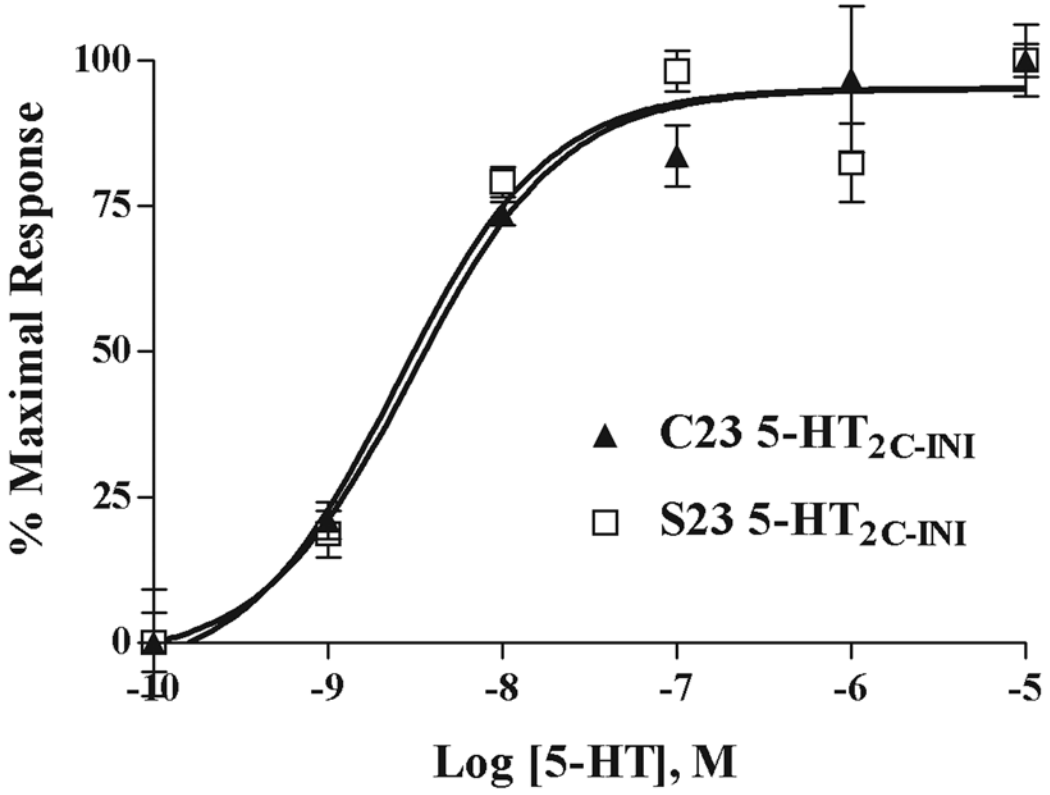

Figure 24: 5-HT stimulation of phospholipase C at C23 and S23 5-HT_{2C-INI} receptors in HEK293 cells
HEK293 cells were transiently transfected with C23 or S23 5-HT_{2C-INI} cDNA and stimulated with 5-HT for 30 minutes.

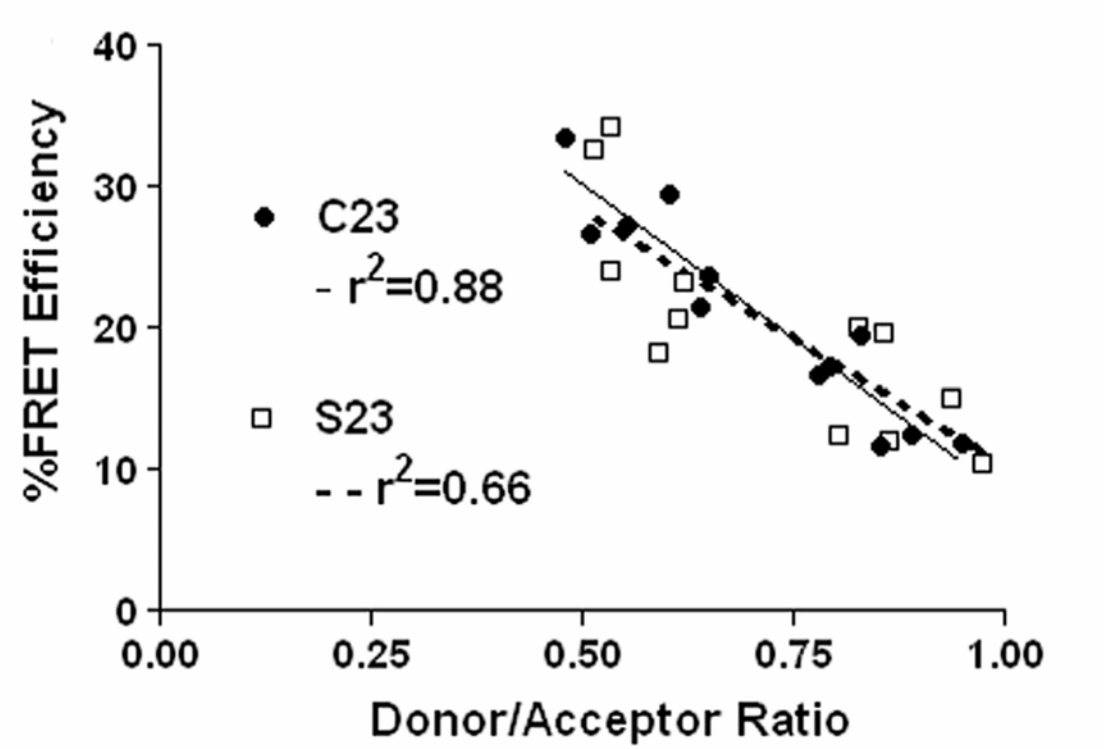

Figure 25: Homodimerization of C23 and S23 5-HT$_{2C\text{-}INI}$ receptors
FRET was measured on the plasma membrane of HEK293 cells expressing C23 or S23 5-HT$_{2C\text{-}INI}$/CFP and 5-HT$_{2C\text{-}INI}$/YFP receptors. Relationship between FRET efficiency and donor/acceptor ratio (donor fluorescence divided by acceptor fluorescence) measured in 12 cells expressing C23 or S23 5-HT$_{2C}$ receptors.

the mean FRET efficiency for each group was calculated (Table 6). This was done to provide a meaningful comparison of C23 and S23 FRET efficiencies from cells with similar donor/acceptor ratios. The results presented in Table 7 indicate that there was no difference in FRET efficiencies for C23 and S23. FRET efficiency was independent of acceptor fluorescence (Fig. 26), indicating that FRET was independent of receptor expression level.

Discussion

The human 5-HT_{2C} receptor mRNA undergoes A-to-I editing at 5 positions termed A, B, C, D, and E in the second intracellular loop of the receptor (Burns et al., 1997; Niswender et al., 1998), a region involved in G-protein coupling (Niswender et al., 1999; Arora et al., 1995; Visiers et al., 2001). This receptor also has a number of SNPs in its promoter region (Yuan et al., 2000; Deckert et al., 2000) and one in the coding region (Lappalainen et al., 1995). The present study focused on the SNP in the coding region which converts a cysteine to a serine at the 23rd amino acid in the N-terminus of the receptor. This polymorphism occurs at a frequency of 13% in the Caucasian population (Lappalainen et al., 1995) and has been associated with numerous disease symptoms (for a recent review see Sanders-Bush et al., 2003). Therefore, this paper addresses the functional consequences of the combination of RNA editing and the C23S polymorphism in the human 5-HT_{2C} receptor. A recent study by Okada et al (2004) demonstrated that the C23S SNP in the 5-HT_{2C} receptor was functional, but here we report that the C23S SNP has no functional consequences, even when expressed in the VSV backbone, the most prominent isoform in the human brain. These negative data, which were

Table 6: Relationship between FRET efficiency and donor/acceptor ratio
FRET efficiencies measured in 12 HEK293 cells expressing C23 5-$HT_{2C\text{-}INI}$/CFP + 5-$HT_{2C\text{-}INI}$/YFP or S23 5-$HT_{2C\text{-}INI}$/CFP + 5-$HT_{2C\text{-}INI}$/YFP (data from Figure 6a) were divided into three groups based on their donor/acceptor ratios (D/A). Data represent the mean ± S.E.M for the number of cells per group as indicated (n).

C23			S23		
D/A	**%FRET**	**n**	**D/A**	**%FRET**	**n**
0.90 ± 0.03	12.1 ± 0.2	3	0.89 ± 0.04	12.6 ± 0.9	4
0.74 ± 0.04	19.8 ± 1.3	5	0.72 ± 0.07	20.2 ± 0.2	4
0.54 ± 0.03	29.3 ± 1.5	4	0.55 ± 0.02	28.7 ± 2.9	4

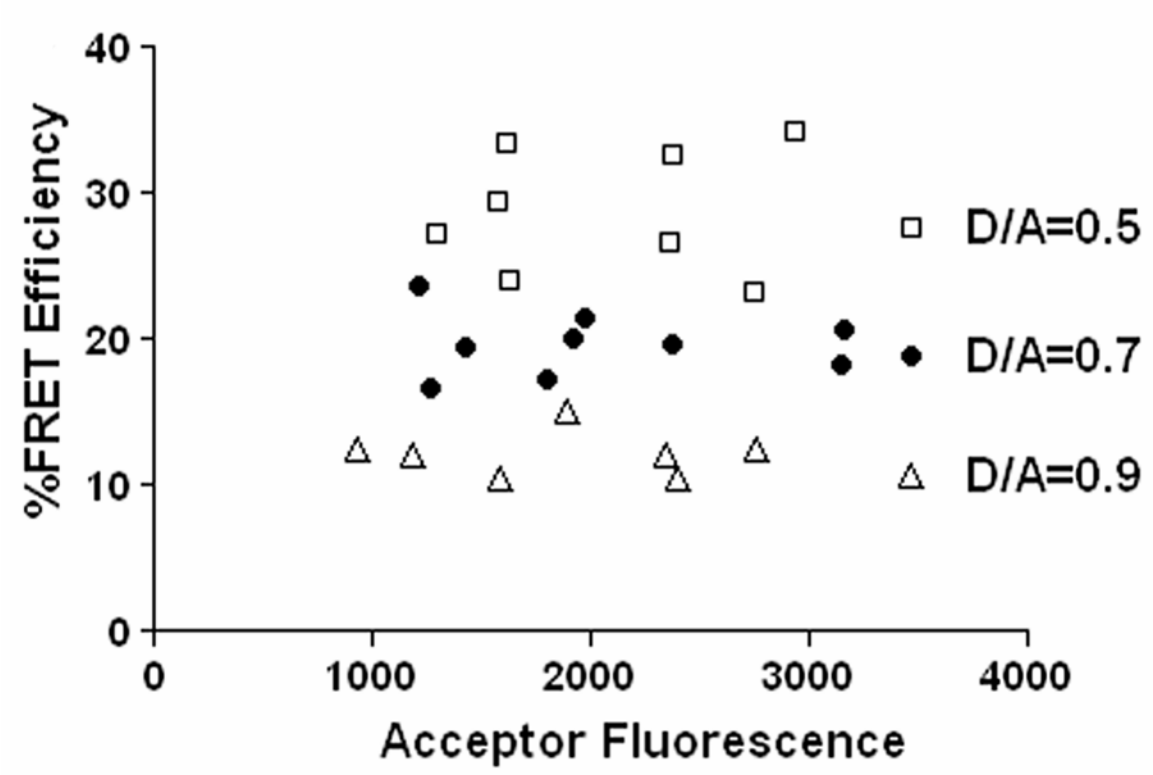

Figure 26: Relationship between FRET efficiency and acceptor fluorescence for C23 and S23 5-$HT_{2C\text{-}INI}$ receptors

reproduced in three cell lines and two laboratories, may suggest a different strategy for future genetic studies.

Non-synonymous SNPs change the amino acid coding potential of a protein which may lead to an alteration in conformation of the protein. This amino acid change can lead to an unstable protein that may be retained in the endoplasmic reticulum, thereby decreasing the amount of receptor on the surface available to interact with agonist (Wenkert et al., 1996; Morello et al., 2000). The current study demonstrated that S23 5-$HT_{2C\text{-}INI}$ receptors were localized very similar to C23 5-$HT_{2C\text{-}INI}$ receptors. This was first examined by immunofluorescence combined with confocal imaging in HEK293 cell transients. In order to answer more quantitatively whether the C23S SNP altered cell surface expression, cellular distribution was evaluated by cell surface biotinylation and isolating biotinylated surface proteins with streptavidin beads. Probing immunoblots with a human 5-HT_{2C} receptor antibody revealed equivalent levels of cell surface biotinylated protein in cells expressing the C23 or S23 5-$HT_{2C\text{-}INI}$ or 5-$HT_{2C\text{-}VSV}$ receptors. All isoforms of the protein migrated at similar molecular weights as diffuse bands revealing that glycosylation is intact. This agrees with previous studies of the 5-HT_{2C} receptor (Backstrom et al., 1997). All together, these data suggest that protein processing and targeting are not altered by the C23S polymorphism.

The present study found that NIH-3T3 fibroblasts and HEK293 cells expressing S23 5-HT_{2C} receptors were able to bind all ligands tested with similar affinities to that of C23 5-HT_{2C} receptors. In NIH-3T3 fibroblasts with equivalent receptor densities, competition binding curves for the agonists 5-HT, DOI, and m-CPP were shallow and best fit by a two-site model, suggesting that both C23 and S23 receptors can bind agonists

in the G-protein coupled and uncoupled states. In order to test this hypothesis, a non-hydrolyzable GTP analog, Gpp(NH)p, was added. This addition of Gpp(NH)p shifted agonist competition curves to a single low affinity state, confirming that agonists were binding to the receptors with high and low affinity and that agonist high affinity binding was not altered in cells expressing the S23 5-HT_{2C} receptors. In an independent study, HEK293 cells were transiently transfected with C23 or S23 5-$HT_{2C\text{-}INI}$ receptor cDNA and agonist competition binding experiments were performed. Again, none of the ligands tested showed a significant difference between C23 and S23 receptors (data not shown). Okada et al. (2004) demonstrated increased high affinity binding to 5-HT and m-CPP at S23 5-$HT_{2C\text{-}INI}$ receptors. Although statistically significant, the differences found were small and may not be biologically significant. Both mutagenesis and biochemical studies with a variety of class A GPCRs suggest that receptor activation by ligand binding involves disruption of strong ionic interactions in transmembrane helices 3 and 6 (reviewed in Kroeze et al., 2003). These studies are consistent with our data showing that a SNP in the N-terminus of the 5-HT_{2C} receptor does not alter binding. It is very unlikely that a SNP in the N-terminus would change the binding affinity of the 5-HT_{2C} receptor unless there was an extensive conformational change. If a conformational change such as this did take place, one might expect that trafficking or G-protein coupling would be altered, but we did not observe this. Therefore, our data lead us to conclude that the S23 allele does not alter ligand binding.

The 5-HT_{2C} receptor has been shown to exhibit prominent constitutive activity (Barker et al., 1994), defined as the ability of a receptor to bind its cognate G-protein and activate signaling in the absence of agonist. Constitutive activity in the 5-HT_{2C} receptor

has been shown to be dramatically reduced in the 5-HT_{2C} receptor as a consequence of RNA editing (Niswender et al., 1999; Herrick-Davis et al., 1999). In the current studies, we found that COS-7 cells transiently transfected with increasing concentrations of C23 or S23 5-HT_{2C} receptor cDNA elicited [^{3}H]-IP production in the absence of agonist. Basal activity was greater in cells expressing 5-$HT_{2C\text{-}INI}$ than 5-$HT_{2C\text{-}VSV}$ receptors in agreement with previous studies (Niswender et al., 1999; Herrick-Davis et al., 1999). However, there was no significant difference in basal activity between C23 and S23 receptors, whether in the INI or VSV backbone. To address the possibility that a functional consequence could be cell-type dependent, we examined constitutive activity in HEK293 cells and found no difference at multiple receptor densities. Okada and his group (Okada et al., 2004) found that S23 5-$HT_{2C\text{-}INI}$ receptors had increased constitutive activity compared to wildtype receptors using in vitro reconstitution of receptor expressed in Sf9 insect cells with squid Gαq and bovine Gβγ subunits. In the current study, Okada's differences found utilizing purified proteins in vitro are not reproduced in intact cells; our studies showed that C23 and S23 5-HT_{2C} receptors have similar activities in intact mammalian cells.

In the present study, PLC activation was examined in NIH-3T3 transients to see how C23 and S23 5-$HT_{2C\text{-}INI}$ receptor downstream signaling compared. Activation with 5-HT and DOI gave similar dose response curves at C23 and S23 receptors with no differences in potency or maximal response. This was also replicated in stable cell lines expressing the INI or VSV isoform (data not shown). In addition, HEK293 cells transiently expressing 5-$HT_{2C\text{-}INI}$ receptor showed no difference in the potency or maximal response to 5-HT. Taken together, these data suggest that the C23 and S23 5-

HT_{2C} receptors have comparable signaling capabilities. However, it is possible that there could be differences in other pathways that the 5-HT_{2C} receptor is known to activate such as PLD or PLA_2.

The 5-HT_{2C} receptor has been reported to form homodimers/oligomers (Herrick-Davis et al., 2004), as has been found for other G-protein-coupled receptors (reviewed in Angers et al., 2002). 5-HT_{2C} receptor dimerization can be visualized on the plasma membrane of living cells using FRET combined with confocal microscopy (Herrick-Davis et al., 2004). FRET occurs when the light emitted from a laser-excited donor is transferred to an acceptor, resulting in excitation of the acceptor and quenching of the donor. In order for this interaction to occur, the donor and acceptor must have overlapping emission and excitation spectra, and they must be within 1-10nm of each other with their dipoles oriented appropriately for energy transfer (Förster, 1948). When the acceptor is removed by photobleaching, the donor becomes de-quenched and FRET is measured as an increase in donor fluorescence. In the present study, FRET efficiencies were measured by acceptor photobleaching on the plasma membrane of cells expressing C23 or S23 5-$HT_{2C\text{-}INI}$ receptors to determine if the C23S polymorphism alters the ability of the receptor to form homodimers/oligomers. FRET can result from specific protein:protein interactions, such as dimer/oligomer formation (receptors in a clustered distribution), or from high levels of donor and acceptor in close enough proximity to produce FRET because they are tightly packed in a small region of membrane (random proximity effect). Recent studies examining these two models have suggested that FRET resulting from random proximity of donor and acceptor is dependent on the amount of acceptor expressed on the plasma membrane, while FRET resulting from clustered

proteins should be independent of acceptor expression levels and dependent on the ratio of donor to acceptor (Kenworthy and Edidin, 1998; Wallrabe et al., 2003). In the present study, FRET efficiency was dependent on the donor/acceptor ratio and independent of acceptor expression, suggesting that FRET resulted from receptors in a clustered distribution on the plasma membrane and not from receptor over-expression. When FRET efficiencies were compared in cells with similar donor/acceptor ratios, there was no difference in the amount of FRET measured on the plasma membrane of cells expressing C23 or S23 5-$HT_{2C\text{-}INI}$ receptors. These results suggest that the C23S polymorphism has no effect on 5-HT_{2C} receptor homodimerization. However, it is not possible to determine whether 5-HT_{2C} receptor homodimers are formed once the receptors reach the plasma membrane or if they form intracellularly and are transported to the plasma membrane as homodimers.

The current study is important to the field given the number of association studies examining the C23S polymorphism with neuropsychiatric disorders. This SNP occurs at a frequency 13% in the Caucasian population (Lappalainen et al., 1995) and has been reported at even higher frequencies in other populations (Lerer et al., 2001; Masellis et al., 1998). The C23S polymorphism has been found to be positively associated with disease symptoms and drug responses in schizophrenia, unipolar depression, bipolar disorder and Alzheimer's disease (for recent review, see Sanders-Bush et al., 2003). The underlying causes of these associations are unknown. Since there appears to be no biologically relevant difference in the function of the C23S SNP, positive associations may be due to linkage disequilibrium between this SNP and the causative SNP which could be in another gene or in another position of the 5-HT_{2C} receptor gene.

In summary, using multiple stable and transient cell lines generated from different plasmid constructs and in different laboratories, we have data from three mammalian cell-types showing no functional consequence of the C23S polymorphism in the human 5-HT_{2C} receptor in either the non-edited INI or the edited VSV isoform, which is the principal isoform in the human brain (Niswender et al., 1999). No differences between the C23 versus the S23 allele were detected in a number of experimental parameters, including radioligand binding, immunolocalization, cell surface targeting, receptor dimerization, constitutive activity and agonist-promoted phosphoinositide hydrolysis. These results do not agree with a recent report by Okada et al (2004), which showed that the S23 variant has increased constitutive activity in reconstitution experiments, utilizing 5-HT_{2C} receptors expressed in insect cells. Although constitutive activity was increased in the S23 receptor variant in this unnatural preparation, our results show that this difference is not reproduced in intact mammalian cells. Given that the 5-HT_{2C} receptor is only expressed in brain, it would be interesting to examine the properties of the S23 variant in a neuronal cell line.[1]

[1] This chapter has been pubished in the Pharmacogenomics Journal (2005): Hugh M. Fentress, Ellinor Grinde, Joseph E. Mazurkiewicz, Jon R. Backstrom, Katharine Herrick-Davis, and Elaine Sanders-Bush. Pharmacological Properties of the Cys23Ser Single Nucleotide Polymorphism in Human 5-HT_{2C} Receptor Isoforms. *Pharmacogenomics J*. (in press)

CHAPTER III

GENETIC ANALYSIS OF 5-HT_{2C} RECEPTORS IN UNIPOLAR DEPRESSION

Introduction

Unipolar depression, also known as major depressive disorder (MDD), is a severe psychiatric disease with lifetime prevalence between 13-19% (Lehtinen and Joukamaa, 1994). The importance of a genetic component is widely accepted (Kendler et al., 1994), but the mode of inheritance is complex and non-Mendelian. Pharmacological studies have suggested that depression is associated with an impairment of brain neurotransmitters; a role for 5-HT is based upon the efficacy of selective serotonin reuptake inhibitors (SSRIs) in the treatment of depression. In addition, many antidepressants such as fluoxetine, norfluoxetine, citalopram, amitriptyline, and mianserin have high affinity for the 5-HT_{2C} receptor (Palvimaki et al., 1996; Fentress et al., 2005), although anatogonism at this receptor is not predictive of antidepressant utility. Behavioral studies by Cryan and Lucki (2000) showed that, like fluoxetine, selective 5-HT_{2C} receptor agonists are able to decrease immobility and increase swimming in the rat forced swim test, a model of antidepressant efficacy. More evidence for the involvement of the 5-HT_{2C} receptor in depression is that blockade of 5-HT_{2C} receptors has been shown to potentiate the effects of SSRIs in rats (Cremers et al., 2004). All together, these studies suggest that genetic alterations in 5-HT_{2C} receptors may increase the susceptibility of an individual to develop MDD or an endophenotype within depression.

A non-synonymous SNP in the coding region of the 5-HT_{2C} receptor has been identified that converts a cysteine (Cys) to a serine (Ser) at the 23rd amino acid position (Cys23Ser) (Lappalainen et al., 1995). The Cys23Ser polymorphism has been shown to associate with individuals who have late onset Alzheimer's disease with visual hallucinations and hyperphagia (Holmes et al., 1998), in addition to schizophrenia patients with tardive diskinesia (Segman et al., 2000). Recently, a genetic component of MDD was identified (Sullivan et al., 2000). However, there have been only two association analyses of the Cys23Ser SNP in MDD. One of these studies, performed on a population of European subjects, found a positive association between the occurrence of the Ser23 allele and MDD (Lerer et al., 2001). This SNP has also been reported to occur at higher frequencies in African American patients (Masellis et al., 1998; Glatt et al., 2004). Since MDD is a complex disorder, we hypothesized that the Cys23Ser SNP may be associated with an endophenotype of depression. The term "endophenotype" is defined as an internal phenotype that fills the gap between available descriptors, the genes, and the elusive disease processes (Gottesman and Shields, 1973). Therefore, the goal of this study was to perform exploratory analyses on the frequency of non-synonymous SNPs in the 5-HT_{2C} receptor in depression and their association with endophenotypes within the disease.

Methods

Depressed Subjects

110 depressed patient samples were collected through collaboration with Dr. Richard Shelton at Vanderbilt University, and were obtained using proper informed consent and protocols approved by the Institutional Review Board. All subjects were persons with DSM-IV MDD (unipolar type). Participants were self-referred or referred by professionals to the clinic. About 80% of the patients were Caucasian, 15% were African-American, and 5% were underrepresented minorities. This reflects the distribution of the population in the Nashville, TN area. Patients were (1) over the age of 18; and (2) willing and able to give written informed consent.

Any subjects were excluded if they met any of the following: (1) bleeding disorder; (2) evidence of any medical disorder or condition that would exclude participation, in the judgment of the investigator; (3) current treatment with catecholaminergic antihypertensive medication (including reserpine, beta-blockers, clonidine, alphamethyldopa, etc.; diuretics, ACE inhibitors and calcium channel inhibitors were allowed); (4) history of significant endocrine disease (including Cushing's Disease, Nelson's disease, or other HPA abnormality, or hypothyroidism); (5) pregnancy or lactation; or (6) clear indication of secondary gain (e.g., court ordered treatment or compensation issues).

Persons diagnosed with major depression were determined to be free of: (1) a history of bipolar affective disorder (bipolar I, II, or mixed type) or cyclothymia; (2) any history of non-affective psychotic Axis I disorders, including schizophrenia, schizoaffective disorder, delusional disorder, psychotic disorder due to a substance or

medical condition, or psychotic disorder NOS; (3) current nonpsychotic Axis I disorder; e.g., generalized anxiety disorder; panic disorder, phobic disorder, obsessive-compulsive disorder, eating disorder, etc., if currently present and if the predominant aspect of the clinical presentation. In addition, all patients were free of (1) antisocial, borderline, or schizotypal Axis II personality disorder; (2) subnormal intellectual potential (estimated IQ below 80); or (3) history of substance abuse in the past six months or substance dependence in the past twelve months. If suicide risk or psychosis was present, steps were taken to ensure the safety of subjects (e.g., hospitalization, if appropriate). After a positive MDD diagnosis, several patient interviews were conducted, enabling a secondary diagnosis of either atypical or melancholic depression. In addition, the MDD endophenotypes of each patient were recorded for subsequent SNP association studies (Table 7).

African Subjects

Normal African subjects were recruited from Accra, Ghana. Recruitment was done at the clinics of Department of Medicine at the Korle Bu Teaching Hospital of University of Ghana Medical School, the Mamprobi Hypertension Clinic, and the Kaneshie Market. Subject recruitment protocols were approved by the Institutional Review Board of Meharry Medical College and the Ethical Committee of the University of Ghana School of Medicine.

Table 7: Endophenotypes examined in Major Depressive Disorder patients

MDD Endophenotypes
MDD subtype
MDD age of onset
Recurrent or single episode MDD
MDD severity
Past/present antidepressant treatment
Patient history of other psychiatric disease
Patient history of substance abuse
Presence of personality disorders
Family history of psychiatric disease
Family history of substance abuse
Depressed mood
Guilt
Suicide (ideation and attempts)
Early/middle/late insomnia
Hypersomnia (hours of additional sleep)
Napping
Anhedonia
Retardation
Agitation
Psychic or somatic anxiety
Change in appetite
Energy
Libido
Hypochondriasis
Weight loss/gain
Degree of insight

DNA Extraction

DNA was extracted blood samples (6-12 mL) using the Puregene genomic DNA extraction kit per manufacturer's instructions (Gentra Systems, Minneapolis, MN).

DNA Analysis

For DNA genotyping, one set of PCR amplification primers (Urogentec, West Chester, PA) and a single sequencing primer (DNA core, Vanderbilt University, Nashville, TN) were designed and prepared for each SNP. The PCR primers consisted of one biotinylated primer and one unbiotinylated primer that were designed to amplify a region of the 5-HT_{2C} receptor genomic DNA of less than 200 nucleotides, containing the SNP of interest. Following amplification of the target region, the biotinylated amplicons were annealed to streptavidin-coated agarose beads and separated from unbiotinylated strands by vacuum filtration. The isolated biotinylated strands were then washed and transferred to 96-well plates and buffers (Pyrosequencing AB, Uppsala, Sweden), sequencing primer, and polymerase were added to each well. The sequencing primer was designed to anneal just 5′ (~10 nucleotides) of the polymorphism. The target sequence downstream of the sequencing primer was entered into the pyrosequencing computer (with "false" nucleotides as controls). Based upon this sequence, dNTPs were automatically released into each well one nucleotide at a time. If a nucleotide was complementary to the biotinylated strand, it was incorporated and caused the release of pyrophosphate. This pyrophosphate was converted to ATP by sulfurylase, and this ATP was used as energy by luciferase to convert luciferin to oxyluciferin, generating light (Fig. 27). Each flash of light was detected by the pryrosequencing camera and used to

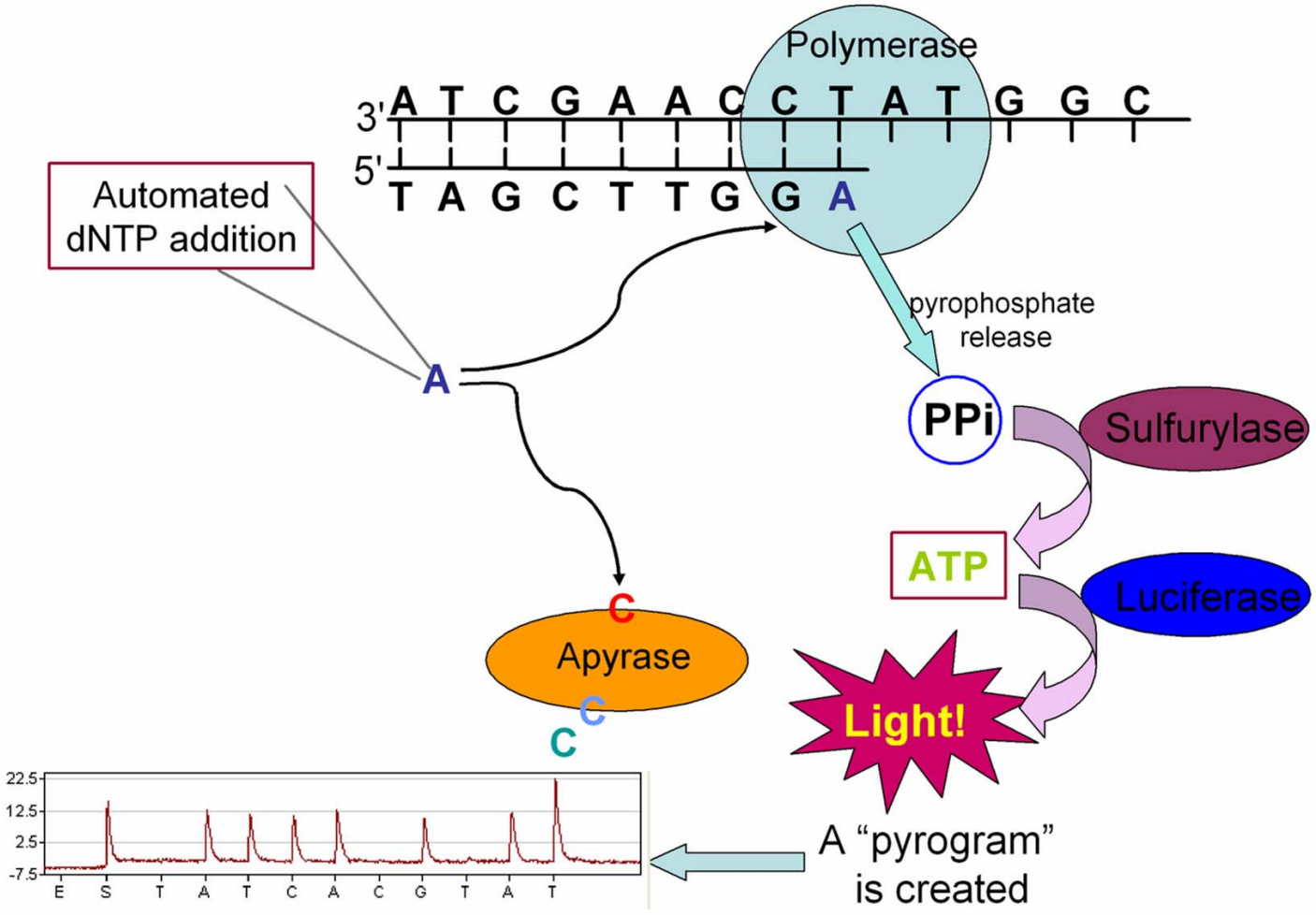

Figure 27: Pyrosequencing method for genotypic analysis

generate a mini sequence report (pyrogram, Fig. 28) that was analyzed for the presence of SNPs.

Statistical Analysis

Genotypes and allele frequencies were compared across all patient endophenotypes and drug treatment groups by Fisher's exact test and logistic regression analysis.

Results and Discussion

In the present study of 110 depressed patients, the allele frequency of the Cys23Ser polymorphism in the 5-HT_{2C} receptor was not different from that published in the literature. There were no homozygous females in our population while there were eight hemizygous males (Table 8). The allele frequency of the polymorphic Ser23 allele was 12% which is not different from the initial value reported for normal controls (Lappalainen et al., 1995). However, this SNP occurs at varying frequencies in different populations (Table 9). Recently it was reported that the Cys23Ser SNP occurs at 40% in African-American populations (Masellis et al., 1998; Glatt et al., 2004). In order to evaluate this further, African samples were obtained from a collaborator Scott Williams (Vanderbilt University) and analyzed for the polymorphism. If this polymorphism truly occurs at three times the frequency in African populations relative to Europeans, it could dramatically skew the association analyses from mixed samples especially if there are large numbers of African-Americans. We found the allele frequency of the Ser23 allele

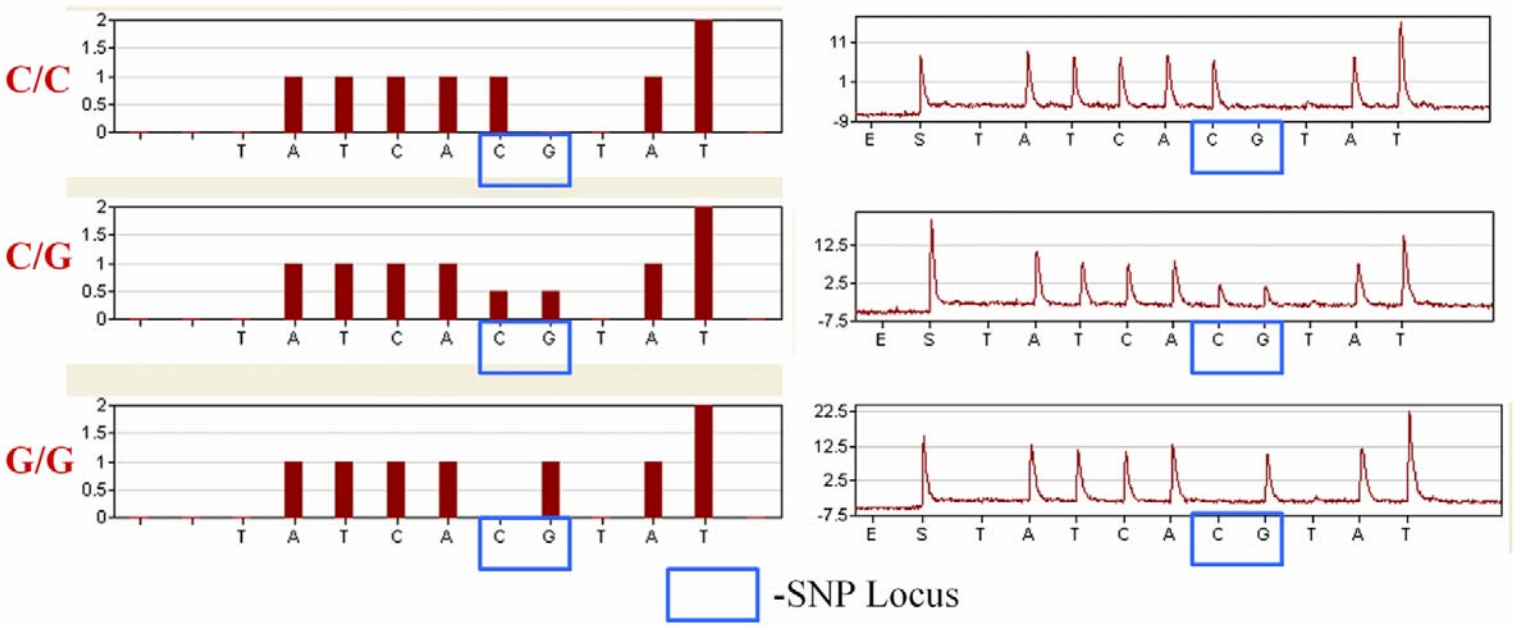

Figure 28: Pyrogram and mini-sequence report for the C23S SNP
Left panels represents (computer-generated) outcome for each genotype.
Right panels represents the actual pyrograms for each genotype.

Table 8: Cys23Ser genotype in MDD patients
The HTR2C gene is on the X chromosme, therefore the Cys/- genotype represents hemizygous males for Cys23; Ser/- genotype represents hemizygous males for Ser23. Percent equals the percentage of the respective genotype for the population as a whole.

5-HT_{2C} Receptor Genotype	**Number of Patients**	**Percent**
Cys/-	34	30.9
Ser/-	8	7.2
Cys/Cys	53	48.2
Cys/Ser	15	13.7
Ser/Ser	0	0
Total	110	100

Table 9: Frequency of Cys23Ser SNP in different populations

Population	Frequency	Reference
Caucasian	13%	Lappalainen et al., 1995
African-American	40%	Masellis et al., 1998; Glatt et al., 2004
Japanese	1.9%	Kusumi et al., 2004
German	16.9%	Lerer et al., 2001; Stefulj et al., 2004
Slavic	16.1%	Stefulj et al., 2004
Belgian	21.1%	Lerer et al., 2001
Bulgarian	10.3%	Lerer et al., 2001
Greek	24.6%	Lerer et al., 2001
Italian	14.2%	Lerer et al., 2001
Scottish	9.2%	Lerer et al., 2001
Swedish	19.2%	Lerer et al., 2001

to be 39% in the African population (Table 10), agreeing with the previous reports in African-Americans (Masellis et al., 1998; Glatt et al., 2004). Over 50% of the subjects had a Ser23 allele and there were 4 homozygous females in the sample as opposed to zero in the depressed population. An examination of the ethnic distribution of our depressed patients (Table 11) revealed that the majority were Caucasian (85%), making it unlikely that there were enough non-Caucasians to cause stratification.

Major depressive disorder is a complex disease, involving many genes and neurotransmitter systems in combination with environmental factors. Therefore, it is unlikely that a SNP in one gene will cause depression. However, it is more probable that a single SNP may be responsible for an endophenotype within depression (Table 7). Thus, we examined the association of the Cys23Ser polymorphism in the 5-HT_{2C} receptor with endophenotypes of unipolar depression. There were positive associations of the minor allele (Ser23) with decreased early and late insomnia (Table 12). The association with middle insomnia was weaker and not significant (Table 12). These results suggest that the Ser23 is protective and decreases insomnia in depressed patients. 5-HT_{2C} receptor antagonists produce dose dependent increases in slow wave sleep (SWS) in humans and rats (Sharpley et al., 1990; Dugovic and Wauquier, 1987) while 5-HT_{2C} agonists inhibit SWS in rats (Dugovic, 1992). Futhermore, the 5-HT_2 antagonist ritanserin has been found to have different functional responses in depressed patients compared to controls. For example, the dose dependent increase in SWS observed with ritanserin in normal individuals is reduced in depressed patients (Staner et al., 1992) suggesting an upregulation in 5-HT_2 receptors in patients with depression, thereby leading to a blunted functional response (Smith et al., 2002). Based on these

Table 10: Cys23Ser genotype in African subjects

The HTR2C gene is on the X chromosme, therefore the Cys/- genotype represents hemizygous males for Cys23; Ser/- genotype represents hemizygous males for Ser23. Percent equals the percentage of the respective genotype for the population as a whole.

5-HT_{2C} Receptor Genotype	**Number of Patients**	**Percent**
Cys/-	34	36
Ser/-	21	22
Cys/Cys	12	13
Cys/Ser	24	25
Ser/Ser	4	4
Total	95	100

Table 11: Ethnicity of MDD patients
Other category represents patients of mixed descent. Percent equals the percent of the total population.

Ethnicity	Number of Patients	Percent
Caucasian	94	85
African-American	7	6
Asian	5	5
Hispanic	1	1
Other	3	3
Total	110	100

Table 12: Cys23Ser SNP associations with endophenotypes in MDD patients
Analyses were performed on the entire depressed population (110 patients). Coefficients were derived by ordinal logistic regression. The coefficients represent the direction and magnitude of the associations. p values were determined by the Wald test. Asterisks (*) represent significance of the minor allele (Ser23) compared to patients with major allele (Cys23).

Endophenotype	Coefficients	p value
Early Insomnia	-1.75	0.009*
Middle Insomnia	-1.12	0.086
Late Insomnia	-1.52	0.041**
Somatic Anxiety	1.61	0.015***

pharmacological studies, one possible interpretation of our results is that depressed patients with the Ser23 allele have decreased functional 5-HT_{2C} receptors which might translate into increased SWS and decreased insomnia.

Upon further analysis, we also found an association of Ser23 with increased somatic anxiety (Table 12). Somatic anxiety is defined as physiologic concomitants of anxiety such as dry mouth, gas, indigestion, diarrhea, constipation, dizziness and sweating. m-chlorophenylpiperazine (mCPP), a metabolite of the antidepressants trazodone and nefazodone is a non-selective 5-HT_{2C} receptor partial agonist that causes anxiety and sympathetic nervous system arousal in humans (Abi-Saab et al., 2002; Broocks et al., 2001). The anxiety like effect of mCPP in rats is blocked by specific 5-HT_{2C} antagonists (Blackburn et al., 1997). When 5-HT_{2C} receptor antagonists are administered alone, they produce anxiolytic-like behavior in various animal models (Kennett et al., 1996, 1997). Kuhn et al. (2004) recently found differences in mCPP response in healthy subjects with and without the Cys23Ser SNP. Subjects with the Cys23 allele had region cerebral blood flow (rCBF) increased in the left medial prefrontal cortex and decreased in the left anterior cingulate and right medio-temporal cortex, whereas subjects with the Ser23 allele showed an increase in rCBF in the left medio-temporal cortex and a reduction of rCBF in the right medial prefrontal cortex (Kuhn et al., 2004). Together, these data suggest that the Cys23Ser polymorphism is functionally relevant in humans, perhaps related to anxiety phenotypes.

Since 5-HT_{2C} Cys23Ser SNP occurs at various frequencies depending upon the ethnic population (Table 9), we reanalyzed the patient data using Caucasians only. The Caucasian depressed patients continued to have strong associations of the Ser23 allele

with early and late insomnia (Table 13). Additionally, we were now able to attain an association with middle insomnia (Table 13). However, upon examining the endophenotype of somatic anxiety, the positive association was lost in the Caucasian patients (Table 13). The loss of the association with somatic anxiety could be due to the reduced number of patients with the Ser23 allele after removing the non-Caucasian patients. Alternatively, there may be increased anxiety among the other ethnicities and when these individuals are excluded, the association disappears.

Taken together, the association data suggest that the Cys23Ser SNP in the human 5-HT_{2C} receptor may have functional consequences. However, this conclusion conflicts with our functional data in cell lines. One possible explanation for this discrepancy is that the Cys23Ser SNP is in linkage disequilibrium with the causative SNP which could be in the HTR2C gene or another gene. Therefore, the associations that were found could be due to a SNP in a gene that is closely linked to the Cys23Ser SNP.

There are several polymorphisms in the promoter region of the HTR2C gene (Table 2). Two of these SNPs, -697 G/C and -759 C/T, have been shown to have functional consequences and to be associated with disease states (Table 3). Therefore, we examined the occurrence of these SNPs in the depressed patient set. The frequencies of the -697 G/C and -759 C/T polymorphisms were similar to the values previous reported in the literature (Table 14). Analysis of the -697 G/C promoter SNP with endophenotypes in depressed Caucasians revealed positive associations of the minor allele (C) with somatic anxiety, early insomnia, and increased appetite (Table 15). The -759 minor allele (T) was associated with suicide attempts and the total score for the Hamilton Rating Scale for Depression (HRSD) items 1-17 in Caucasian depressed

Table 13: Cys23Ser SNP associations with endophenotypes in Caucasian MDD patients

Analyses were performed on Caucasian depressed patients (94 patients). Coefficients were derived by ordinal logistic regression. The coefficients represent the direction and magnitude of the associations. p values were determined by the Wald test. Asterisks (*) represent significance of the minor allele (Ser23) compared to patients with the major allele (Cys23).

Endophenotype	Coefficients	p value
Early Insomnia	-2.42	0.003*
Middle Insomnia	-2.00	0.014**
Late Insomnia	-2.05	0.03***
Somatic Anxiety	1.07	0.14

Table 14: Promoter SNP genotypes and frequencies in MDD
The HTR2C gene is on the X chromosme, therefore the G/Y and C/Y genotypes represent hemizygous males for the major G and C alleles, respectively; C/Y and T/Y genotypes represent hemizygous males for the minor C and T alleles, respectivley. Percent equals the percentage of the respective genotype for the population as a whole.

5-HT$_{2C}$ Receptor Genotype		**Number of Patients**		**Percent**	
-697 G/C	**-759 C/T**	**-697 G/C**	**-759 C/T**	**-697 G/C**	**-759 C/T**
G/-	C/-	28	35	27	32
C/-	T/-	14	8	14	7
G/G	C/C	31	48	30	44
G/C	C/T	27	19	26	17
C/C	T/T	3	0	3	0
Total		103	110	100	100

Table 15: -697 G/C SNP associations with endophenotypes in Caucasian MDD patients

Analyses were performed on Caucasian depressed patients (94 patients). Coefficients were derived by ordinal logistic regression. The coefficients represent the direction and magnitude of the associations. p values were determined by the Wald test. Asterisks (*) represent significance of the minor allele (C) compared to patients with the major allele (G).

Endophenotypes	Coefficients	p value
Early Insomnia	-0.93	0.015*
Middle Insomnia	-0.68	0.078
Increased Appetite	0.87	0.034**
Chronic Depressed Episode	0.86	0.07
Somatic Anxiety	1.5	0.001***

patients (Table 16). These data suggest that there may be genetic linkage between the Cys23Ser and the -697 G/C polymorphisms in the 5-HT_{2C} receptor given that they associate with the same endophenotypes in MDD.

Table 16: -759 C/T SNP associations with endophenotypes in Caucasian MDD patients

Analyses were performed on Caucasian depressed patients (94 patients). Coefficients were derived by ordinal logistic regression. The coefficients represent the direction and magnitude of the associations. p values were determined by the Wald test. Asterisks (*) represent significance of the minor allele (T) compared to patients with the major allele (C).

Endophenotpes	Coefficients	p value
Suicide Attempts	1.73	0.014*
HRSD Score 1-17	1.82	0.03**
Increased Appetite	0.84	0.09
Somatic Anxiety	0.89	0.08

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

The identification of only 30,000 genes with the completion of sequencing the human genome reveals the importance of mechanisms for generating molecular and genetic diversity. One way to achieve molecular diversity is through post-transcriptional events such as RNA editing. The 5-HT_{2C} receptor is the only GPCR that has been shown to undergo RNA editing, which generates functionally distinct protein isoforms from a single gene. Another way to achieve diversity is through genetic modifications: SNPs, deletions, insertions, and repeats. SNPs are the most common type of genetic variation and by definition occur at a frequency greater than 1% (Wang et al., 1998). Within the coding region of the human 5-HT_{2C} receptor gene are two SNPs (Lappalainen et al., 1995; Gibson, et al., 2004) whereas three have been reported in the promoter region (Yuan et al., 2000). When I initiated my research, the Cys23Ser polymorphism had been extensively examined in association studies, but the protein had not been characterized functionally. In this thesis, I report the results of functional analyses of this SNP in a variety of assays.

Despite extensive investigations in three different cell types, we did not find any evidence of altered function of the Cys23Ser SNP; however, we did find positive associations with this SNP in depressed patients, including the subphenotypes of insomnia and anxiety. Therefore, the Cys23Ser polymorphism may be in linkage

disequilibrium with another SNP, leading to the observed associations with depressive symptoms.

5-HT_{2C} receptors with the Cys23Ser SNP did not have any difference in cellular distribution, even in edited backbones. This was demonstrated by immunofluorescence and confirmed by surface biotinylation combined with western blotting. The banding pattern of the receptors were very similar, migrating as broad bands around 50 kDa, indicating that glycosylation is intact. Although these results suggest that trafficking and cell targeting is not altered in receptors with the Cys23Ser polymorphism, it is still possible that there are small changes that are beyond the sensitivity of these assays. In the immunofluorescence experiments, receptors that have a CFP tag fused to their C-terminals were overexpressed in HEK293 cells. Although these receptors were still able to signal, the tag may change the conformation without altering function, but eliminating any differences in conformation that may be present in native receptors. Morello et al. (2002) showed that treatment of cells expressing the mutant vasopressin receptors with antagonists caused increased surface expression, presumably via binding and stabilizing partially folded mutant receptors. Pilot experiments of possible rescue of functional receptors by pretreatment of cells expressing 5-HT_{2C} receptor variants with 5-HT_{2C} antagonists were negative. It would be interesting to pursue such studies in more direct assays of cell surface receptors; however, there was very little 5-HT_{2C} receptor protein on the cell surface in our biotinylation experiments. The large intracellular pool of receptors may be due to the receptor's high constitutive activity. The prominent intracellular localization, combined with the limitations of the assays, make subtle changes difficult to accurately quantitate.

Our inability to find differences in binding, signaling, and constitutive activity at Cys23Ser 5-HT_{2C} receptors could be due to a number of reasons. We used NIH-3T3 fibroblasts that are derived from mouse and COS-7 cells, derived from monkeys. HEK293 cells were used in some studies but these cells are derived from the human embryonic kidney. Since the 5-HT_{2C} receptor is only expressed in the brain, it would be interesting to examine the properties of receptor variants in a neuronal cell line. Another potential caveat is the fact that we only examined one signaling pathway of the 5-HT_{2C} receptor. This receptor has also been shown to activate PLD and PLA_2 (McGrew et al., 2002; Berg et al., 1996, 1998) and interact with the G_{13} protein (Price et al., 2001). In order to definitively conclude that no functional consequences result from the Cys23Ser polymorphism, examination of these pathways should be conducted. New technology now allows us to examine many pathways and genes at once. SuperArray Bioscience corporation has focused DNA microarrays with human G-protein subunits and their downstream signaling and effector proteins, making gene expression profiling of G-protein signaling feasible. Alternatively, Kinexus offers technology to track 31 phosphorylation sites of different proteins (as an indirect index of activation) in lysates from cells or tissues. Future studies should use these methods to further compare the function of the Cys23Ser SNP in 5-HT_{2C} receptor isoforms. If an alteration is found, more experiments could then be performed to determine the mechanism of the alteration.

Although no functional consequences of the Cys23Ser SNP were found in our in vitro studies, we did find associations with the Ser23 allele in MDD. Subjects with Ser23 had increased anxiety and reduced early and late insomnia. Because the frequency of this SNP varies across ethnic populations, we re-analyzed the data with Caucasians only;

some of the associations changed in this analysis. These changes could be a result of the small sample size or an altered frequency of the SNP in the population that was removed. This suggests that one has to be careful when analyzing association data on this SNP with mixed populations. The fact that we found associations in MDD and no change in functional assays in cells implies that the Cys23Ser polymorphism in the human 5-HT_{2C} receptor may be in linkage disequilibrium with a functional SNP. Likely candidates are the promoter polymorphisms in the 5-HT_{2C} receptor. Although our data show that the -697 G/C promoter SNP is closely linked to the Cys23Ser SNP, it is not in complete linkage disequilibrium which leaves the possibility that other genes may be involved. The first genes that should be examined are those adjacent to the HTR2C gene on the X-chromosome which includes LOC286528 (similar to heat shock protein C182) and IL13RA2 (interleukin 13 receptor, alpha 2) (Figure 29). According to HapMap, this region of the X-chromosome has a very high degree of linkage disequilibrium in both Caucasian and African populations, suggesting that our hypothesis of the Cys23Ser SNP being in linkage disequilibrium with another SNP is likely correct. However, analyses of the Perlegen Genome Browser revealed that African subjects have smaller blocks of linkage disequilibrium. Therefore, future studies to locate other polymorphisms closely linked to the Cys23Ser SNP should use the Perlegen database of African subjects.

Analyses of mitochondrial DNA and nuclear DNA markers have shown that Africa is the most genetically diverse region of the world (Tishkoff and Williams, 2002). As a result, we feel that the African subjects would be a powerful resource to screen for novel polymorphisms in the 5-HT_{2C} receptor. Normally, there is an average of one relatively common SNP and several less common SNPs (1% or less) in GPCRs of

average length (1000-1500 coding base pairs) (Sadee et al., 2001). Therefore, a SNP discovery project may be necessary since the Cys23Ser SNP is the only common SNP in coding region of the 5-HT_{2C} receptor, while the closely related 5-HT_{2A} receptor has five

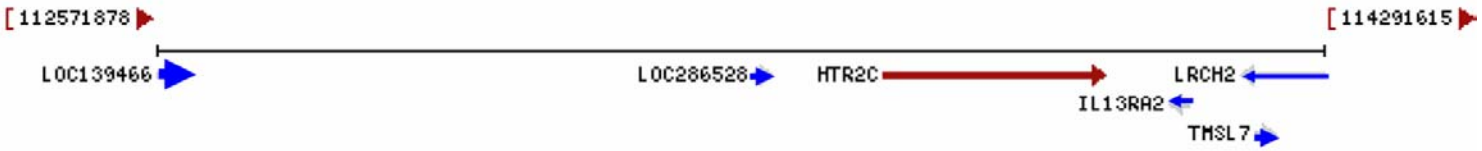

Figure 29: Human X-chromosome band q24
LOC139466, peptidyl prolyl isomerase H (cyclophilin H); LOC286528, similar to HSPC182 protein; HTR2C, serotonin 2C receptor; IL13RA2, interleukin 13 receptor, alpha 2; LRCH2, leucine-rich repeats and calponin homology (CH) domain containing 2; TMSL7, thymosine-like 7

SNPs in its coding region**.** However, since the 5-HT_{2C} receptor undergoes RNA editing, generating up to 24 different protein isoforms, this receptor may have reached its requisite level of diversity via this mechanism. In most SNP screenings, investigators only examine the exons of genes. In the case of the 5-HT_{2C} receptor, the introns are also important because the intron upstream of the edited sites is necessary for RNA editing of the transcripts. Consequently, potential SNPs in the intron forming the RNA duplex may disrupt editing, increasing the risk for disease.

In conclusion, the current work may change the way genetic association studies are performed, since it appears the Cys23Ser SNP in the 5-HT_{2C} receptor is functionally silent. This chapter outlines potential future experiments that will enhance our knowledge and understanding of the 5-HT_{2C} receptor and its genetic alterations, and as a result may eventually lead to novel drug design and treatments based upon genetic backgrounds.

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