IN VIVO FUNCTIONAL CONSEQUENCES OF THE FULLY EDITED 5-$HT_{2C\text{-}VGV}$ RECEPTOR

By

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TO

My mother Maria JP da Silva without whom none of this would be possible

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LIST OF ABBREVIATIONS

5-HT	Serotonin, 5-hyroxytryptamine
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
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
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
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

The indolamine neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) was isolated as a vasoconstrictor released from platelets (Rapport et al., 1948) and subsequently named by Maurice Rapport, Arda Green and Irvine Page of the Cleveland Clinic. The synthesis and metabolism of 5-HT, summarized in Figure 1, has been extensively studied since the first demonstration of 5-HT in brain (Erspamer 1952). 5-HT is synthesized from the amino acid tryptophan, which is acted upon by the rate limiting enzyme tryptophan hydroxylase. Brain tryptophan hydroxylase is not saturated, nor regulated by end product inhibition; as a result, the amount of tryptophan in the brain influences the synthesis of 5-HT. The product of this reaction, 5-hydroxytryptophan, is then metabolized by L-amino acid decarboxylase to form 5-HT. Monoamine oxidase (MAO) is the enzyme involved in the principal route of degradation of 5-HT; it acts on 5-HT to form 5-hydroxyindole acetic acid (5-HIAA) through a 2-step process (Figure 1). 5-HT action is also terminated by Na+ dependent active uptake by the 5-HT transporter, localized in the outer membrane of pre-synaptic serotonergic axon terminals and in platelets. 5-HT is found in enterochromaffin cells throughout the gastrointestinal tract and in platelets, as well as in specific regions of the CNS. Cell bodies of serotonergic neurons are localized in brainstem midline raphe

nuclei. Serotonergic axon projections are widely distributed throughout the brain and spinal cord (Figure 2). 5-HT's primary role is modulation of neural transmission. At the systems level it plays a role in a great deal of physiologic and cognitive functions such as, sleep, learning and memory, temperature regulation, aggression , appetite, motor behavior, cardiovascular and gastrointestinal function (Lentes et al., 1997; Heisler, 2000). 5-HT dysfunction has been implicated in an array of brain disorders including, depression, schizophrenia, and obsessive compulsive disorder, for example (Breier, 1995). 5-HT is able to achieve promiscuous modulation of these wide ranging effects through activation of a large family of 5-HT receptors.

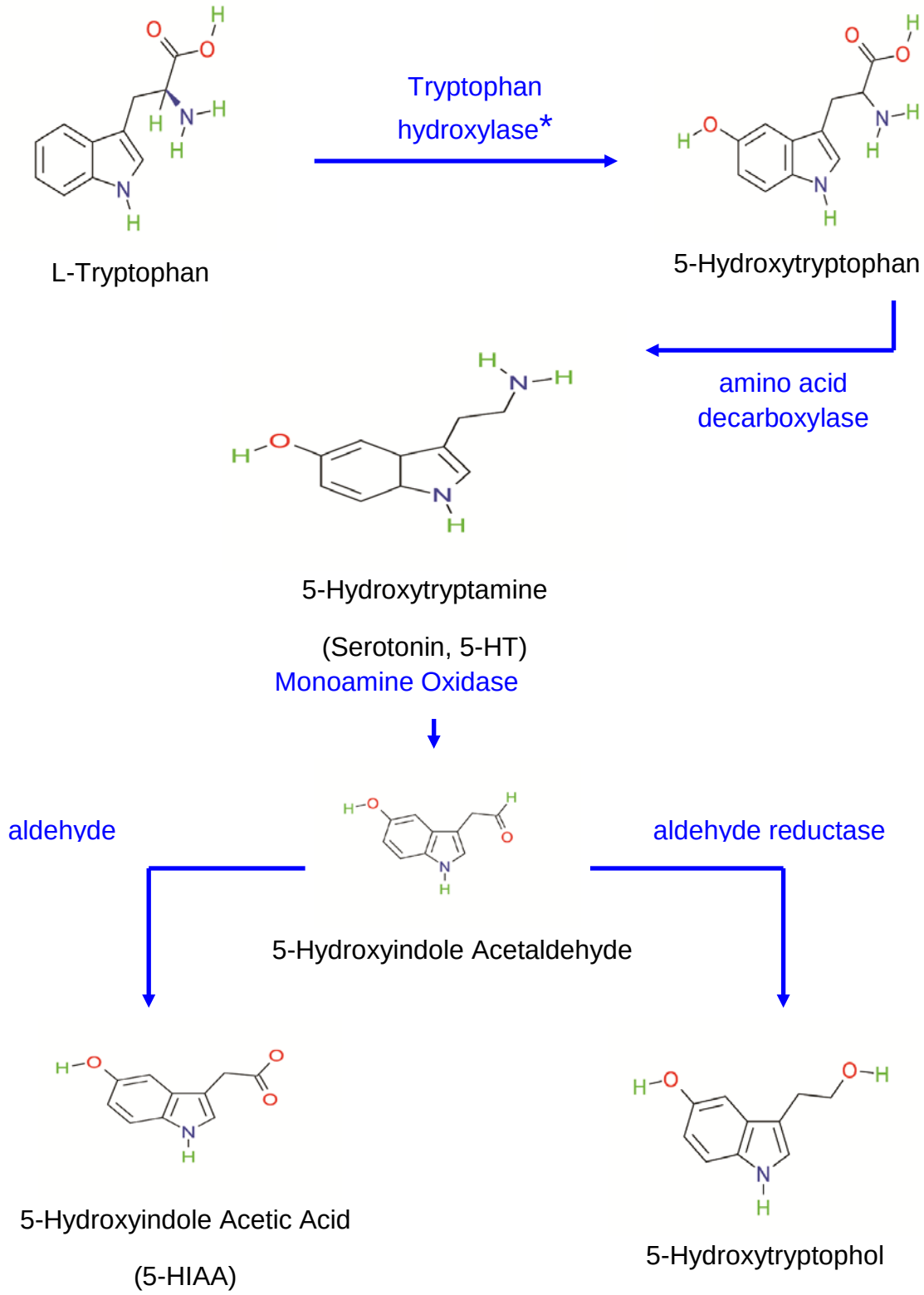

Figure 1. Synthesis and Degradation of Serotonin (5-HT).

The amino acid L-tryptophan is synthesized into 5-HT via the rate limiting enzyme tryptophan hydroxylase. The major metabolite of 5-HT is 5-HIAA. S10ynthesis and degradation enzymes are highlighted in blue.

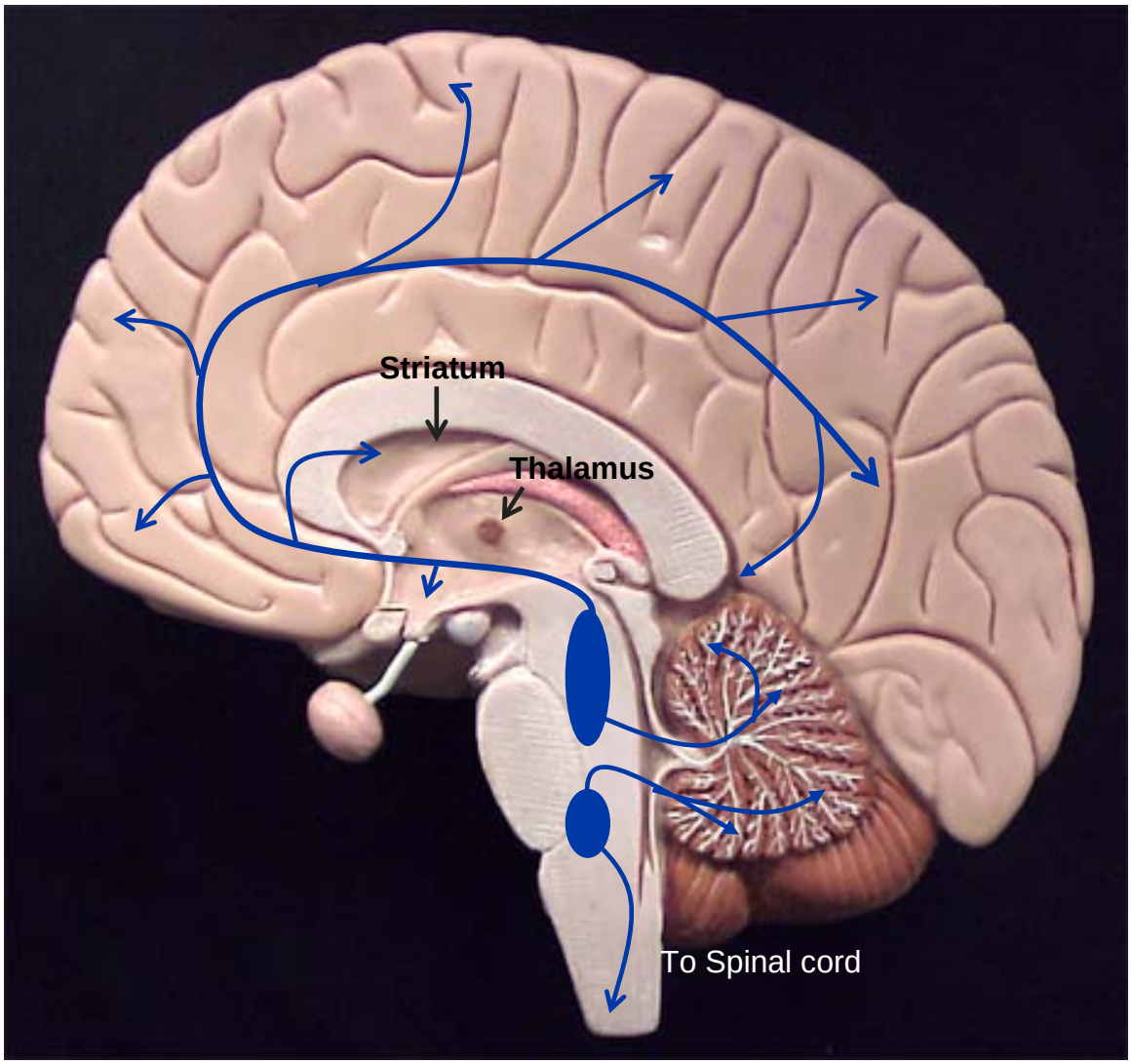

Figure 2: Serotonergic Projections

Serotoninergic projections from the brain stem raphe nuclei (blue ovals) innervate the CNS

Serotonin Receptor Subtypes

Fourteen structurally and pharmacologically distinct receptors are classified into seven receptor families (5-$HT_{1\ to\ 7}$). This scheme originally proposed by Humphrey et al (1993), remains largely unchanged (Table 1). One member of the receptor family, the 5-HT_3 receptor is a ligand-gated ion channel, the remaining members are seven transmembrane spanning , G-protein coupled metabotropic receptors; each comprised of three extracellular and three intracellular loops. The intracellular loops are critical for receptor interaction with heterotrimeric GTP-binding proteins (G-protein), which initiates signal transduction. The different receptor families are distinguished primarily by their downstream effectors (Table 1) (See Barnes and Sharp, 1999 for review). The 5-HT_1 family of receptors consists of 5 receptor subtypes all linked to inhibition of adenylate cyclase; through coupling to the Gi/o family of G proteins (Limbird, 1988). The 5-HT_{1A} receptor is localized in raphe cell bodies where it inhibits cell firing. The 5-$HT_{1B/D}$ receptor functions as an autoreceptor and inhibits neuronal 5-HT release. 5-HT_4 receptors which are distributed throughout the body, activate adenylate cyclase, leading to a rise in cyclic AMP (Hegde and Eglen, 1996). 5-HT_6 and 5-HT_7 receptors are also linked to activation of adenylate cyclase.

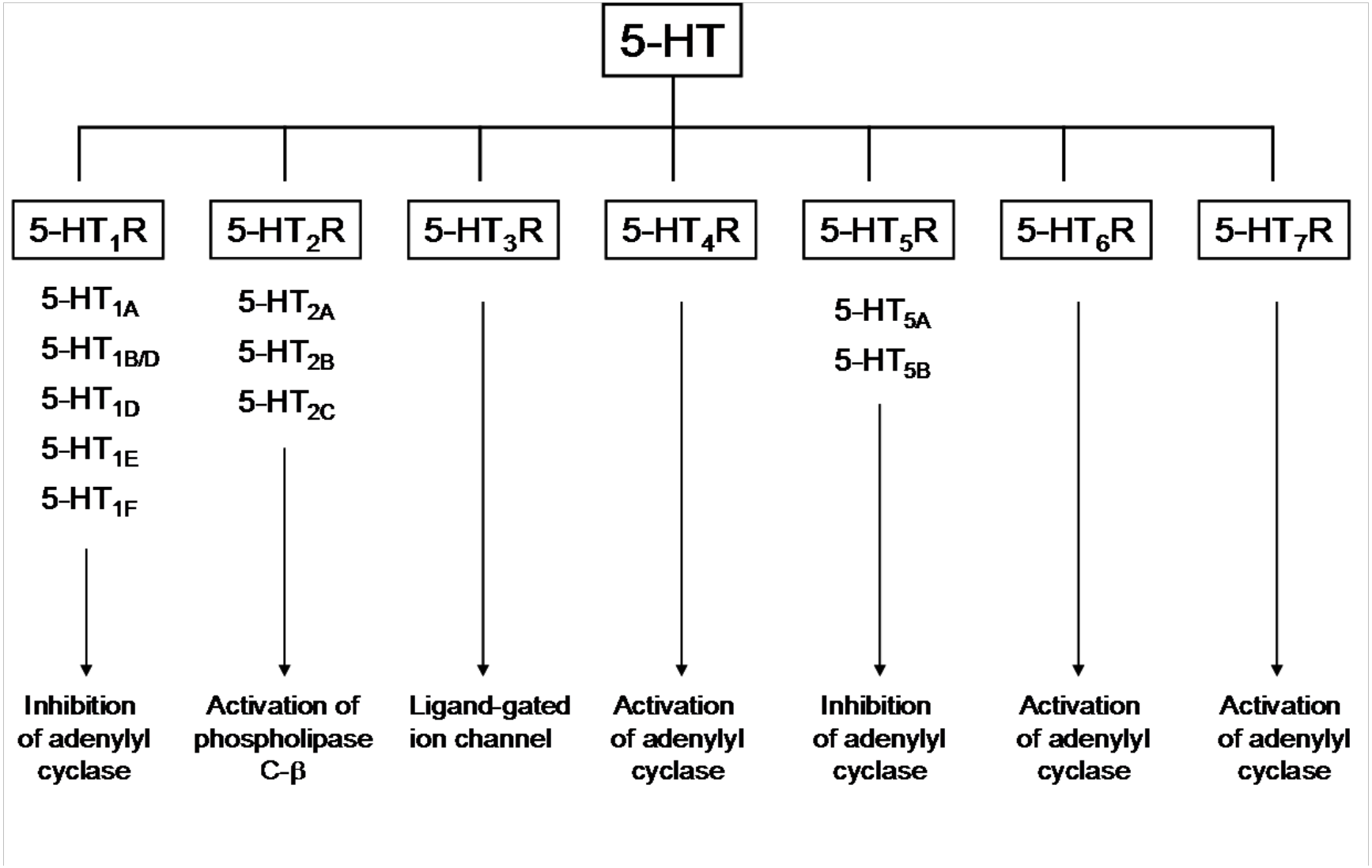

Table 1: Serotonin Family of Receptors

5-HT_2 Receptors

Of particular interest to our laboratory is the 5-HT_2 family of receptors, which encompasses three receptor subtypes, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. The classical signal transduction pathway for these receptors is $G_{\alpha q}$-coupled activation of phospholipase C (PLCβ), cleaving phosphotidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG), which activates protein kinase C (PKC) and inositol triphosphate (IP_3), which promotes intracellular calcium release (Figure 3). 5-HT_2 receptors also couple to other G-proteins (G_{12}/G_{13} and $G_{i/o}$) to activate additional signaling cascades (Figure 4).

5-HT_{2A} Receptor

The 5-HT_{2A} receptor was initially observed in cortical membranes derived from rat. It was detected as a binding site with high affinity for the dopamine antagonist [^{3}H]-spiperone, but with a pharmacological profile more consistent with a 5-HT receptor (Leysen et al., 1978; Peroutka and Synyder, 1979). The 5-HT_{2A} receptor has been shown to be a key target of hallucinogenic drugs. While most hallucinogens have affinity for the 5-HT_{2C} receptor as well, drug discrimination studies have demonstrated that discriminative stimuli elicited by hallucinogenic drugs are dependent on 5-HT_{2A} receptor activation (See Winter, 2009; Fantegrossi et al., 2008). Autoradiography and immunocytochemistry have been used to map 5-HT_{2A} receptor distribution. Receptor autoradiography studies, usually employing "selective" antagonists, localized high binding site

levels in cortical areas, caudate nucleus, nucleus accumbens, hippocampus and olfactory tubercle (Palacios and Dietl, 1988) . There is close concordance between 5-HT_{2A} receptor binding sites, mRNA and immunoreactivity, which suggests that cells expressing these receptors are located in the same regions as the receptors themselves. Evidence also suggests that the 5-HT_{2A} receptor is localized on GABAergic interneurons, cortical pyramidal neurons, and cholinergic neurons which supports its ability to modulate a variety of physiological processes. Activation of the 5-HT_{2A} receptor has been shown to induce hyperactivity, head twitches in rodents, and visual hallucinations in humans.

5-HT_{2B} Receptor

The 5-HT_{2B} receptor, originally found to mediate contractions of the stomach fundus in rats (Vane, 1959), is thought to be present in limited amounts in the mouse and human brain (Loric et al., 1992; Bonhaus et al., 1995).

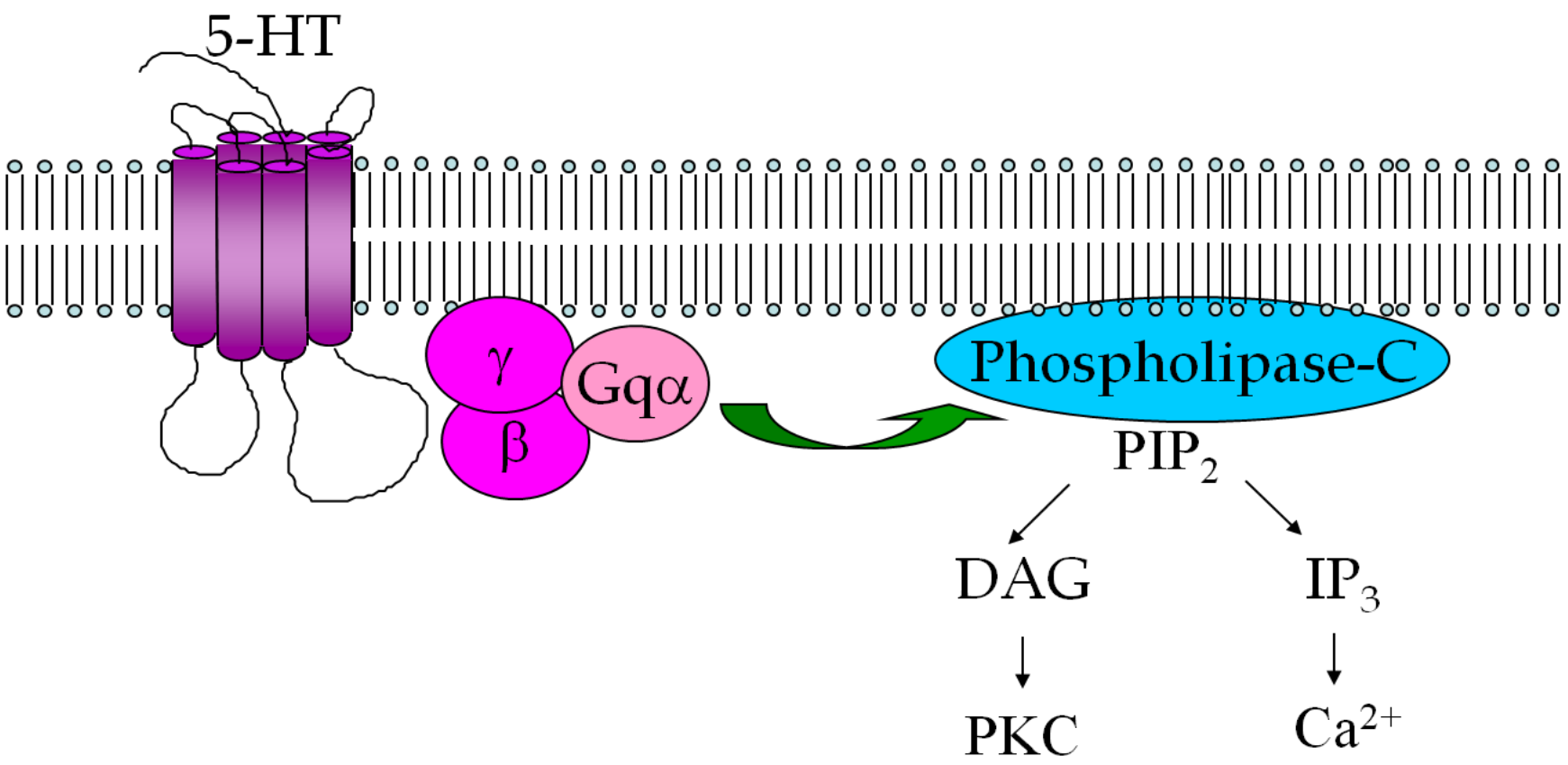

Figure 3: Intracellular Signaling Cascade of the 5-HT_{2C} Receptor.

Gq, heterotrimeric G protein subunit; PIP_2, phosphatidylinositol-1,4-bisphosphate; IP_3, inositol-1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C

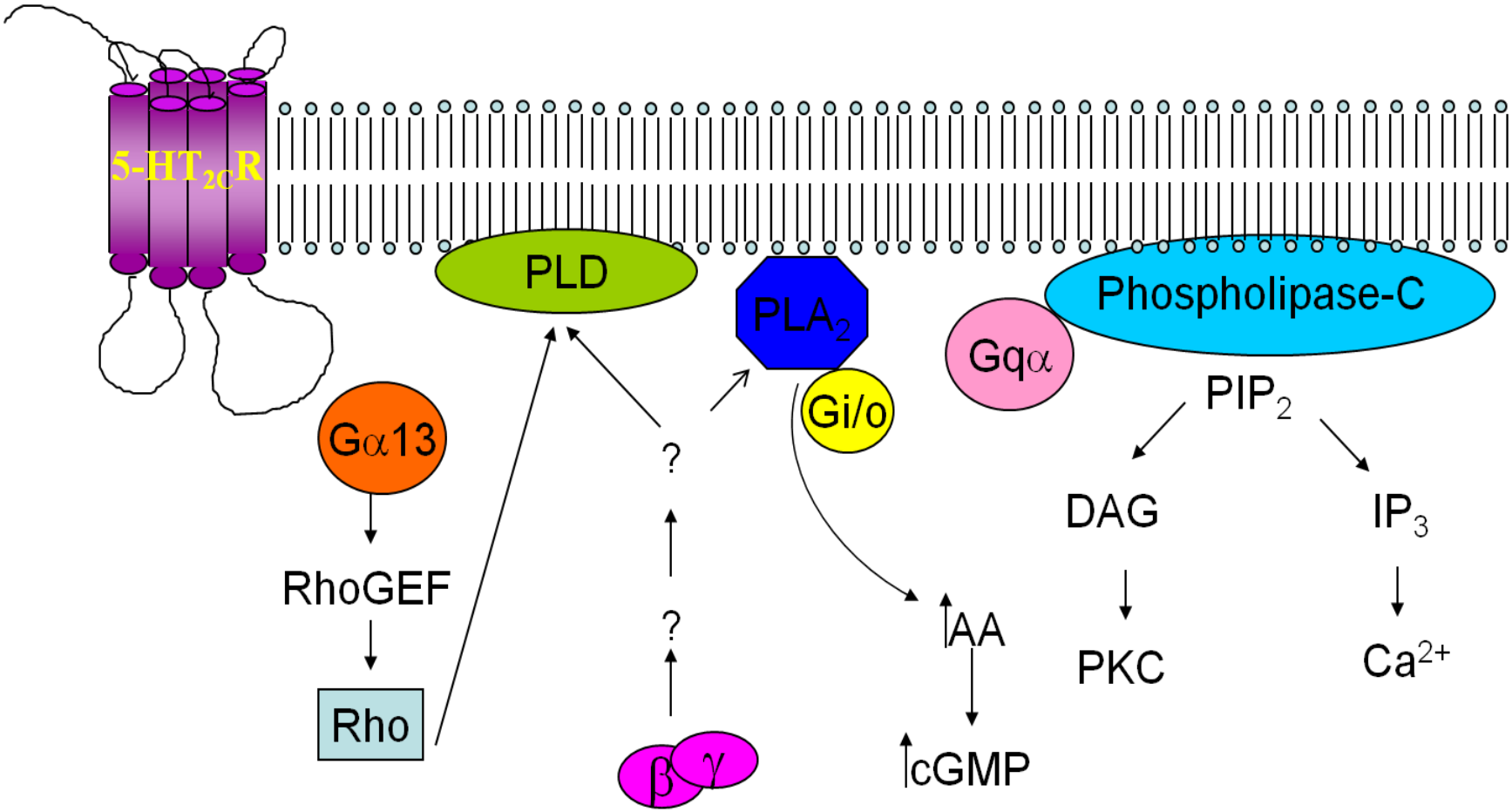

Figure 4: Mutiple Signaling Cascades of the 5-HT$_{2A/2C}$ Receptor.

The 5-HT$_{2A/2C}$ receptor can activate multiple G-proteins, stimulating multiple pathways. PIP2, phosphatidylinositol-1,4-bisphosphate; IP$_3$, 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

5-HT_{2C} Receptors

The 5-HT_{2C} receptor was initially observed as a [^{3}H]-serotonin binding site in the choroid plexus of various species. These binding sites could also be labeled with [^{3}H]-mesulergine and [^{3}H]-LSD, but not by [^{3}H]-ketanserin, an antagonist ligand used to label 5-HT_{2A} receptor binding sites (Pazos et al., 1984a). Unlike the 5-HT_{2A} receptor, the 5-HT_{2C} receptor has a relatively high affinity for 5-HT, which led to it initially being classified as a member of the 5-HT1 family of receptors. By 1991, the 5-HT_{2C} receptor had been cloned in rat, mouse and human, which led to the reclassification of the receptor to the 5-HT2 family where it presently resides. The 5-HT_{2C} receptor is X-linked (human chromosome Xq24, mouse chromosome X D-X F4); this means that males only express a single maternal allele of the gene. There is greater than 80% sequence homology between the human and mouse receptors in the transmembrane regions. The mouse receptor possesses six N-glycosylation sites (Yu et al., 1991), four of these sites are also present in the human receptor (Saltzman et al., 1991).

5-HT_{2C} Receptor Signal Transduction

The 5-HT_{2C} receptor couples to several G-proteins including G_q, G_{12}, G_{13} and G_{15} (Berg et al., 2005); this allows for 5-HT_{2C} receptor mediated activation of a diverse array of signaling pathways. Activation of 5-HT_{2C} receptors increases PLC_β activity (Sanders-Bush et al., 1988) via G_q activation (Figure 3; Chang et

al., 2000). 5-HT_{2C} receptors have also been shown to activate phospholipase D (PLD) (McGrew et al., 2002), ERK MAP Kinase (Werry et al. 2005) and PLA2 (Berg et al., 1996) as well as other downstream secondary signals (Raymond et al., 2001; Hoyer et al., 2002). 5-HT_{2C} receptor activation of PLD in choroid plexus is mediated by coupling to G_{13}, and activation of the small G-protein Rho (Figure 4). 5-HT_{2A} receptor activation of PLA2, via a pertussis-toxin sensitive G-protein(s) (Kurrasch-Orbaugh et al., 2003), leads to the release of arachidonic acid from membrane phospholipids. It is likely that the 5-HT_{2C} receptor shares similar properties. Arachidonic acid has several cellular functions of its own and is metabolized into an array of bioactive compounds.

5-HT_{2C} Receptor Distribution

A variety of ligands have been used to map the distribution of 5-HT_{2C} receptors throughout the CNS (Palacios et al., 1991b). Very high densities are detected in the choroid plexus of all species examined. 5-HT_{2C} receptors are also localized in cortical areas, the limbic system (nucleus accumbens, hippocampus, amygdala) and the basal ganglia, although at a density far below than that of choroid plexus. Unlike other 5-HT_2 receptors, studies have found no evidence for 5-HT_{2C} receptor expression outside of the CNS.

Extended Ternary Complex Model

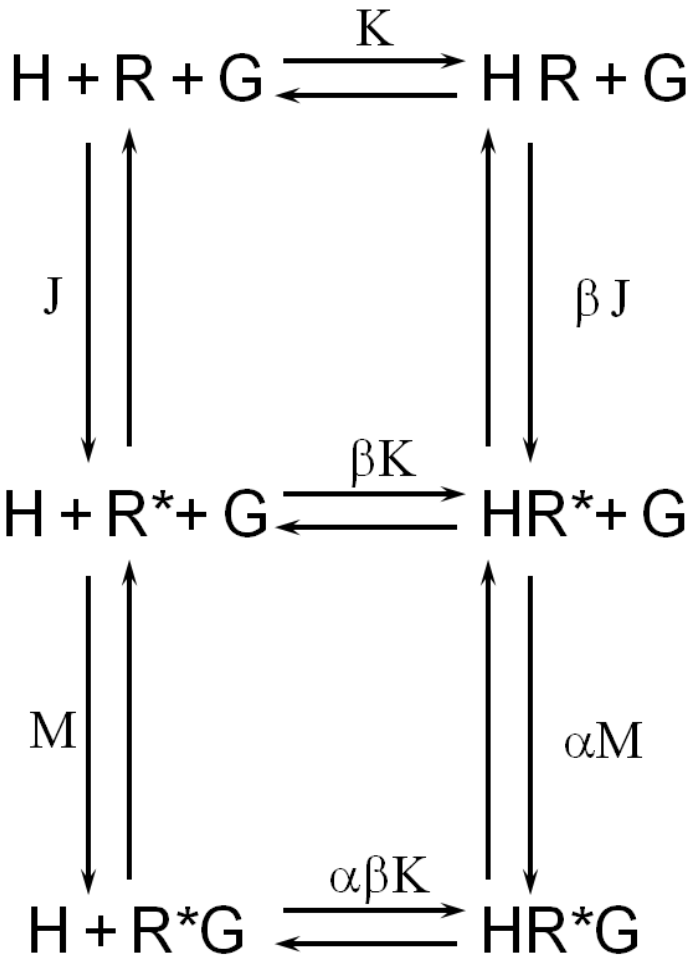

Figure 5: Extended Ternary Complex Model

The Extended Ternary Complex Model allows for the receptor to spontaneously isomerize to an active state, R*, or to couple to G in the absence of ligand. The cooperativity factors (e.g., α, ß) modify the isomerization constant, L, as well as the equilibrium association constants (K_a and K_g) when these isomerizations occur for receptor occupied either by agonist or inverse agonist.

Constitutive Activity of the 5-HT_{2C} Receptor

What further separates the 5-HT_{2C} receptor from the other members of its family is constitutive activity. Simply put this means that the receptor has the ability to couple to and activate G-proteins and downstream signaling cascades, in the absence of a ligand induced conformational change. Constitutive activity has been shown to occur at many GPCRs including the 5-HT_{2C} receptor (Barker et al., 1994). Although originally described *in vitro* (Niswender et al., 1999; see Teitler et al., 2002 for review), constitutive activity has since been shown to play a role *in vivo*, based on the finding that a 5-HT_{2C} receptor inverse agonist/antagonist enhances DA release (De Deurwaerdère et al., 2004). The ternary complex model of receptor-G-protein interactions was revised to predict that GPCRs have the capacity to change spontaneously from an inactive confirmation (R) to an active confirmation (R*). The R* conformation of a receptor is the conformation that allows for coupling to and activation of G-proteins (Samama et al., 1993; Perez et al., 1996; Figure 5). This model has been further revised more recently to account for newer concepts such as functional selectivity.

In Vivo Function of 5-HT_{2C} Receptors

Activation of 5-HT_{2C} receptors is associated with a variety of behavioral responses such as hypophagia, anxiety, penile erection, hyperthermia and hypolocomotion (Koek et al., 1992). These agonist-induced behaviors are antagonized by the 5-HT_{2C} inverse agonist/antagonist SB206553 (Kennett et al.,

1994b, 1996). The 5-HT_{2C} receptor has been shown to regulate release of both mesolimbic and mesocortical dopamine (DA). Activation of 5-HT_{2C} receptors decreases DA release, the inverse agonist/antagonist SB206553 increases DA release (Alex and Pehek, 2007; De Deurwaerdere et al., 2004; Filip and Cunningham, 2003; Navailles et al., 2003). 5-HT_{2C} receptor contributions have also been examined in genetically modified mice unable to express functional 5-HT_{2C} receptors. These mice display hyperphagia, obesity and an increased propensity for convulsions (Tecott et al., 1995). There is also evidence for increased compulsive behavior (Chou-Green et al., 2003a), changes in sleep behavior (Frank et al., 2002) and in the response to stress (Chou-Green et al., 2003b; Akana, 2008) in 5-HT_{2C} receptor null mice. In addition, the 5-HT_{2C} receptor null mice display a hyperactivity phenotype, as well as a decline in the energy cost of locomotor activity thought to contribute to the adult onset obesity (Nonogaki et al., 2003).

Genetic Variation of 5-HT_{2C} Receptors

The HTR2C gene (Figure 6) encodes for the 5-HT_{2C} receptor. As noted above It is X-linked (human chromosome Xq24; mouse chromosome X D-F4), meaning that males possess a single maternal allele. 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. 5).

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. Such polymorphisms may produce an unstable conformation of the protein, resulting in retention in the endoplasmic reticulum and decreased plasma membrane expression (Wenkert et al., 1996). Alterations in the amino acid structure may also have significant 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 ligands, both endogenous and exogenous, 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, as well as other intracellular regions, 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. 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. Gender can be a factor for polymorphisms that occur on genes of the X and Y chromosomes. SNPs and other mutations that occur in this region may have a gene 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 occur among males. Additionally, the X-chromosome also undergoes the phenomena of X-chromosome inactivation – the silencing of one copy of the X-chromosome. X-chromosome inactivation will not change the genotypic frequency of a polymorphism in females, but it can alter the phenotypic consequence of variability of the silenced gene.

Coding SNPs of the 5-HT_{2C} receptor

Two SNPs have 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. 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 codon 4 (Figure 7).

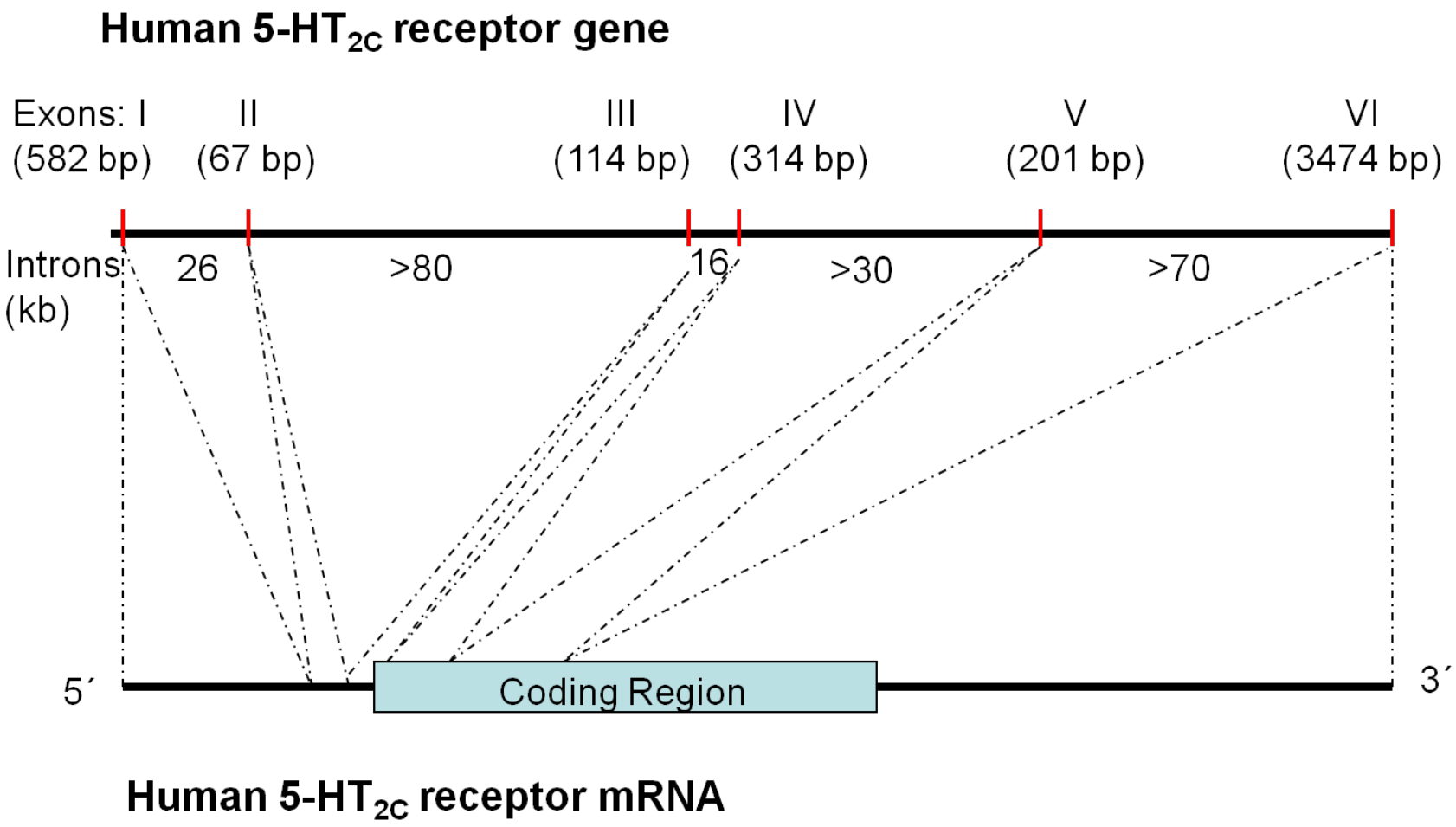

Figure 6: Human 5-HT$_{2C}$ Receptor Gene and Mrna Structure

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

What further separates the 5-HT_{2C} receptor from its cohorts is RNA editing; the 5-HT_{2C} receptor is the only known GPCR to undergo post-transcriptional pre-mRNA editing. First documented in 1986 (Benne et al., 1986), RNA editing describes a cellular process, other than splicing, that results in an mRNA sequence different than that

designated by its DNA (Gott and Emeson 2000). This process involves either modification, deletion or insertion of nucleotides at distinct editing sites. RNA editing of the $5HT_{2C}$ receptor is carried out by a family of three enzymes called Adenosine Deaminases that Act on RNA (ADARs) which convert adenosines to inosines, hence altering the genetic code. In the 5-HT_{2C} receptor this takes place at 5 adenosine residues, or editing sites termed A, B, C, D and E (Burns et al. 1997; Niswender et al., 1999). These adenosines are located within codons 157, 159, and 161 in the mouse (Du et al., 2006), which code for isoleucine, asparagine, and isoleucine (INI), respectively. Editing by ADARs results in 24 possible protein isoforms of the 5-HT_{2C} receptor, ranging from 5-$HT_{2C\text{-}INI}$, the unedited isoform, to 5-$HT_{2C\text{-}VGV}$, the fully edited isoform (Figure 8). Variation in 5-HT_{2C} receptor RNA editing has been implicated in a variety of neuropsychiatric disorders, including depression, bipolar disorder and schizophrenia (Dracheva et al., 2008a; Dracheva et al., 2003; Dracheva et al., 2008b; Gurevich et al., 2002; Iwamoto and Kato, 2008; Iwamoto et al., 2005; Niswender et al., 2001; Sodhi et al., 2001). The most consistent and reproducible finding is an increase in 5-HT_{2C}

receptor mRNA editing found post-mortem in the prefrontal cortex of suicide victims (Table 2). Although editing is decreased in the prefrontal cortex of persons with schizophrenia, this is less robust and not reproducible.

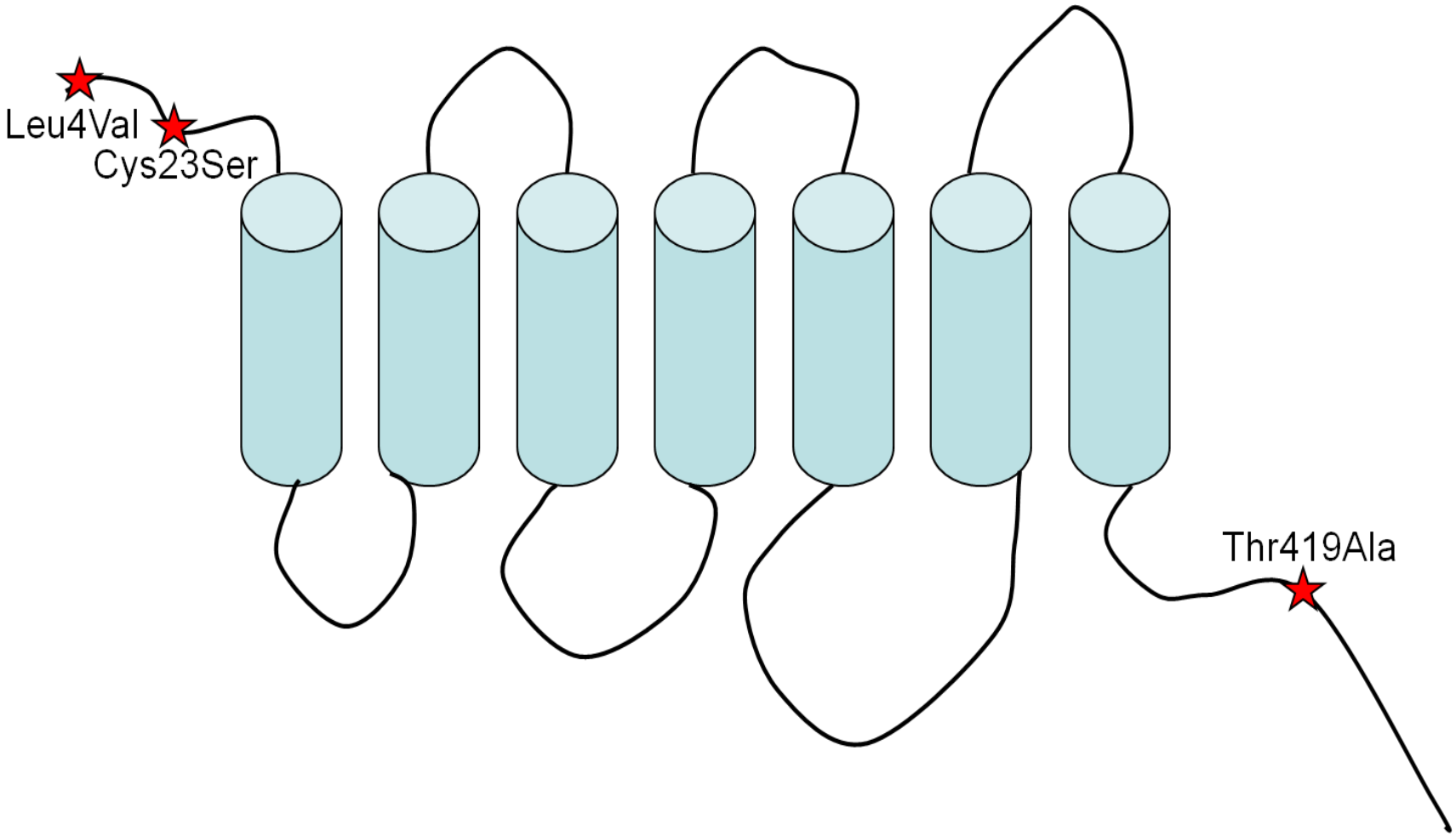

Figure 7: 5-HT$_{2C}$ Receptor Amino Acid Mutations

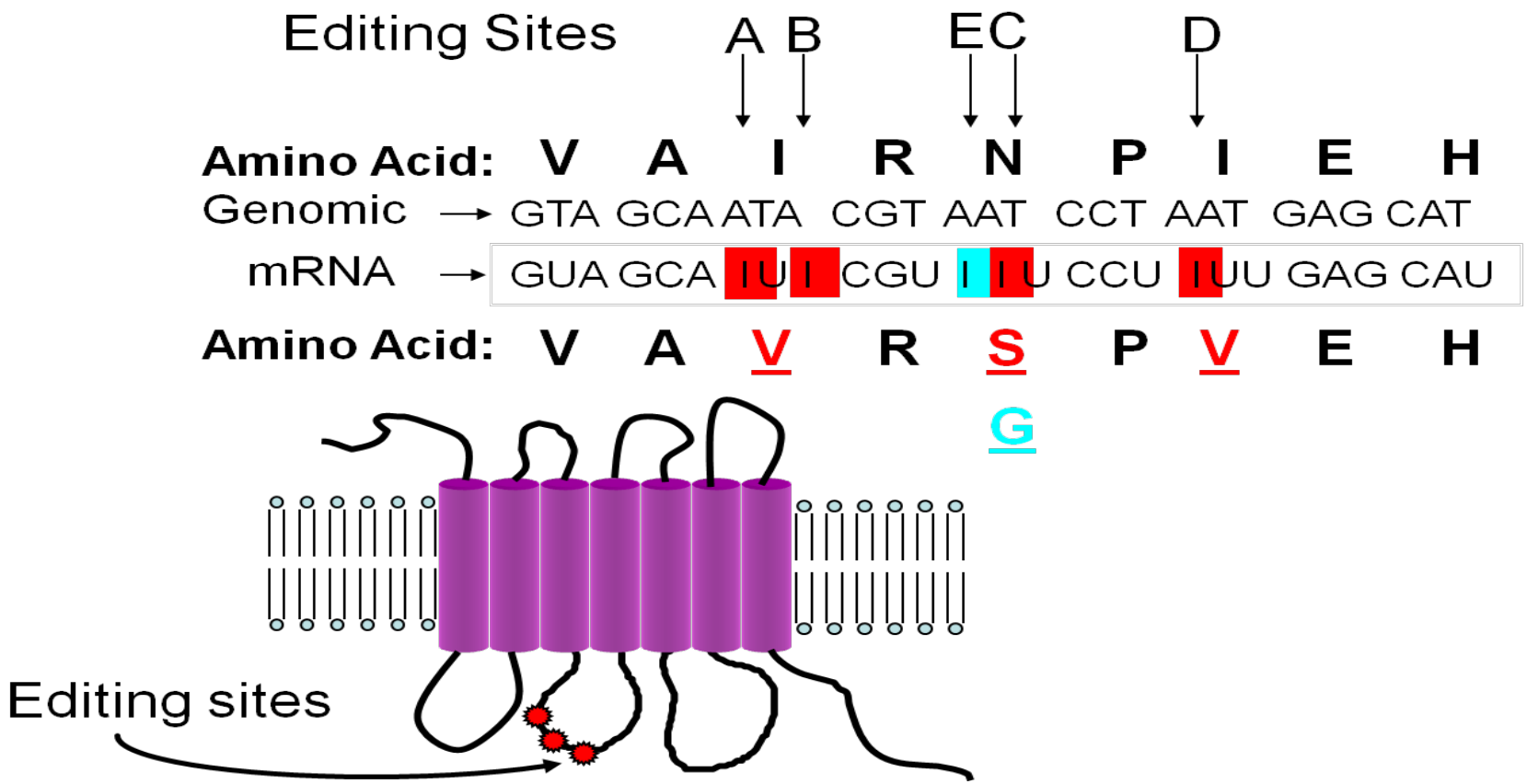

Figure 8: 5-HT$_{2C}$ Receptor Editing Sites.

The genomic and edited mRNA sequences and their corresponding amino acid sequences are shown. Editing sites are highlighted in red and blue. The receptor schematic highlights the editing region in the second intracellular loop.

Distribution of 5-HT_{2C} Receptor mRNA Isoforms

While it is not possible to selectively label individual protein isoforms, it is possible to quantify the proportion of individual mRNA isoforms in the brain. In mice, the most abundant isoform is VNV isoform which accounts for approximately 40% of the total $5HT_{2C}$ receptor mRNA, the VSV isoform accounts for ~20% (Figure 9). Those two isoforms along with INI, INV, VNI, and VSI account for 80 to 90% of the mRNA. The remaining isoforms are rarely expressed, being observed at 0-3%. There are however, considerable strain differences in the editing profiles of mice (Englander et al., 2005; Hackler et al., 2006). In the human brain the VSV isoform is the most abundant in terms of mRNA at 38% percent (Niswender et al., 2001). This is significant considering that the VSV has been shown to result in reduced functionality of the receptor in cell culture (Niswender et al., 1999).

Effects of 5-HT_{2C} Receptor Editing *in vitro*

A decade ago, Colleen Niswender a graduate student in the laboratory of Ron Emeson at Vanderbilt, examined the effects of 5-HT_{2C} receptor editing on receptor function in cell culture. Her work demonstrated that editing occurring at sites that encode for the second intracellular loop of the 5-HT_{2C} receptor, has the potential to disrupt G-protein coupling and therefore signaling downstream of 5-HT_{2C} receptor activation. Her work and the work of others shows that the VSV isoform has four-fold reduced constitutive activity and a four-fold reduction in

Table 2: Evidence for 5-HT_{2C} Receptor RNA Editing in Human Brain Disorders

Postmortem association studies of 5-HT_{2C} mRNA editing and various neurological disorders. Results are highly variable, however, the most consistently reproducible finding is increased editing in suicide victims regardless of associated neurological disorder.

Disorder	Editing Pattern	Reference
Schizophrenia	No Change	Dracheva et al., 2003
Schizophrenia	Decreased B editing; increase INI isoform	Sodhi et al., 2001
Schizophrenia / Depression with suicide	Increased editing at A&D editing	Niswender et al., 2001
Suicide with mood disorders	Increased E, decreased D site editing	Gurevich et al., 2002
Suicide	Increased editing	Dracheva et al., 2008

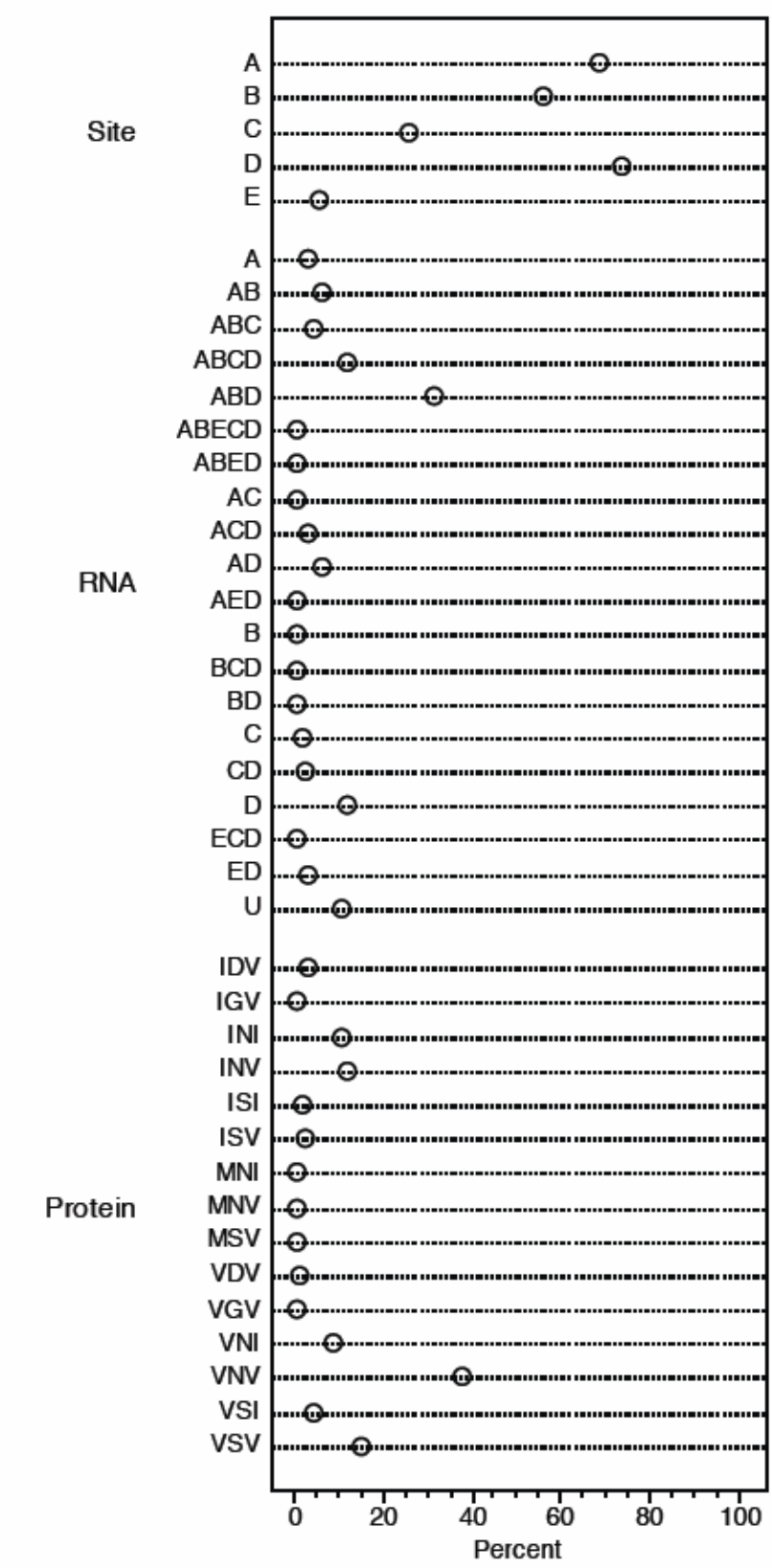

Figure 9: 5-HT$_{2c}$ Mrna Isoform Distribution In JAX 129S1 Mice.

Editing site (top), RNA (middle), and protein (bottom) isoform distribution in 129 mice.

Courtesy of David Airey, Vanderbilt University

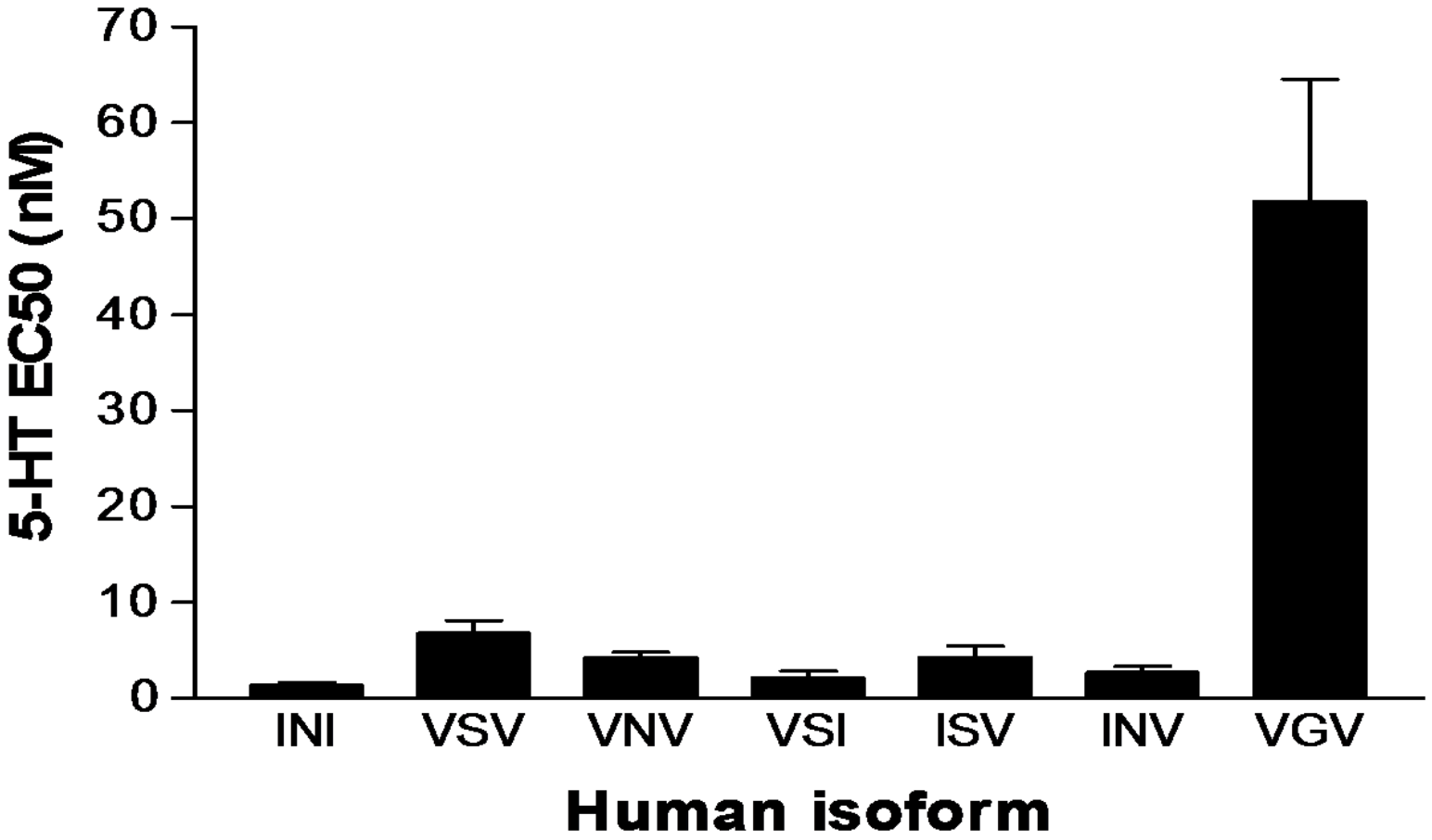

Figure 10: RNA Editing Changes EC_{50} Values for PI Hydrolysis

EC_{50}, effective concentration at 50% response; PI, Phosphoinositide (Niswender et al., 1999).

potency to initiate intracellular signaling cascades relative to the unedited INI isoform (Figure 10; Niswender et al.,1999; Wang et al., 2000; see also Burns et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Price and Sanders-Bush 2000; Visiers et al., 2001; Berg et al., 2001;Price et al., 2001; McGrew et al., 2004). Furthermore the fully edited human VGV isoform shows a 40-fold decrease in 5-HT potency (Figure 10) and a complete loss of constitutive activity of the receptor, which is an indirect measure of G-protein coupling efficiency. Niswender et al. (1999) also showed that 5-HT_{2C} receptor editing eliminates agonist high affinity binding, and inferred that G-protein coupling is compromised. The high affinity state of the receptor is the conformation that occurs after the dissociation of GDP and prior to the association of GTP (R*, Figure 11). GTP analogs eliminate the preferential binding of 5-HT to the high affinity state of the receptor as illustrated in figure 12a. This effect is lost in 5-$HT_{2C\text{-}VGV}$ receptors. The curves for 5-$HT_{2C\text{-}INI}$ receptors show two binding sites, brought to light by the addition of Gpp(NH)p, while only a single low affinity state exists in the cell line expressing 5-$HT_{2C\text{-}VGV}$ receptors(Figure 12b).

5-HT_{2A} Receptor vs. 5-HT_{2C} Receptor

While the 5-HT_{2C} and 5-HT_{2B} receptors remain largely indistinguishable pharmacologically, numerous studies have elucidated the opposing relationship of 5-HT_{2A} and 5-HT_{2C} receptors in a variety of physiological responses such as locomotion, impulsivity, and dopaminergic modulation despite their 51% amino acid homology (Winstanley et al., 2004; Fletcher et al., 2007; Robinson et al.,

2008; Halberstadt et al., 2009). A number of radioligands are available for each receptor, two of the more commonly used antagonist ligands are [^{3}H]-ketanserin, a 5-HT$_{2A}$ receptor antagonist, and [^{3}H]-mesulergine, a 5-HT$_{2C}$ receptor antagonist. Both ligands are often used in membrane binding and autoradiographic studies, but conditions must be adjusted to mask binding to the other site. [^{125}I]-DOI is an agonist radioligand with relatively equal affinity for both receptors and assays using this ligand must be further differentiated using a selective antagonist. There are a few truly selective antagonist ligands for the 5-HT$_{2A}$ receptor, and fewer still for the 5-HT$_{2C}$ receptor (Table 3). To further complicate differentiation of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, the selectivity of drugs may vary from species to species, a complication often ignored.

Specific Aims

Given the wide variety of processes the 5-HT$_{2C}$ receptor modulates and evidence for changes in editing in affective disorders, examining the impact of 5-HT$_{2C}$ receptor editing *in vivo* is of great importance. With that goal in mind the objectives of my project were to:

1. Establish conditions for specifically measuring 5-HT$_{2C}$ receptors in mice

2. Predictably manipulate 5-HT$_{2C}$ receptor mRNA editing in a single mouse strain

3. Evaluate *in vivo* functional consequences of 5-HT_{2C} receptor RNA editing at the level of receptor signaling and secondarily using biochemical and behavioral measures.

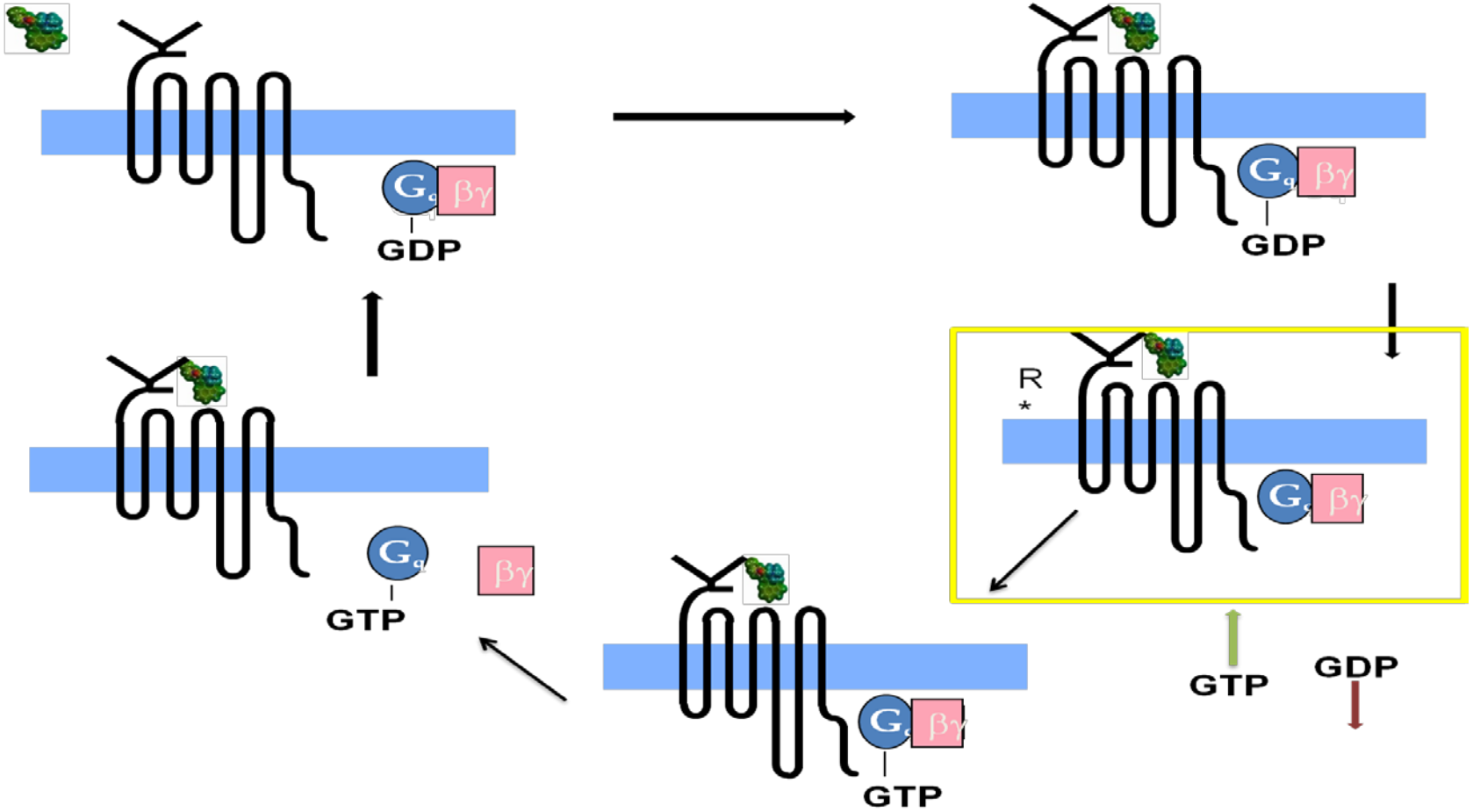

Figure 11: Agonist Activation of 5-HT$_{2c}$ Receptors

Different conformations of the 5-HT$_{2C}$ receptor highlighting the R* conformation with both agonist and G-protein bound to the receptor. We define this as the high affinity state of the receptor.

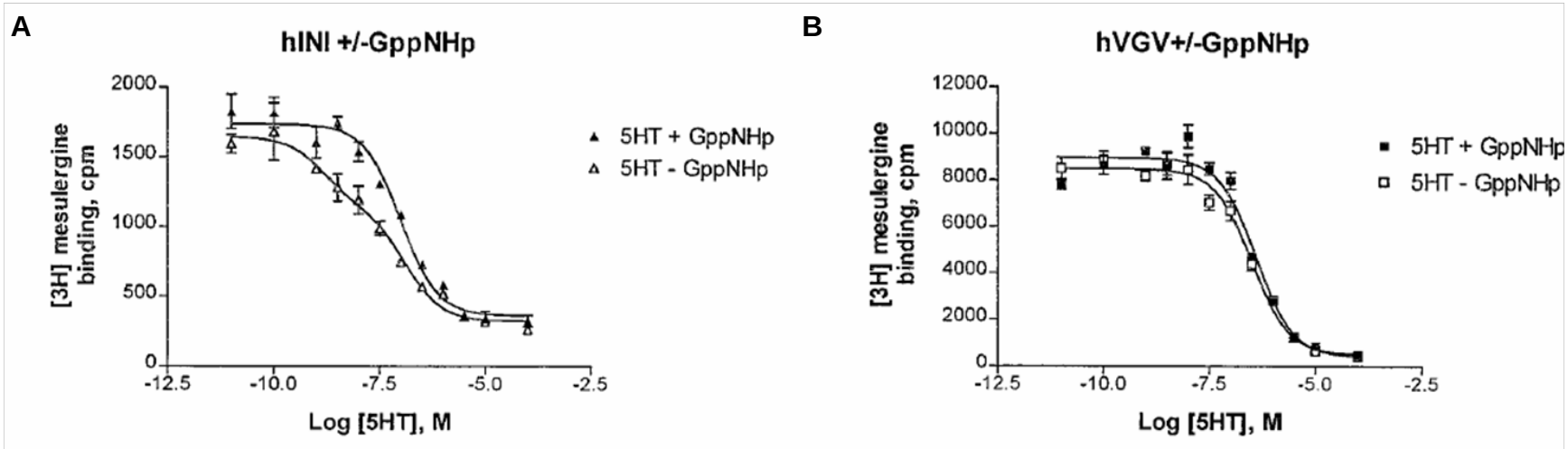

Figure 12. 5-HT$_{2C}$ Editing Eliminates Agonist High Affinity Binding

5-HT affinity for human edited 5-HT$_{2C}$ receptors and modulation by the GTP analogue Gpp(NH)p. Competition binding analyses are shown for 5-HT$_{2C\text{-}INI}$ (*A) and 5-HT$_{2C\text{-}VGV}$ (B) receptor cell lines in the presence (closed symbols) and absence (open symbols) of 100 µM Gpp(NH)p.* Increasing concentrations of 5-HT were incubated with [^{3}H]mesulergine (From Niswender et al., 1999)

Table 3
Affinity (K_i nM) of Various 5-HT_2 Ligands at Human Receptors

	5-HT_{2A}	**5-HT_{2B}**	**5-HT_{2C}**
5-HT_{2A} receptor			
Spiperone (antagonist)	1.59	>1,000	884
M100907 (antagonist)	0.84	261	99.7
Ketanserin (antagonist)	1.13	911	88.1
5-HT_{2B} receptor			
5-MeOT(agonist)	392	8.46	52.4
α-Methyl-5-HT	13	10.1	2.69
SB204741(antagonist)	>10,000	94.9	>2,000
5-HT_{2C} receptor			
SB242084 (antagonist)	851	144	0.478
RS102221 (antagonist)	>1,000	>1,000	2.64
RO60-0175 (agonist)	446	4.29	26.9
WAY629 (agonist)	>2,000	N/A	56
5-$HT_{2B/2C}$ receptors			
SB200646 (antagonist)	>5,000	31.6 (rat)	100
mCPP (agonist)	142	30.3	147
SB221284 (antagonist)	549	2.45	2.18
SB206553 (antagonist)	>1,000	5.49	7.59
MK212 (agonist)	>17,000(rat)	616	891
Non-Selective			
DOI (agonist)	45	20	18.6

K_i values (nM) taken from PDSP database (http://pdsp.med.unc.edu/pdsp.php). Values were calculated using competition binding vs. established standard antagonist if available; [^{3}H]-Ketanserin for 5-HT_{2A}, [^{3}H]-5HT for 5-HT_{2B}, and [^{3}H]-Mesulergine for 5-HT_{2C}. All data is from cloned human receptors except where noted. * Some ligands have significant affinity for non-serotonin receptors.

Chapter II: Ligand Specificity in Mice

Introduction

In order to examine the functional properties of the 5-HT_{2C} receptor *in vivo* and *ex vivo*, we must first be able to selectively label and activate the receptor. As discussed in Chapter I, while there are relatively selective ligands at each human receptor, the selectivity of these and most other ligands in mice has been virtually unexplored. This is disturbing considering the volume of pharmacological data generated using mice. We used competition binding to determine ligand affinities at both mouse 5-HT_{2A} and mouse 5-HT_{2C} receptors *ex-vivo*. Competition binding works by using a competing test ligand to compete with a radioligand incubated in parallel. With increasing concentrations of a test ligand, I can generate a competition curve that can be used to determine the IC50 and K_i of each test ligand. Determining ligand selectivity *in vivo* is far more difficult; we attempted to do so using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), which has been used for a few decades to block and inactivate various receptor binding sites including but not limited to: alpha-adrenergic (Adler et al., 1985; Ribas et al., 2001), dopaminergic (Meller et al, 1985; Stanwood et al., 2000) and serotonergic (Battaglia et al., 1987; Gozlan et al., 1994; Vinod et al., 2001). The irreversible blockade occurs in two steps. The first step consists of EEDQ

reacting with a carboxylic group in the receptor protein, forming a mixed anhydride corresponding to an activated ester (Figure 16). In the second step, this intermediate can react with any nucleophillic group in its vicinity to form an irreversible bond (Martel et al., 1969; Meller et al., 1988). Even though it has no chemical specificity EEDQ does not affect all receptor types, for example mouse 5-HT_{2A} receptors are more sensitive to EEDQ inactivation than mouse 5-HT_{2C} receptors (my observations), and D2 receptors are more sensitive than D1 (Cameron and Crocker, 1988).

Materials and Methods

Animals

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Naive male Jax® 129S1 mice, 3 to 6 months of age, were used for all experiments. Mice were housed in groups of 3 to 5 in a temperature controlled colony room (ambient temperature 22-23°C, 12:12 light: dark cycle). Food and water were available ad libitum, and all testing occurred between 1200 and 1700 during the light phase.

Drugs and Radioligands

SB206553 hydrochloride [3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2-b:4,5-b']di pyrrole-1(2H)-carboxamide hydrochloride] and spiperone hydrochloride [8-[4-(4-fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazas piro[4,5]decan-4-one hydrochloride] were purchased from Tocris Bioscience (Ellisville, MO, USA) and dissolved in deionized water. M100907 [α-(2,3-dimethoxyphenyl)-1-(2-(4-fluorophenylethyl))-4-piperidine methanol] was a gift from Marion Merrill-Dowell (Cincinnati, OH) and was dissolved in 0.02% tartaric acid. All drugs were administered subcutaneously (s.c.) at a volume of 10ml/kg.

Radioligand Binding

Mice were anesthetized using isoflurane and decapitated. Brains were removed, placed in a slicing mold, and sliced coronally rostral to the optic chiasm. Tissue anterior to the cut was homogenized in 20ml of ice cold binding buffer (50mM Tris base, 10mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4). Homogenates were centrifuged at 15,000 x g for 20 min. at 4°C. The resulting pellet was re-suspended in fresh binding buffer and incubated at 37°C for 15 minutes, then centrifuged a second time. The final pellet was re-suspended in sufficient binding buffer to yield a protein concentration 0.6 to 1mg/ml.

Competition binding was performed with 4nM [^{3}H]-mesulergine in the presence of increasing concentrations of an unlabeled competitor at 37°C for 60 min. IC_{50} values were determined by fitting data to a sigmoidal curve with

variable slope using GraphPad Prism® (GraphPad Software, Inc., USA). The procedure for 5-HT_{2A} receptor competition binding was identical with the following exceptions; binding was carried out in membranes derived from 5-HT_{2C} receptor knock-out mice on a C57B6 background and binding sites were labeled with 4nM [^{3}H]-ketanserin. IC_{50} values were converted to K_i using the transformation of Cheng-Prusoff (Cheng and Prusoff, 1973).

5-HT_{2C} receptor binding assays were run on the same day as the membrane preparation to avoid a decrease in signal due to freezing. Assays were carried out in triplicate and incubated for 90 min in a 37°C water bath at final volume of 600 µl, consisting of 500 µl of membrane suspension (300-500 µg protein), 50 µl of radioligand, and 50 µl of antagonist to mask alternative binding sites as appropriate. Non-specific binding was determined in the presence of 100 µM methysergide. Reactions were terminated by the addition of ice-cold Tris buffer (50 mM tris base pH 7.4 at 25°C), and membranes were collected by vacuum filtration (Brandel harvester) using Whatman GF/B glass fiber filters presoaked in 0.3% polyethylenimine. Filters were rinsed 3 times with cold Tris buffer, and the bound radioactivity was determined by liquid scintillation spectrometry after soaking overnight in scintillation cocktail (Aquasol-2, PerkinElmer, Boston, MA USA). Data were analyzed using nonlinear regression (GraphPad Prism, Graphpad Inc., USA). Total density for EEDQ assay was estimated in each membrane preparation using a single saturating concentration of 5nM [^{3}H]-Ketanserin.

EEDQ Receptor Inactivation Protection Assay

SB206553 (2, 3, 4, and 6mg/kg, 10ml/kg) and M100907 (0.25mg/kg, 10ml/kg) were administered subcutaneously (s.c.) 30 minutes prior to EEDQ. EEDQ was dissolved in 20% ETOH and administered s.c. at 10mg/kg and 10ml/kg. Animals were left in home cages and sacrificed 24 hours later (Figure 17). Single concentration binding assays were conducted as previously described.

Results and Discussion

Competition Binding

K_i values were determined for spiperone, M100907, and SB206553 at both the 5-HT_{2A} and 5-HT_{2C} receptors (Table 4). Unlike in human and rat derived membranes, M100907 was not as selective for 5-HT_{2A} receptor in mouse tissue. Based on the competition curves it became obvious that we could not eliminate all [^{3}H]-ketanserin binding to 5-HT_{2A} receptor using M100907, without interfering with the 5-HT_{2C} receptor binding signal (Figure 13). We turned to spiperone, which while toxic *in vivo,* served our needs *in vitro*. 1µM Spiperone added to our binding buffer blocks all [^{3}H]-ketanserin 5-HT_{2A} receptor binding sites, while eliminating no [^{3}H]-mesulergine binding to 5-HT_{2C} receptors (Figure 14). 10µM SB206553 allowed us to eliminate [^{3}H]-mesulergine binding to 5-HT_{2C} receptors

without diminishing [^{3}H]-ketanserin binding to 5-HT$_{2A}$ receptors (Figure 15). Masking antagonists as determined in competition binding were added to buffer solution for saturation binding experiments: 1 µM spiperone to block 5-HT$_{2A}$ receptor; 10 µM SB206553 to block the 5-HT$_{2C}$ receptor. We used these ligand concentrations, as appropriate, in our binding buffers in all subsequent binding experiments.

EEDQ Inactivation Protection Assay

Although 10 mg/kg EEDQ very effectively inactivated 77% of 5-HT$_{2A}$ receptors (Figure 18), it was ineffective at the 5-HT$_{2C}$ receptor (Figure 19). As a result, while we could not determine the *in vivo* selectivity of M100907, we have clearly established 3mg/kg of SB206553 as selective for 5-HT$_{2C}$ receptor over 5-HT$_{2A}$ receptors in JAX 129S1 mice. Doses of 3 and 4 mg/kg of SB206553 failed to protect 5-HT$_{2A}$ receptors from EEDQ inactivation (Figure 20).

Predictably Manipulating 5-HT$_{2C}$ receptor mRNA editing

We initially attempted to manipulate 5-HT$_{2C}$ mRNA editing using the SSRI fluoxetine in the same manner as Englander et al. (2005), but were unsuccessful (data not shown). Fortunately we were able to make use of transgenic mice created by the Emeson group at Vanderbilt University that solely express the fully edited 5-HT$_{2C\text{-}VGV}$ isoform of the receptor.

Ligand	Species					
	Mouse		**Human**		**Rat**	
	$5\text{-}HT_{2A}$	$5\text{-}HT_{2C}$	$5\text{-}HT_{2A}$	$5\text{-}HT_{2C}$	$5\text{-}HT_{2A}$	$5\text{-}HT_{2C}$
M100907	2.15±0.42	93±4.35	0.84	99.72	1.92	104.07
Spiperone	3.37±0.31	3676 ±340	1.59	884	1.07	1700
SB206553	15000±5000	139.5±19.2	1659	7.59	N/A	N/A

Table 4. $5\text{-}HT_2$ Ligand Affinities Across Species

K_i (± S.E.M) for M100907, Spiperone, and SB206553 in mouse, human, and rat tissue. K_i were determined using radioligand competition binding assays. K_i data for human and rat are courtesy PDSP K_i database (http://pdsp.med.unc.edu/indexR.html). Each mouse data point represents the average of 4 experiments performed in triplicate.

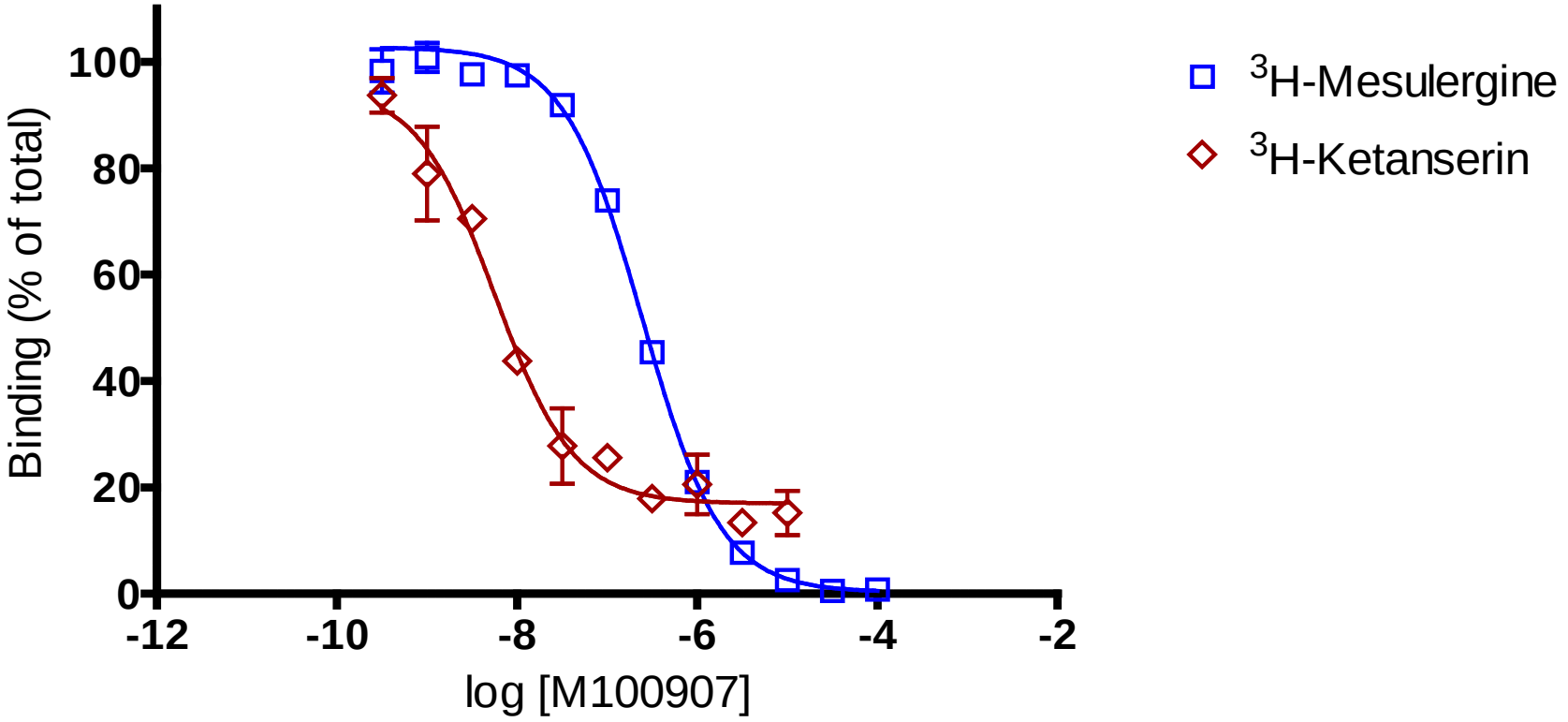

Figure 13. M100907 Competition Binding

Representative plots of increasing concentrations of M10097 versus constant concentrations of [^{3}H]-Mesulergine and [^{3}H]-Ketanserin.

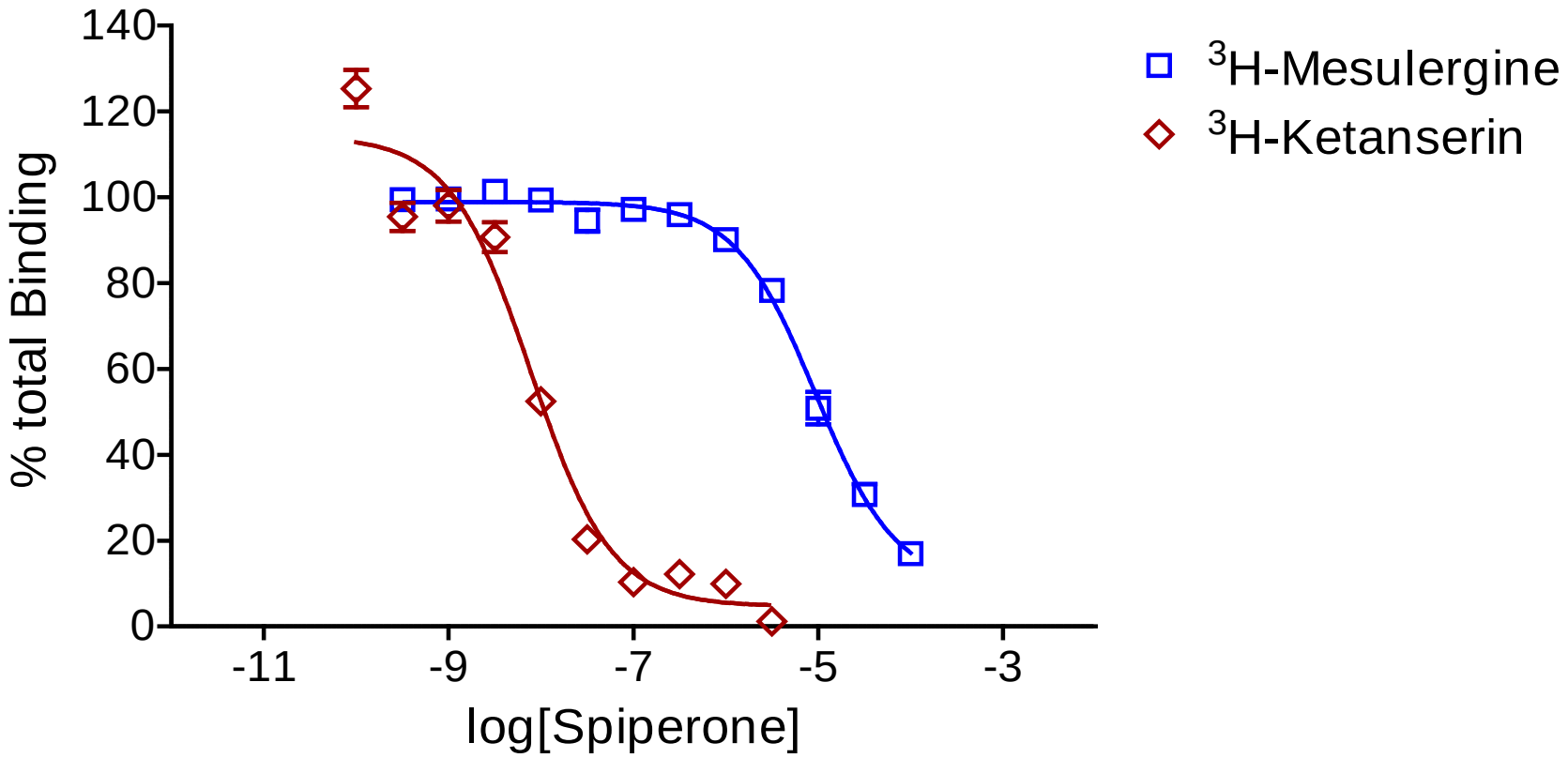

Figure 14. Spiperone Competition Binding

Representative plots of increasing concentrations of Spiperone versus constant concentrations of [^{3}H]-Mesulergine and [^{3}H]-Ketanserin.

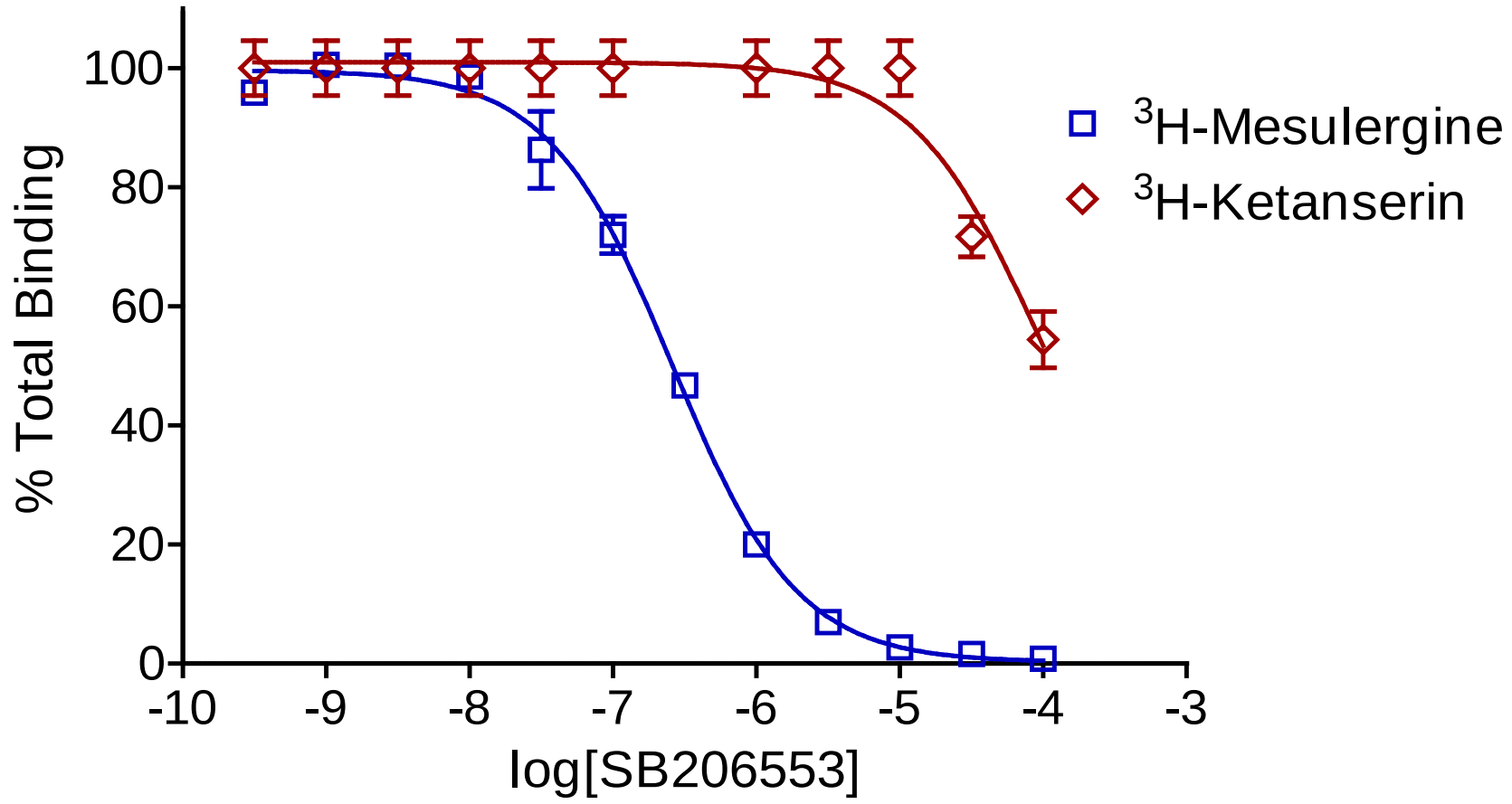

Figure 15. SB206553 Competition Binding

Representative plots of increasing concentrations of SB206553 versus constant concentrations of [^{3}H]-Mesulergine and [^{3}H]-Ketanserin.

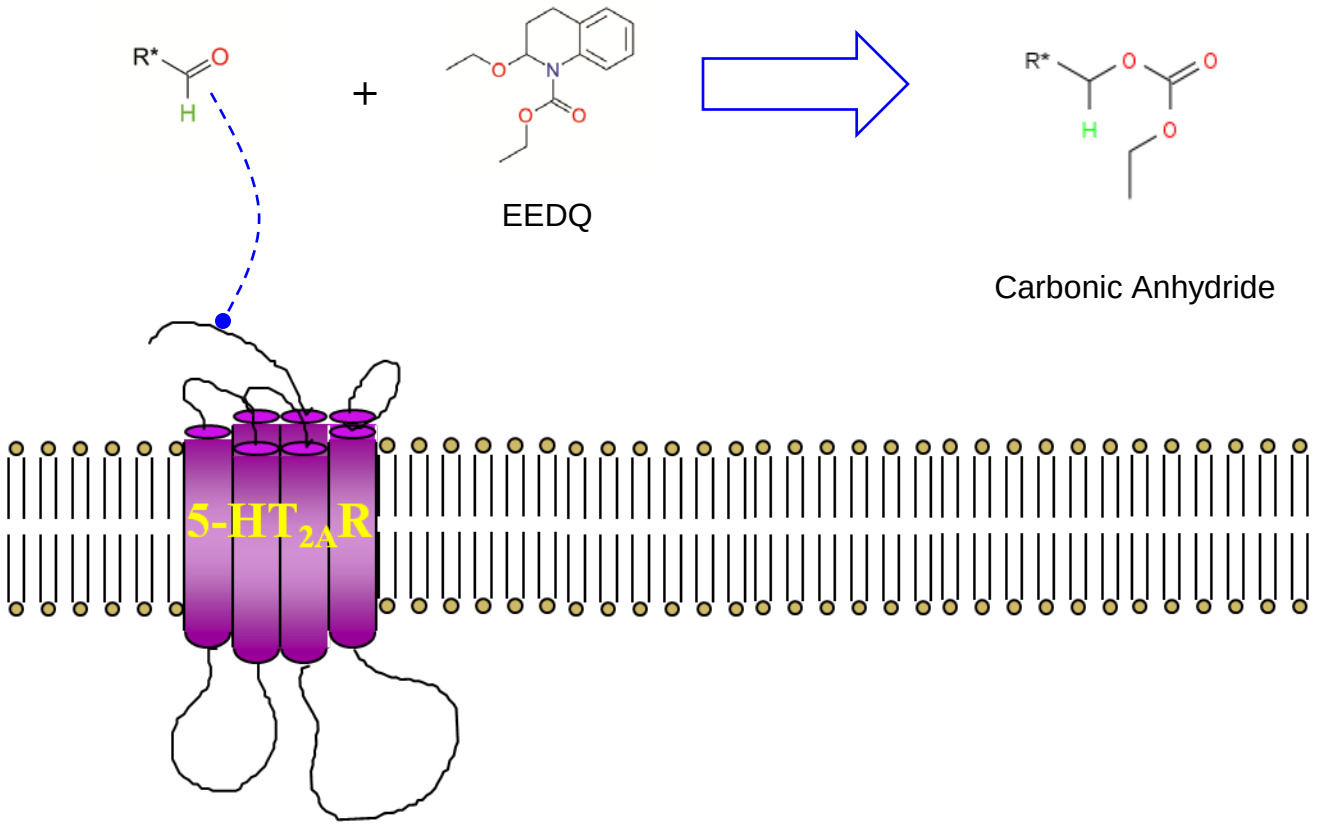

Figure 16. EEDQ Irreversibly Inactivates 5-HT_{2A} Receptors

EEDQ reacts with a carboxyl group important for receptor activation, which is most likely located at or near the active binding site of the 5-HT_{2A} receptor. This reaction produces a highly reactive mixed carbonic anhydride which then reacts with any nucleophilic group to form an irreversible bond. (See Chang et al. (1970) for chemical details).

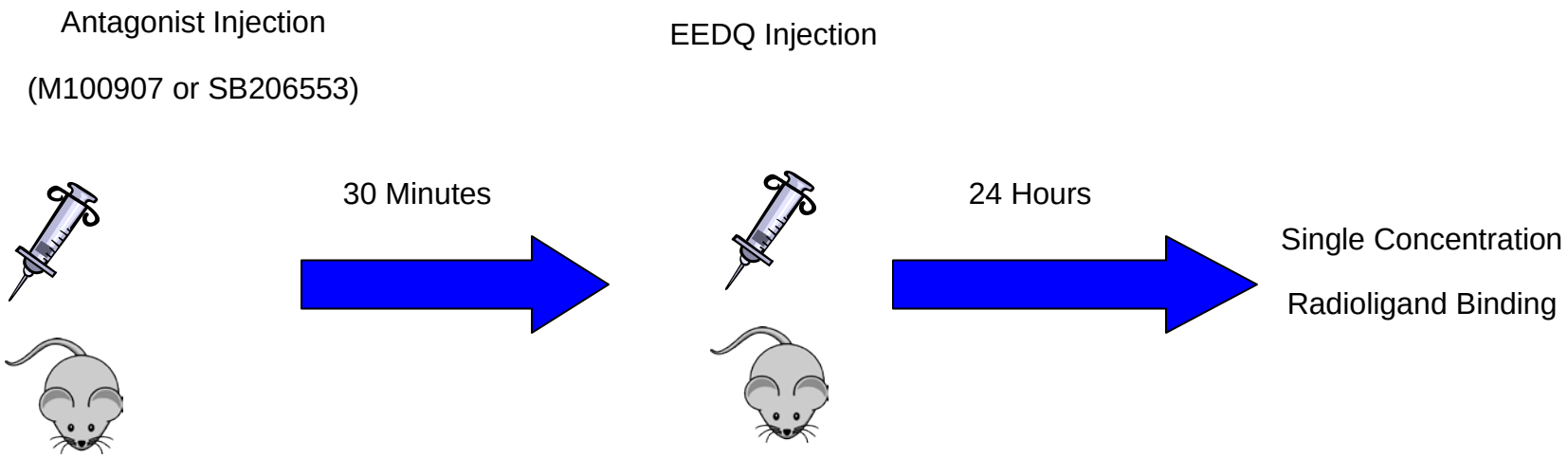

Figure 17. EEDQ Protection Assay Methods

Mice were injected with either the 5-HT_{2A} antagonist M100907 (0.25mg/kg) or the 5-HT_{2C} antagonist/inverse agonist SB206553 (3mg/kg). 30 minutes later animals were injected with 10mg/kg EEDQ. 24 hours after EEDQ injections animals were sacrifice and single concentration antagonist radioligand binding was assayed the same day.

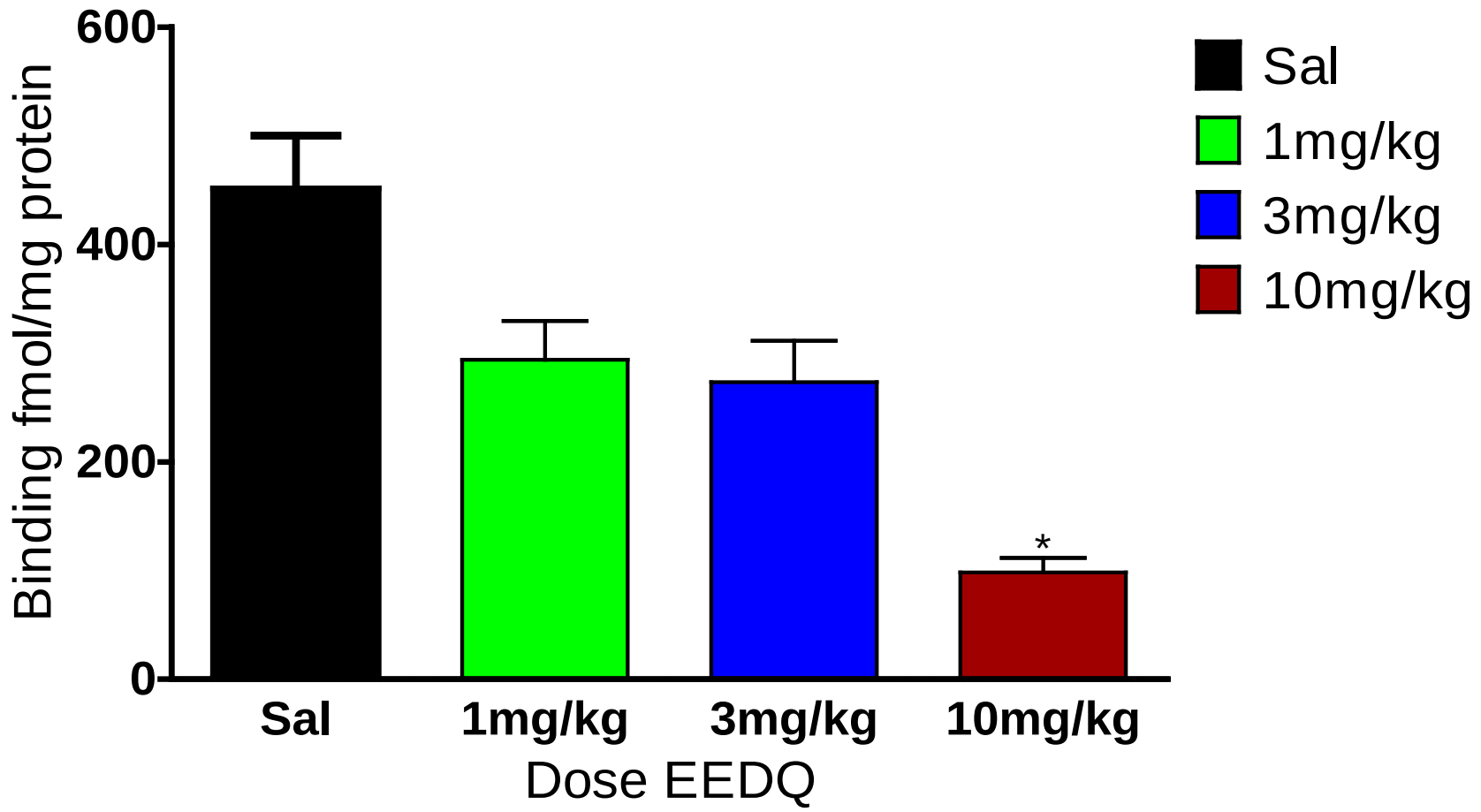

Figure 18. EEDQ Dose Response at 5-HT$_{2A}$ Receptors

[^{3}H]-Ketanserin binding 24 hours after 1, 3, and 10 mg/kg EEDQ administration (S.C.). 10mg/kg EEDQ eliminated 77% of control [^{3}H]-ketanserin binding. ***** $F(3,8) = 10.93$ $p<0.01$.

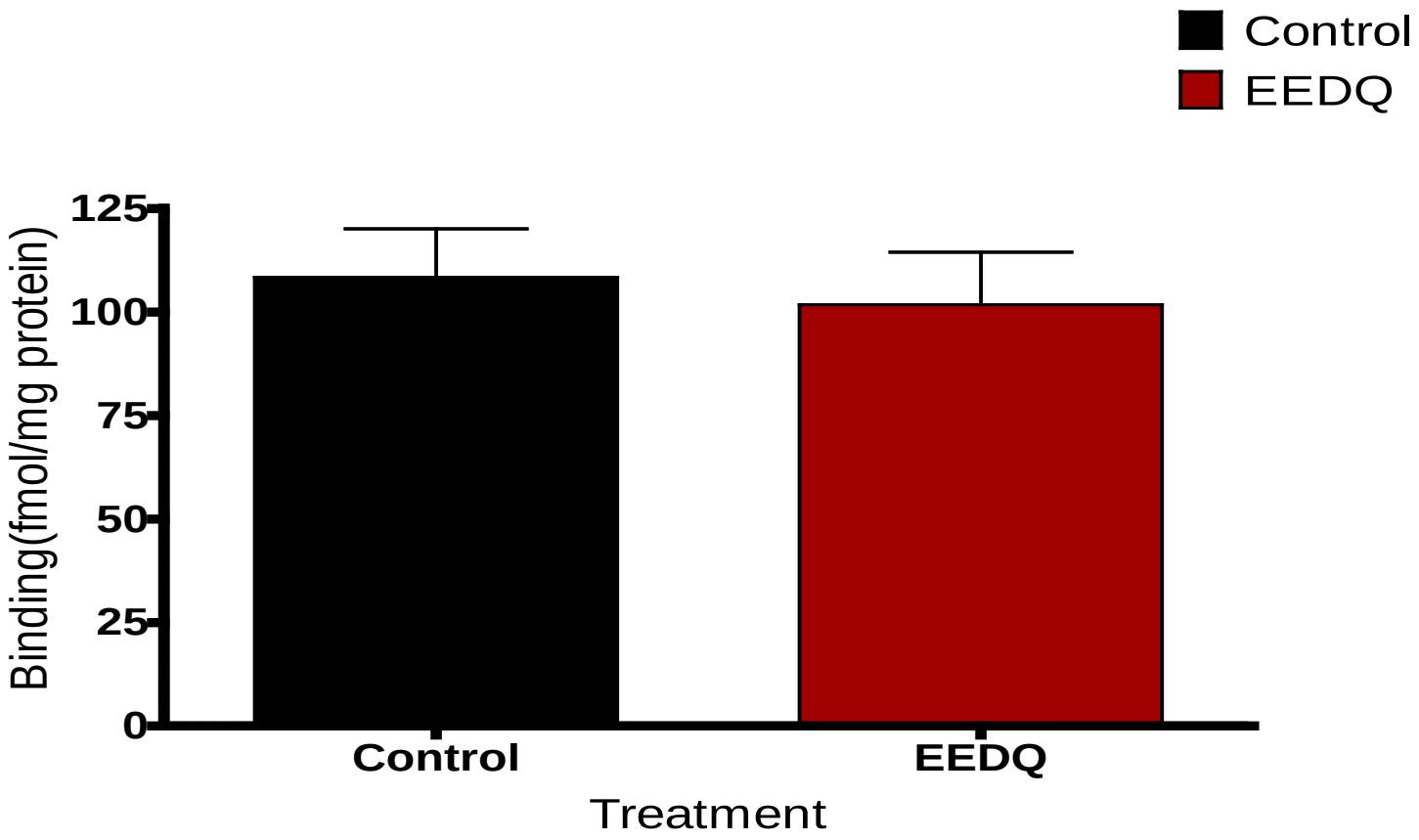

Figure 19. EEDQ is Ineffective at 5-HT_{2C} Receptors

10 mg/kg was ineffective in reducing [^{3}H]-mesulergine binding in mouse derived membranes.

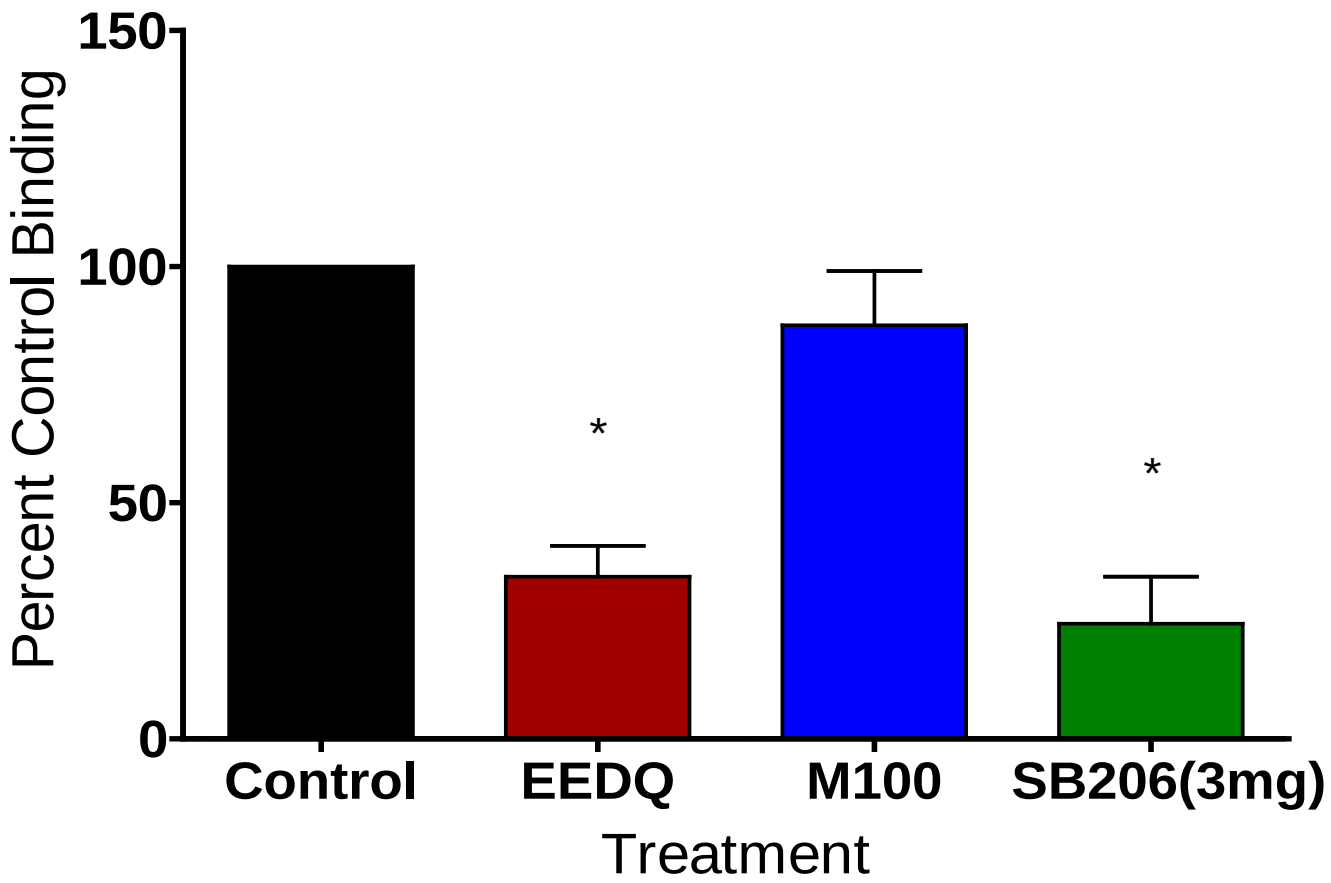

Figure 20. SB206553 does not occupy 5-HT$_{2A}$ Receptors

[^{3}H]-ketanserin binding 24 hours after EEDQ and 30 minute pretreatment with M100907 (0.25mg/kg) or SB206553 (3mg/kg) followed by EEDQ administration. M100907 completely blocks the effect of EEDQ, while SB206553 has no effect on EEDQ inactivation of 5-HT$_{2A}$ receptors.

Chapter III: *In Vivo* Function Of the 5-$HT_{2C\text{-}VGV}$ Receptor

Introduction

The serotonin 2C ($5HT_{2C}$) receptor modulates a number of neurophysiological functions including appetite, mood, sexual behavior, and locomotion (Buhot, 1997; Giorgetti and Tecott, 2004; Millan, 2005; Olivier et al., 1998), and is implicated in the etiology of psychiatric disorders such as depression, anxiety, anorexia-nervosa, and obsessive-compulsive disorder (Berg et al., 2008b; Delgado and Moreno, 1998; Flaisher-Grinberg et al., 2008; Griebel, 1995). Consequently, the $5HT_{2C}$ receptor has received considerable attention as a target for pharmacological treatment of these conditions (Halford et al., 2007; Meltzer et al., 2003; Millan, 2005; Morabito and Emeson, 2009; Serretti et al., 2004).

A distinctive feature of the G-protein coupled $5HT_{2C}$ receptor is its ability to be modified post-transcriptionally by RNA editing (Burns et al., 1997). RNA editing is an enzymatic process that converts adenosine to inosine at 5 nucleotide positions in the 5th exon of the $5HT_{2C}$ receptor RNA transcript, encoding the second intracellular loop, a region important for G-protein coupling. Selective RNA editing can generate up to 24 protein isoforms of the $5HT_{2C}$ receptor potentially adding immense diversity to the function of the receptor *in vivo*.

In vitro studies in transfected cells expressing a single isoform show that differentially edited $5HT_{2C}$ receptor isoforms have unique signaling features, with increased editing generally leading to decreased function. For example, the edited isoform valine-serine-valine (at amino acid positions 157, 159, 161 in humans) has four-fold reduced constitutive activity and four- to five-fold reduced serotonin potency to activate phospholipase C (PLC) relative to the non-edited isoleucine-asparagine-isoleucine isoform (INI) (Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999). The function of the fully edited valine-glycine-valine (VGV) isoform is reduced even further.

Since the first report identifying and characterizing RNA editing of the $5HT_{2C}$ receptor transcripts in rat brain (Burns et al., 1997), many laboratories have observed a large repertoire of differentially edited $5HT_{2C}$ receptor RNA isoforms uniquely distributed in the mammalian brain. Recent studies using animal models suggest that RNA editing of the $5HT_{2C}$ receptor is a dynamic process. For example, chronic stress increases RNA editing of the $5HT_{2C}$ receptor in both mice (Englander et al., 2005) and rats (Iwamoto et al., 2005). Variation in $5HT_{2C}$ receptor RNA editing has also been implicated in a variety of neuropsychiatric disorders, with the most reproducible finding being an increase in RNA editing in brains of suicide victims (Dracheva et al., 2008a; Dracheva et al., 2008b; Gurevich et al., 2002; Niswender et al., 2001).

Despite these findings, defining the *in vivo* functional consequences of $5HT_{2C}$ receptor RNA editing has been limited due to the technical constraints presented by the immense diversity of receptor isoforms in brain. Here we take

advantage of mutant mice solely expressing a single isoform, the fully-edited or non-edited $5HT_{2C}$ receptor (Canal et al., 2009; Kawahara et al., 2008), to characterize for the first time the signal transduction consequences of RNA editing of the $5HT_{2C}$ receptor *in vivo*. We show that G-protein coupling to the fully edited $5HT_{2C\text{-}VGV}$ receptor in brain is nearly abolished. However, the functional consequences at the level of behavior in mice solely expressing the $5HT_{2C\text{-}VGV}$ receptor do not reproduce these signaling alterations, because of a marked increase in $5HT_{2C}$ receptor binding site density.

Materials and Methods

Animals

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Naive adult mice, 3 to 6 months of age, were used for all experiments. Mice were housed in groups of 2 to 5 in a temperature controlled, colony room (ambient temperature 22-23°C, 12:12 light: dark cycle). Food and water were available ad libitum, and all testing occurred between 1200 and 1700 during the light phase. Unless otherwise indicated, male mice were used.

VGV mutant mice were generated by the laboratory of Dr. Ronald Emeson, Vanderbilt University by introducing guanosine residues instead of

adenosine at all five edited sites (Morabito et al., 2007), and were maintained on a mixed JAX® 129S1/Taconic® 129S6 background. Four genotypes of mice were used in the experiments examining functional characteristics of the *in vivo* expressed $5HT_{2C\text{-}VGV}$ receptor: wild-type males and females (WT), heterozygous females (VGV/X), and hemizygous (the $5HT_{2C}$ receptor is X-linked) males (VGV/Y), expressing solely the VGV isoform. Heterozygous female VGV/X mice, with approximately one-half of the RNA isoforms encoding the fully edited $5HT_{2C\text{-}VGV}$ receptor, were used to assess the possible contribution of gene dosing. Using the method of pyrosequencing (Canal et al., 2009), we verified that VGV/X females had VGV as their major isoform; VGV/X females (N=4, average of 60 sequences per mouse) had an average of 62% of RNA isoforms that translate to VGV. Control mice (N=4, average of 60 sequences per mouse) had less than 1% VGV isoforms. We also verified that the increase in the percentage of the VGV isoform in VGV/X did not alter the remaining distribution of isoforms (data not shown).

INI mutant mice were created by introducing multiple mutations into intron 5 to disrupt base-pairing with exon 5 and prevent RNA editing and were maintained on a 129S6 background. Two genotypes of mice were used in the experiments examining receptor density characteristics of the *in vivo* expressed $5HT_{2C\text{-}INI}$ receptor: WT males and hemizygous mutant male (INI/Y) littermates, solely expressing the INI isoform.

The genotypes of all mice were determined by PCR analysis of genomic DNA from tail samples using the RED Extract-N-Amp tissue PCR kit (Sigma, St.

Louis, MO, USA), with the following primers: 5' GGG CAA ATA TTC TGA AAA GAT GTT 3' (reverse) and 5' AAT ATC AAT AGG TAA TTA TAC C 3' (forward).

Drugs and Radioligands

MK212 hydrochloride [6-Chloro-2-(1-piperazinyl) pyrazine hydrochloride], purchased from Tocris Bioscience (Ellisville, MO, USA), was dissolved in deionized water, and injected subcutaneously (s.c.) at a volume of 10 ml/kg. [N^6-methyl-^{3}H]-Mesulergine ([^{3}H]-mesulergine) was purchased from GE Healthcare/Amersham (Buckinghamshire, UK). (±)-1-(2,5,-dimethoxy-4-[^{125}I]iodophenyl)-2-aminopropane ([^{125}I]-DOI) was purchased from Perkin Elmer (Boston, MA, USA). Spiperone and methysergide were purchased from Sigma-Aldrich (St. Louis, MO, USA); SB204741 was purchased from Tocris.

GTP-Sensitive High-Affinity Agonist Binding In Membranes

Agonists, such as DOI, bind preferentially to $5HT_{2C}$ receptors existing in a high-affinity state, a receptor conformation that is coupled to G-protein. *In vitro*, $5HT_{2C\text{-}VGV}$ receptors show no basal coupling to G-proteins (Herrick-Davis et al., 1999; Niswender et al., 1999). Mice were briefly anesthetized using isoflurane and decapitated. Unless otherwise indicated, brains were removed, placed in a slicing mold, and sliced coronally at the level of the optic chiasm. Tissue anterior to the cut, defined here as frontal cortex, was assayed. Tissue was

homogenized in 20 ml of ice cold binding buffer (50mM Tris, 10mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4). Homogenates were centrifuged at 15,000 x g for 20 min. at 4°C. Supernatant was decanted, and the pellet was resuspended in fresh binding buffer and incubated at 37°C for 15 minutes to dissociate endogenous ligand from receptors. After a second centrifugation, supernatant was decanted, and the pellet re-suspended in fresh, cold binding buffer. Binding assays were performed on the same day as the membrane preparation to avoid a decrease in signal due to freezing.

[^{125}I]-DOI saturation binding was performed on membranes from VGV/Y and WT mice to test potential differences in the proportion of receptors in the agonist high-affinity conformation. Assays were carried out in triplicate and incubated for 90 min in a 37°C water bath at final volume of 600 µl, consisting of 500 µl of membrane suspension, 1 µM spiperone to mask 5-HT_{2A} and 5-HT_7 binding sites and increasing concentrations of [^{125}I]-DOI. Nonspecific binding was determined in the presence of 100 µM methysergide. Data were analyzed using nonlinear regression (GraphPad Prism 5.02, Graphpad Inc., USA). In a separate experiment, GTP sensitivity was evaluated using a single concentration of [^{125}I]-DOI (1 nM) in the absence and presence of 200 µM guanylyl-5′-imidodiphosphate (Gpp(NH)p), a nonhydrolyzable analog of GTP that prevents G-protein coupling. Total density was estimated in each membrane preparation using a single saturating concentration of 30nM [^{3}H]-mesulergine.

Samples were incubated for 90 min in a 37°C water bath, and the reactions were terminated by the addition of ice-cold 50 mM Tris buffer.

Membranes were collected by vacuum filtration (Brandel harvester) using Whatman GF/B glass fiber filters presoaked in 0.3% polyethyleneimine. Filters were rinsed 3 times with cold Tris buffer, and the bound radioactivity was determined by liquid scintillation spectrometry after soaking filters overnight in scintillation cocktail (Aquasol-2, PerkinElmer, Boston, MA USA).

[^{125}I]-DOI and [^{3}H]-Mesulergine Autoradiography

Mice were anesthetized with isoflurane inhalation, followed by decapitation. Brains were quickly removed and frozen in 2-methylbutane on dry ice, dried briefly with a Kimwipe (Kimberly-Clark, Roswell, GA, USA), wrapped in aluminum foil and stored at -80˚C. Brains were brought to -20˚C in a cryostat (Leica, Wetzlar, Germany), sectioned at 20 µm and thaw mounted onto Superfrost glass slides (Fisher, USA), then stored at -80˚C until the autoradiographic assay.

For [^{125}I]-DOI autoradiography, slides were thawed at room temperature for 30 min, followed by incubation for 30 min in assay buffer containing 50 mM Tris, 5 mM $MgSO_4$, 4 mM $CaCl_2$ and 0.5 mM EDTA. Slides were then transferred to assay buffer that contained one of four drug treatments: 1) 0.14 nM [^{125}I]-DOI to label $5HT_{2A}$ and $5HT_{2C}$ receptors; 2) 0.14 nM [^{125}I]-DOI plus 1 µM spiperone to identify the $5HT_{2C}$ receptor; 3) 0.14 nM [^{125}I]-DOI plus 100 µM GTPγS to identify GTP-sensitive high affinity [^{125}I]- DOI binding (Appel et al., 1990); 4) 0.14 nM

[^{125}I]-DOI plus 30 µM methysergide to define non-specific binding. Slides were incubated with radioligand at room temperature for 60 min.

[^{3}H]-mesulergine autoradiography procedures were identical to [^{125}I]-DOI autoradiography with the following exceptions. The assay buffer for [^{3}H]-mesulergine was 50 mM Tris, 10 mM $MgCl_2$, and 0.1 mM EDTA. The binding conditions were: 1) 3 nM [^{3}H]-mesulergine; 2) 3 nM [^{3}H]-mesulergine plus 1 µM spiperone; 3) 3 nM [^{3}H]-mesulergine plus 30 µM methysergide to define non-specific binding. Slides were incubated for 120 min at room temperature.

Slides were washed 4x10 min in ice-cold 50 mM Tris buffer, pH 7.4 and dried with a steady stream of dehumidified air. After air drying for an additional 90 min, slides were placed in autoradiography cassettes and exposed to Kodak Biomax MR film (Carestream Health, Inc., USA) for 24 to 48 hours for [^{125}I]-DOI or 6 to 8 weeks for ^{3}H-mesulergine prior to developing (shorter exposures were required for brain sections from VGV/Y mice to accommodate an increased density of $5HT_{2C}$ receptors). Film was scanned (CanoScan 4400F, Canon, USA) onto a PC, and images were analyzed with Image J software (Abramoff et al., 2004). The average of the minimum gray value (darkest area) from three brain slices containing brain area of interest was converted to µCi/g protein using ^{14}C standards (ARC, Inc., St. Louis, MO) for statistical comparisons of specific [^{125}I]-DOI binding, and ^{3}H standards for statistical comparisons of specific [^{3}H]-mesulergine. Data were compared using 2-tailed unpaired t-tests.

$5HT_{2C}$ Receptor Agonist Impact On Locomotor Activity In VGV/Y And VGV/X

$5HT_{2C}$ receptor agonists induce hypolocomotion in a $5HT_{2C}$- and dose-dependent manner (Fone et al., 1998; Gleason et al., 2001; Stiedl et al., 2007). Dose-response studies were conducted to assess the locomotor effects of MK212, a selective $5HT_{2C}$ receptor agonist (Fletcher et al., 2009), in WT, VGV/Y and female VGV/X. Locomotor activity was measured in Plexiglas activity chambers (11x11x11in; LxWxH; Med Associates St. Albans, Vermont), equipped with 16 infrared beams to monitor and record beam breaks in the x-y-z coordinates. VGV/Y and VGV/X mice were tested with saline and MK212 at doses of 0.01, 0.03, 0.1, 0.3 and 1.0 mg/kg. Twenty min after s.c.injection, individual mice were placed inside the activity chamber and distance traveled was recorded for 10 minutes using Activity Monitor software version 5 (Med Associates, St. Albans, Vermont, USA). Dose-response data were analyzed by two-way ANOVA for independent groups.

Biogenic amine levels

DA and 5-HT levels were quantified by previously described high-performance liquid chromatography (HPLC) electrochemical detection methods (Vanderbilt Neurochemistry Core). Animals were anesthetized with isoflurane and rapidly decapitated after cervical dislocation. Brains were removed and placed in a chilled stainless steel mold for dissection – the tissue slice 1.7 to 3.6 relative to bregma is referred to as cortex; striatum represents slice 1.70 to -0.82

relative to bregma with the cortex removed by freehand dissection. Tissue was frozen rapidly on dry ice and stored at -80°C until assay. Thawed samples were homogenized in 250µl acetonitrile and centrifuged at 13,000x g for 30 minutes. The acetonitrile fraction was transferred to a clean tube, washed twice with 125 µl heptane and then evaporated using a stream of nitrogen. The sample was suspended in 75µl of the HPLC mobile phase (37.5 mmole H_3PO_4, pH 8.5) and 50µl was injected into the equilibrated HPLC. Dopamine turnover was measured by the ratio DOPAC/DA and serotonin turnover was measured by the ratio 5HIAA/5HT. Each measure was predicted by a mixed ANOVA model that incorporated between-subject fixed effects for genotype (VGV or WT) and drug (saline or SB206553), a within-subject fixed effect for brain area (cortex or striatum), and a random effect to account for repeated measures in each mouse.

[^{3}H]-Mesulergine Saturation Binding In Membranes

The procedures for [^{3}H]-mesulergine saturation binding were essentially identical to [^{125}I]-DOI binding in membranes. Membrane homogenates, binding buffer, spiperone at a final concentration of 1µM for VGV membranes and 100 nM for INI membranes, and increasing concentrations of [^{3}H]-mesulergine up to 36 nM were added. Non-specific binding was determined in the presence of 100 µM methysergide. Frontal cortex was used for studies with VGV/Y and WT littermates. Whole brain minus cerebellum was used for studies with INI/Y and WT littermates.

Results

$5HT_{2C\text{-}VGV}$ Receptors In Brain Exhibit Undetectable Agonist High-Affinity Binding

In saturation binding assays of membranes from WT littermates, the binding of the radiolabeled agonist [^{125}I]-DOI was best fit by a two-site equation, as illustrated in the Scatchard plot (Figure 21). Approximately 10% of the binding sites were in a high affinity state (K_d=0.51nM); the density of the low affinity site (137 fmol/mg protein) was consistent with that found in [^{3}H]-mesulergine binding (see below). In contrast, [^{125}I]-DOI binding in membranes from VGV/Y mice was best fit by a one-site equation, as illustrated in the Scatchard plot (Figure 22) with a K_d (29nM) that was consistent with the K_d of the low affinity site in WT mice (20nM). The extremely high density of binding sites in VGV/Y mice was confirmed in [^{3}H]-mesulergine binding experiments (see below).

To corroborate the finding of decreased high-affinity binding sites in brains of VGV/Y mice and to estimate the percentage of G-protein coupled receptors, the binding of a single concentration of [^{125}I]-DOI binding was determined in the absence and presence of Gpp(NH)p to uncouple the receptor-G-protein complex. Approximately four percent (3.6 ±0.42; N=4) of the specific [^{125}I]-DOI binding to $5HT_{2C}$ receptors was Gpp(NH)p sensitive in membranes from WT mice (Figure 24). However, there was essentially no Gpp(NH)p-sensitive $5HT_{2C}$ receptor binding in brains of VGV/Y mice (Figure 23).

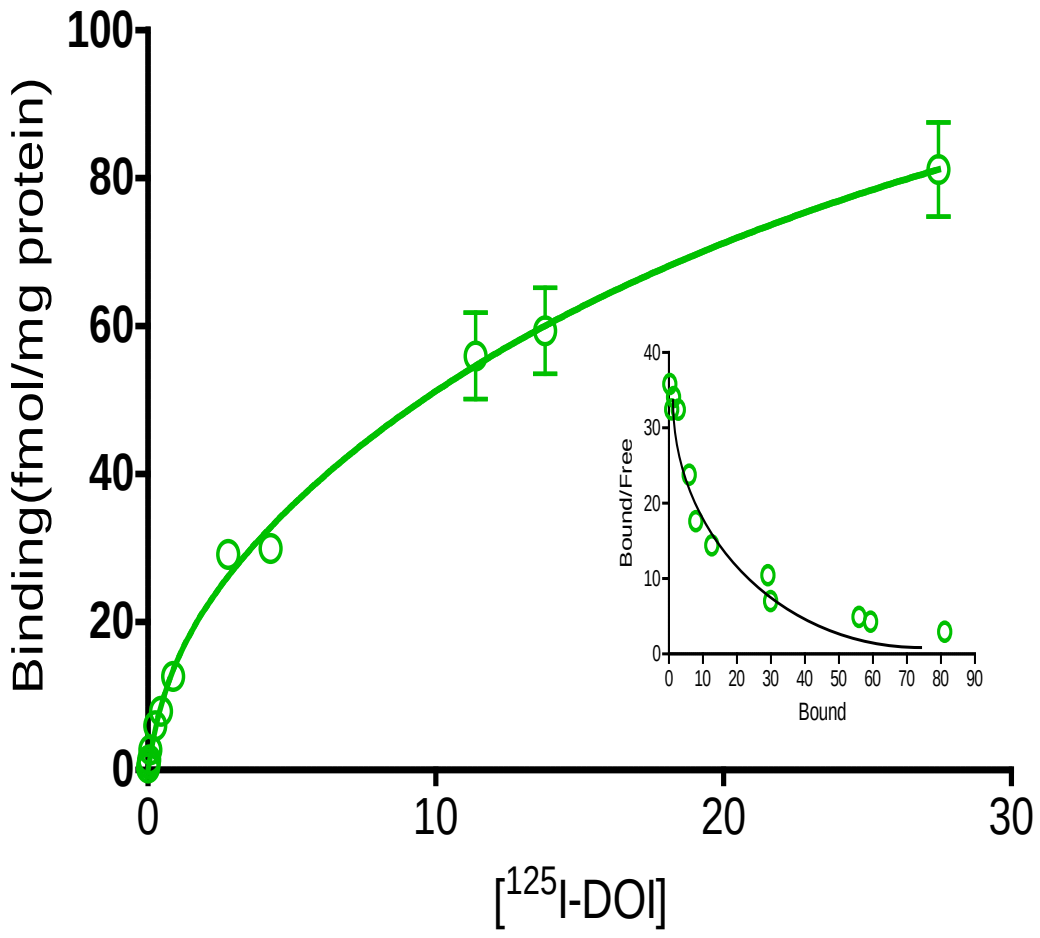

Figure 21. Agonist High Affinity Binding: Membranes From WT Mice.

Representative saturation plots of specific [^{125}I]-DOI binding are shown for membranes from WT mice. Increasing concentrations of [^{125}I]-DOI are plotted on the x-axis and density (fmol/mg protein) is plotted on the y-axis. Data were tested for best fit to one- or two-site models (GraphPad Prism 5.02). Insets show Scatchard transformation of the saturation binding data, bound/free (y-axis) versus bound (x-axis). Scatchard plots illustrate the presence of a high-affinity agonist binding site in WT mice

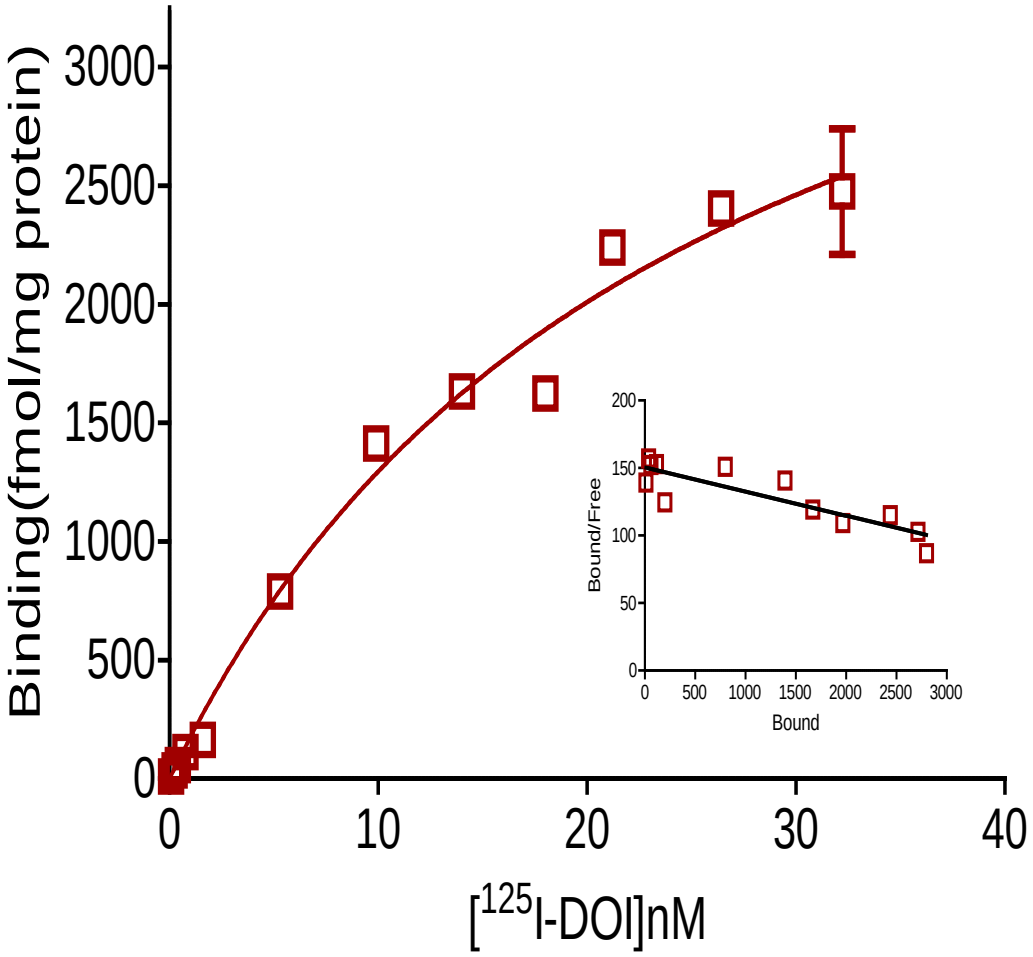

Figure 22. Agonist High Affinity Binding: Membranes From VGV/Y Mice.

Representative saturation plots of specific [^{125}I]-DOI binding are shown for membranes VGV/Y mice . Increasing concentrations of [^{125}I]-DOI are plotted on the x-axis and density (fmol/mg protein) is plotted on the y-axis. Data were tested for best fit to one- or two-site models (GraphPad Prism 5.02). Insets show Scatchard transformation of the saturation binding data, bound/free (y-axis) versus bound (x-axis). Scatchard plots illustrate the absence of high-affinity agonist binding sites in VGV/Y mice

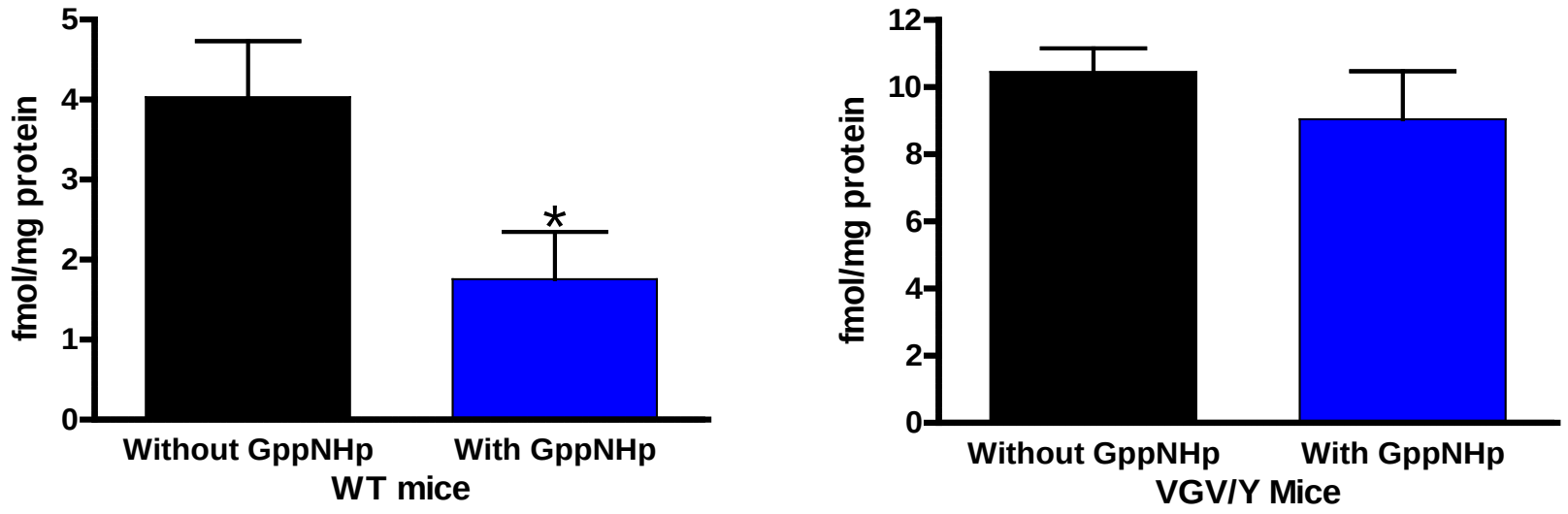

Figure 23. GTP Sensitive Agonist Binding

Gpp(NH)p (200µM) was added to assay buffer containing 1nM [^{125}I]-DOI to eliminate high affinity binding; the [^{125}I]-DOI binding in the absence and presence of Gpp(NH)p is plotted. Membranes from VGV/Y show no detectable GTP-sensitive [^{125}I]-DOI binding to $5HT_{2C}$ receptors. A significant effect of genotype was observed ($F(1,43)=54.66$, $p<.0001$)

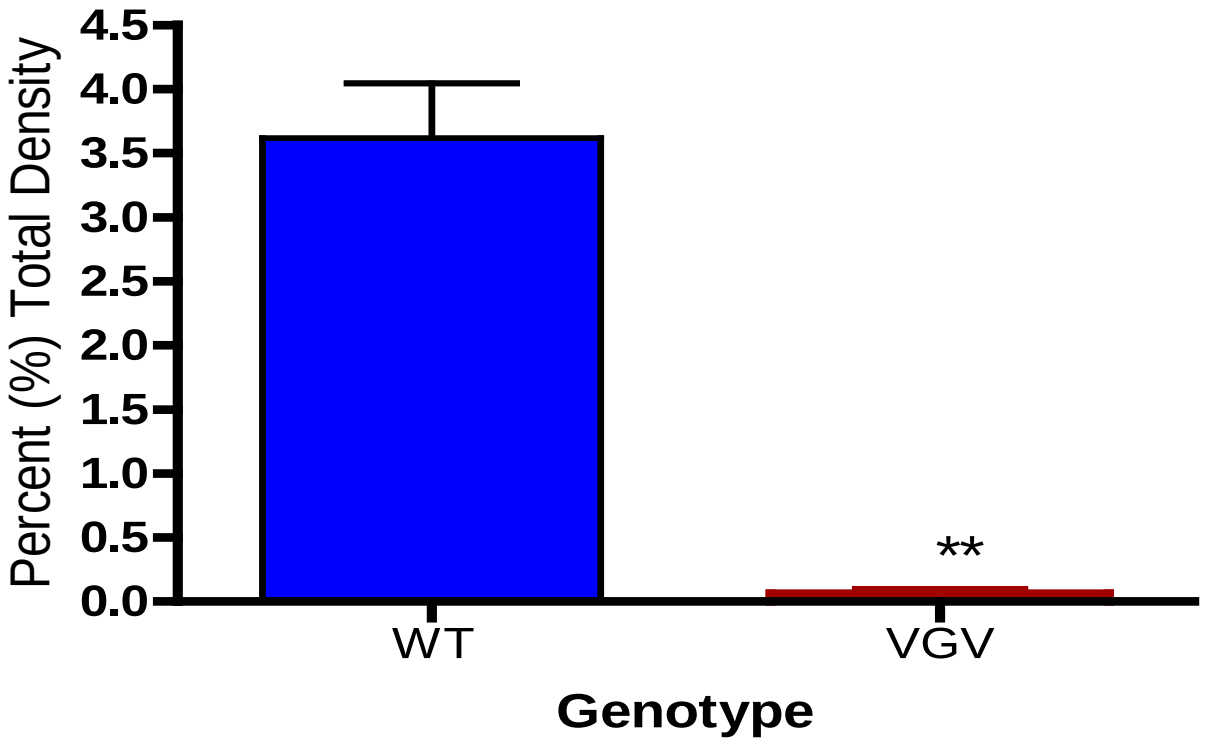

Figure 24. GTP Sensitive Antagonist Binding

Gpp(NH)p (200µM) was added to assay buffer containing 1nM [^{125}I]-DOI to eliminate high affinity binding; the percentage of binding eliminated is plotted. Total receptor density in each preparation (N=4) was estimated using a saturating concentration of [^{3}H]-mesulergine. The values plotted are percentage of total receptor density eliminated by Gpp(NH)p. Membranes from VGV/Y show no detectable GTP-sensitive [^{125}I]-DOI binding to $5HT_{2C}$ receptors. *Denotes significantly different from WT.

$5HT_{2C\text{-}VGV}$ Receptors in Brain Exhibit Low Agonist High-Affinity Binding

[^{125}I]-DOI binding to $5HT_{2C}$ receptors was clearly visible in the choroid plexus in brain slices from WT and VGV/Y; thus the choroid plexus was used for analysis of GTP sensitivity of $5HT_{2C}$ receptor binding. As illustrated in Figure 25, the addition of GTPγS to WT sections incubated with [^{125}I]-DOI decreased specific binding in choroid plexus (45.7 ± 6.52 percent decrease; N=5; $P < 0.05$). However, GTPγS did not significantly alter [^{125}I]-DOI binding in choroid plexus of brains from VGV/Y mice (5.5 ± 3.89 percent decrease; N=6; $P = 0.15$), confirming greatly reduced agonist high-affinity binding at $5HT_{2C\text{-}VGV}$ receptors.

VGV/Y and VGV/X Mice Have Increased Sensitivity to $5HT_{2C}$ Receptor Agonist

Injection of the $5HT_{2C}$ selective agonist, MK212, dose-dependently decreased total distance traveled in all genotypes, yet the potency of MK212 was greater in mutant mice relative to their respective WT littermates. For WT, female VGV/X and VGV/Y mice (Figure 26), a 2-way ANOVA revealed main effects of dose ($F_{5, 135} = 31.77$, $p < 0.0001$) and genotype ($F_{2, 135} = 42.46$, $p < 0.0001$). The potency of MK212 was 10-fold higher in VGV/Y and VGV/X mice relative to WT's (Figure 26; N=10;). For example, 0.1 mg/kg MK212 essentially eliminated locomotor activity in VGV/Y mice (posthoc Fisher least significant difference (LSD) test, saline vs. 0.1 mg/kg, $P < 0.001$), whereas this dose did not reduce locomotor activity in WT mice (posthoc Fisher LSD test, saline vs. 0.1

mg/kg, P = 0.58). There were no gender differences in the motor effects of MK212 in WT mice (data not shown).

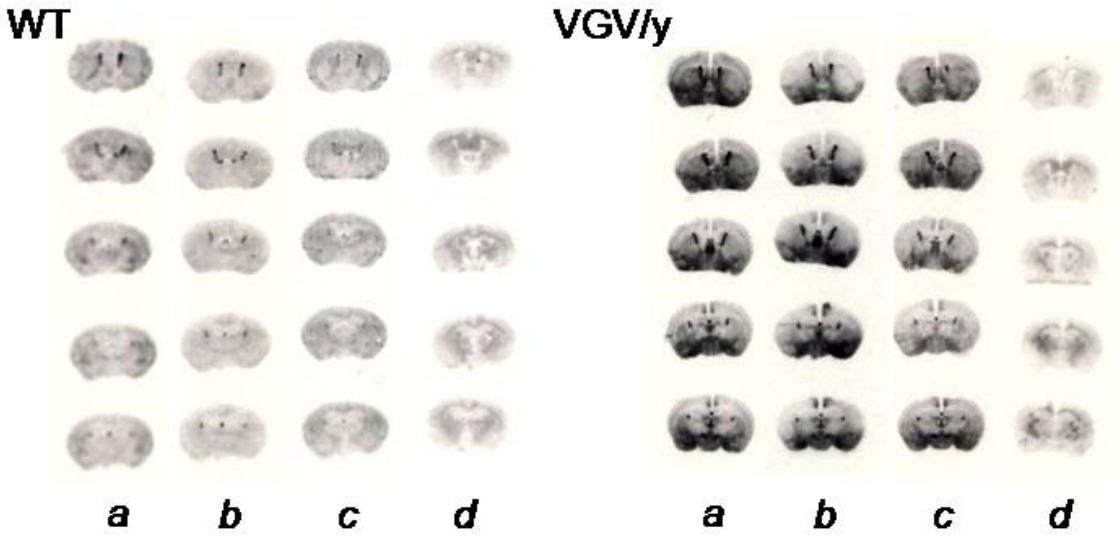

Figure 25. Agonist High Affinity Binding: Autoradiography

Representative [^{125}I]-DOI autoradiography of brain sections from WT and VGV/Y mice. Sections incubated in: a. 0.14 nM [^{125}I]-DOI; b. a plus 1 µM spiperone; c. a plus 100 µM GTPγS; d. a plus 30 µM methysergide. The addition of GTPγS reduced binding in choroid plexus of sections from WT, but not VGV/Y, demonstrating decreased high affinity agonist binding at 5-HT$_{2C\text{-}VGV}$ receptors.

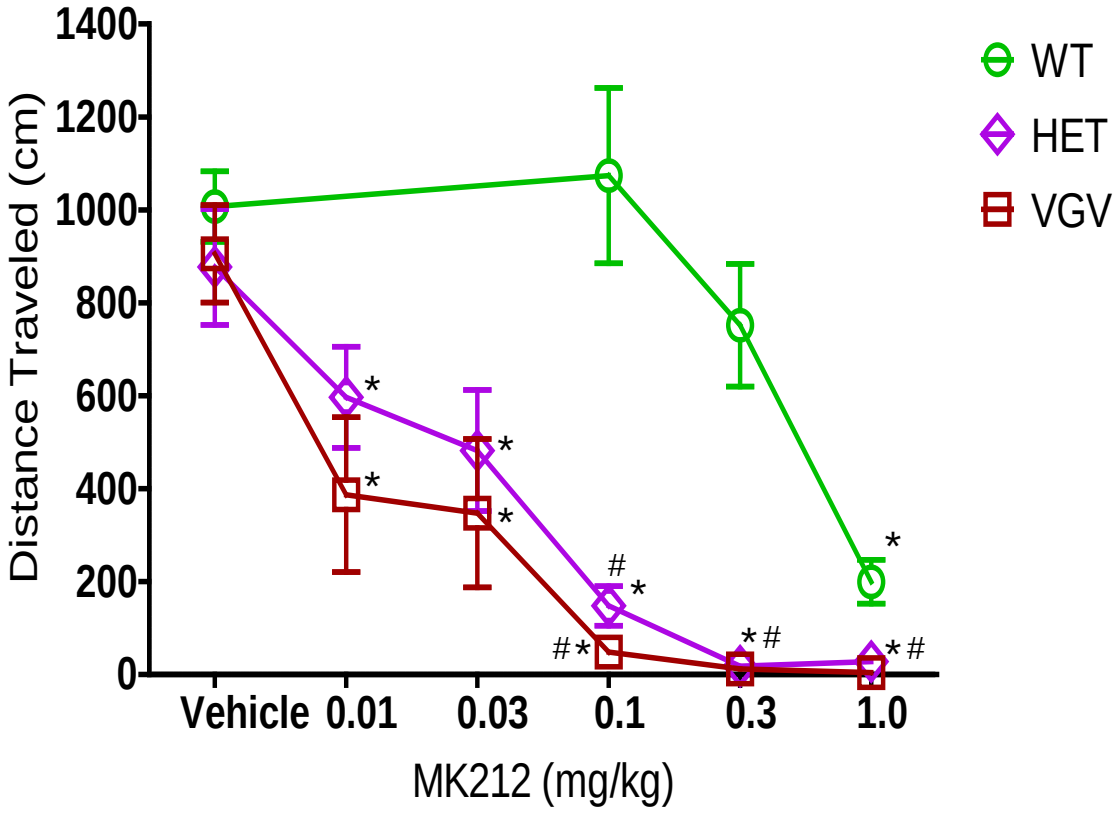

Figure 26. MK212 Dose-Dependent Induction Of Hypolocomotion

Locomotor activity of WT, VGV/X, and VGV/Y mice after single subcutaneous injection of saline or MK212 (0.01, 0.03, 0.1, 0.3 or 1.0 mg/kg). *Denotes significantly different from saline within group. #Denotes significantly different from WT.

VGV/Y Mice Show Massive Increases in $5HT_{2C}$ Receptor Density

[^{3}H]-mesulergine saturation binding was performed to determine the maximum density of $5HT_{2C}$ receptor binding sites in brains of WT, VGV/Y and VGV/X mice (Figure 27) and in WT and INI/Y mice (Figure 28). All plots were best fit by a single site equation. The density of $5HT_{2C}$ receptors in the frontal cortex was increased by 25-fold in VGV/Y mice relative to WT mice (Bmax = 163.3 ± 5.92 fmol/mg protein in WT vs. 4266 ± 259.9 in VGV/Y; N=4). Heterozygous VGV/X mice displayed an approximately 10-fold increase in the density of [^{3}H]-mesulergine binding sites (Bmax = 1754 ± 148.7 fmol/mg protein), which was intermediate to that observed for WT and VGV/Y mice (Figure 27). Conversely, the density of $5HT_{2C}$ receptors in the brain was decreased by 30% in INI/Y mice relative to WT mice (Figure 28; Bmax = 66.94 ± 11.22 fmol/mg protein in WT versus 47.31 ± 8.43 INI/Y; N= 5). The differences in $5HT_{2C}$ receptor density between WT littermates of VGV/Y and INI/Y mice (Figure 27 compared to Figure 28) was likely due to differences in brain areas analyzed, i.e. for studies with VGV/Y mice, frontal cortex was used, whereas whole brain minus cerebellum was used for studies with INI/Y mice. These alterations in receptor density (marked increase in VGV/Y mice and decrease in INI/Y mice) were confirmed by ^{3}H-mesulergine autoradiography (Clinton Canal, personal communication).

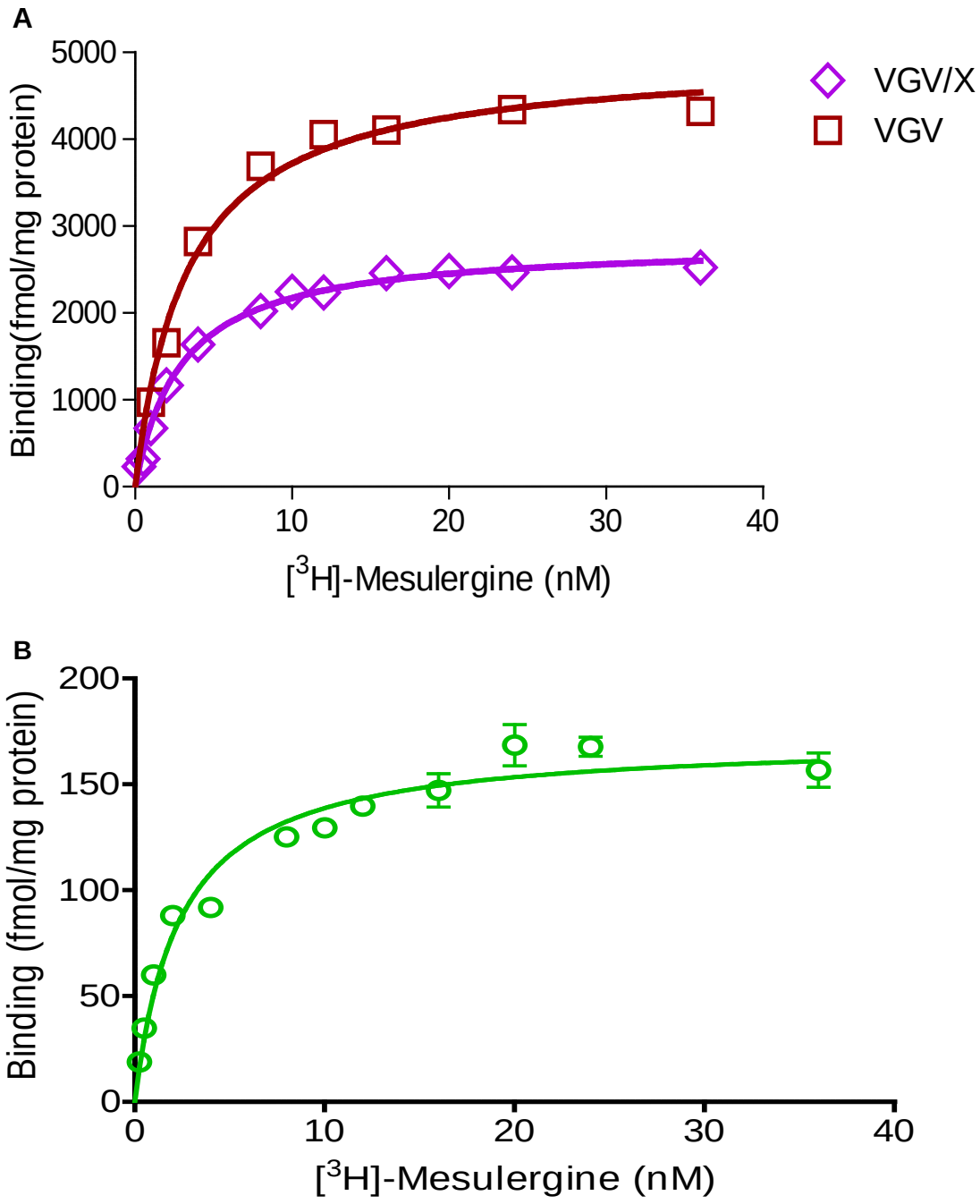

Figure 27. $5HT_{2C}$ Receptor Density in VGV/Y and VGV/X Mice

Representative saturation plots of specific [^{3}H]-mesulergine binding are shown for (A) membranes from VGV/X (HET) and VGV/Y mice or (B) membranes derived from WT mice. Increasing concentrations of [^{3}H]-mesulergine are plotted on the x-axis and density (fmol/mg protein) on the y-axis.

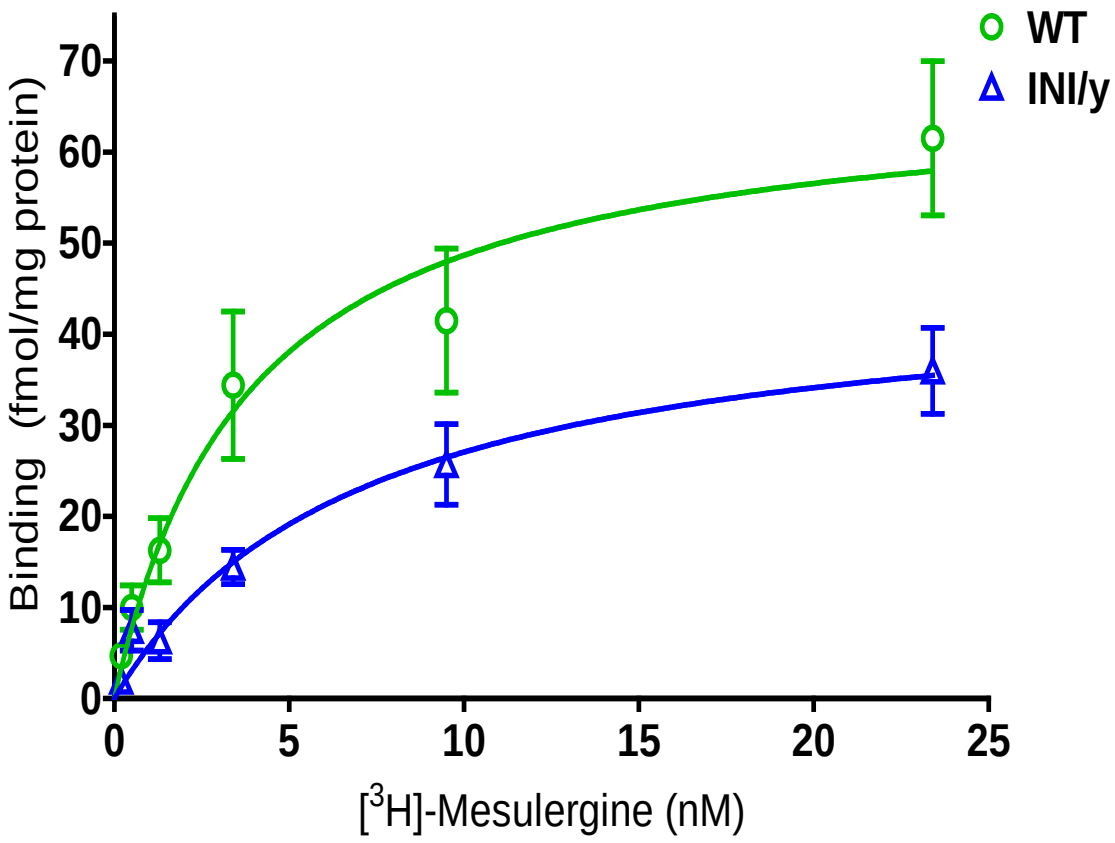

Figure 28. $5HT_{2C}$ Receptor Density in INI MIce

Representative saturation plots of specific [^{3}H]-mesulergine binding are shown for membranes derived from INI/Y and WT mice. Increasing concentrations of [^{3}H]-mesulergine are plotted on the x-axis \and density (fmol/mg protein) on the y-axis. VGV/Y mice had the greatest density, whereas INI/Y mice had the lowest density of $5HT_{2C}$ receptors

VGV/Y Mice Show Large Increases in $5HT_{2C}$ Receptor Autoradiography

Similar to results of saturation binding experiments, [^{3}H]-mesulergine autoradiographs showed tremendous increases in $5HT_{2C}$ receptor density in VGV/Y mice (Figure 29, WT specific binding versus VGV/Y specific binding in all regions examined, P's < 0.05, e.g. 89 percent increase in nucleus accumbens of VGV/Y mice relative to nucleus accumbens of WT; N = 5 WT, N=6 VGV/Y). Conversely, $5HT_{2C}$ receptor density is decreased in INI/Y mice (Figure 30, WT specific binding versus INI/Y specific binding, P < 0.05, e.g. 39 percent decrease in INI/Y choroid plexus relative to WT choroid plexus; N = 6 WT, 5 INI/Y). Addition of 300 nM SB204741 did not alter [^{3}H]-mesulergine binding in WT, VGV/Y or INI/Y brain sections, suggesting $5HT_{2B}$ receptors did not contribute significantly to [^{3}H]-mesulergine binding (data not shown). There was a widespread increase in $5HT_{2C}$ receptor binding in thalamus, amygdala, posterior cingulate cortex, piriform cortex (Figure 31) and nucleus accumbens as well as other olfactory structures (data not shown) in VGV/Y brains compared to WT. Decreases in $5HT_{2C}$ receptor binding in INI/Y brains were seen in the same brain structures (Figure 31). A visual comparison of ^{3}H-mesulergine (plus spiperone) binding shows a similar distribution of the $5HT_{2C}$ receptor in brains of WT and VGV/Y mice, suggesting that the overexpression in the mutant mice does not reflect an aberrant distribution of the $5HT_{2C}$ receptor.

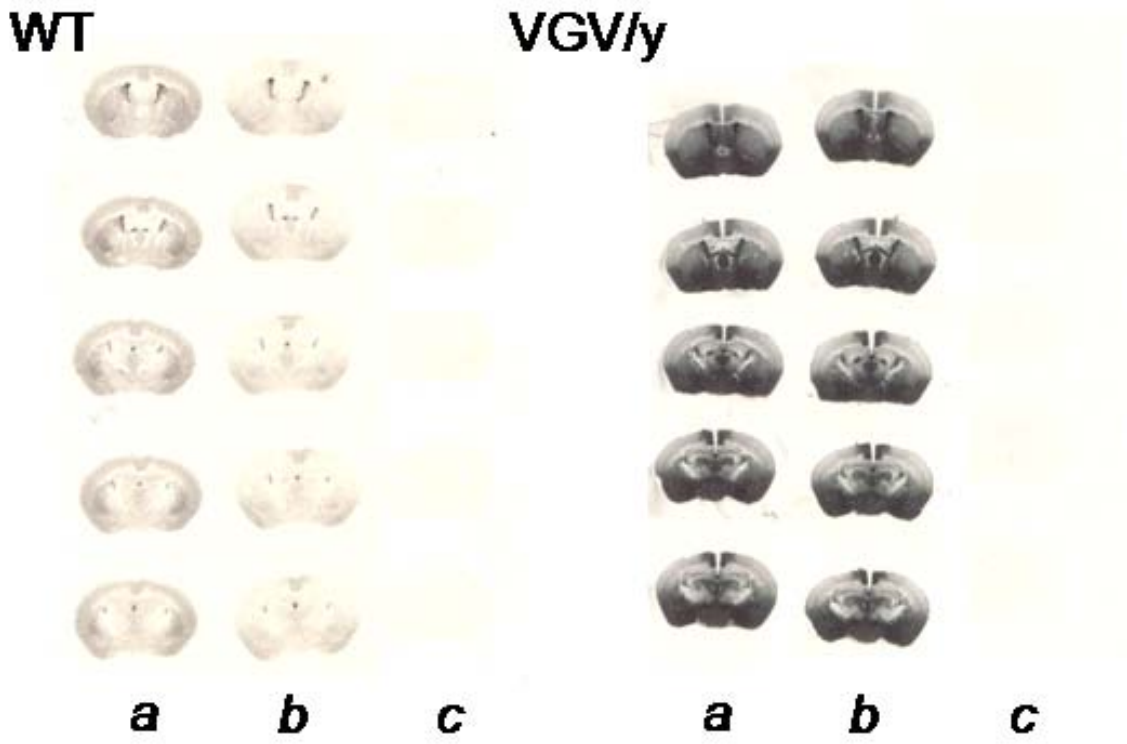

Figure 29: VGV/Y [^{3}H]-Mesulergine Autoradiography

Representative [^{3}H]-mesulergine autoradiography of brain sections from WT and VGV/Y mice. 1. Sections incubated in 3 nM [^{3}H]-mesulergine; 2. plus 1 µM spiperone; 3. plus 30 µM methysergide

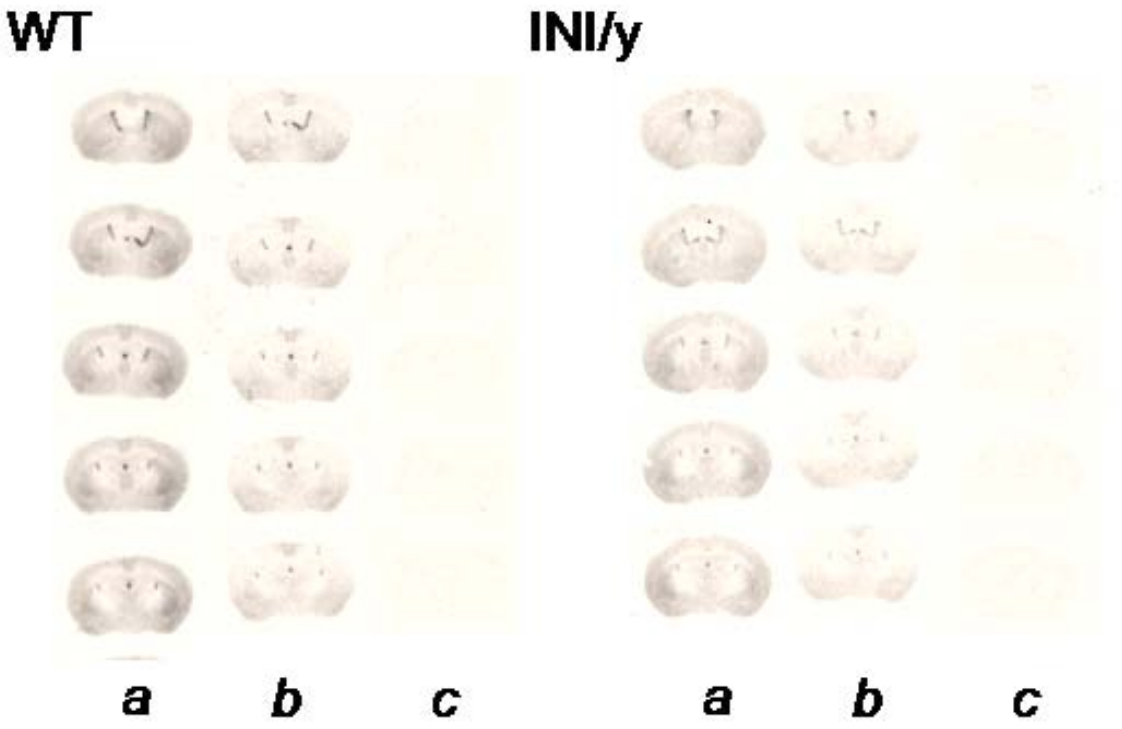

Figure 30: INI/Y [^{3}H]-Mesulergine Autoradiography

Representative [^{3}H]-mesulergine autoradiography of brain sections from WT and INI/Y mice. 1. Sections incubated in 3 nM [^{3}H]-mesulergine; 2. plus 1 μM spiperone; 3. plus 30 μM methysergide. These data are courtesy of Dr. Clinton Canal.

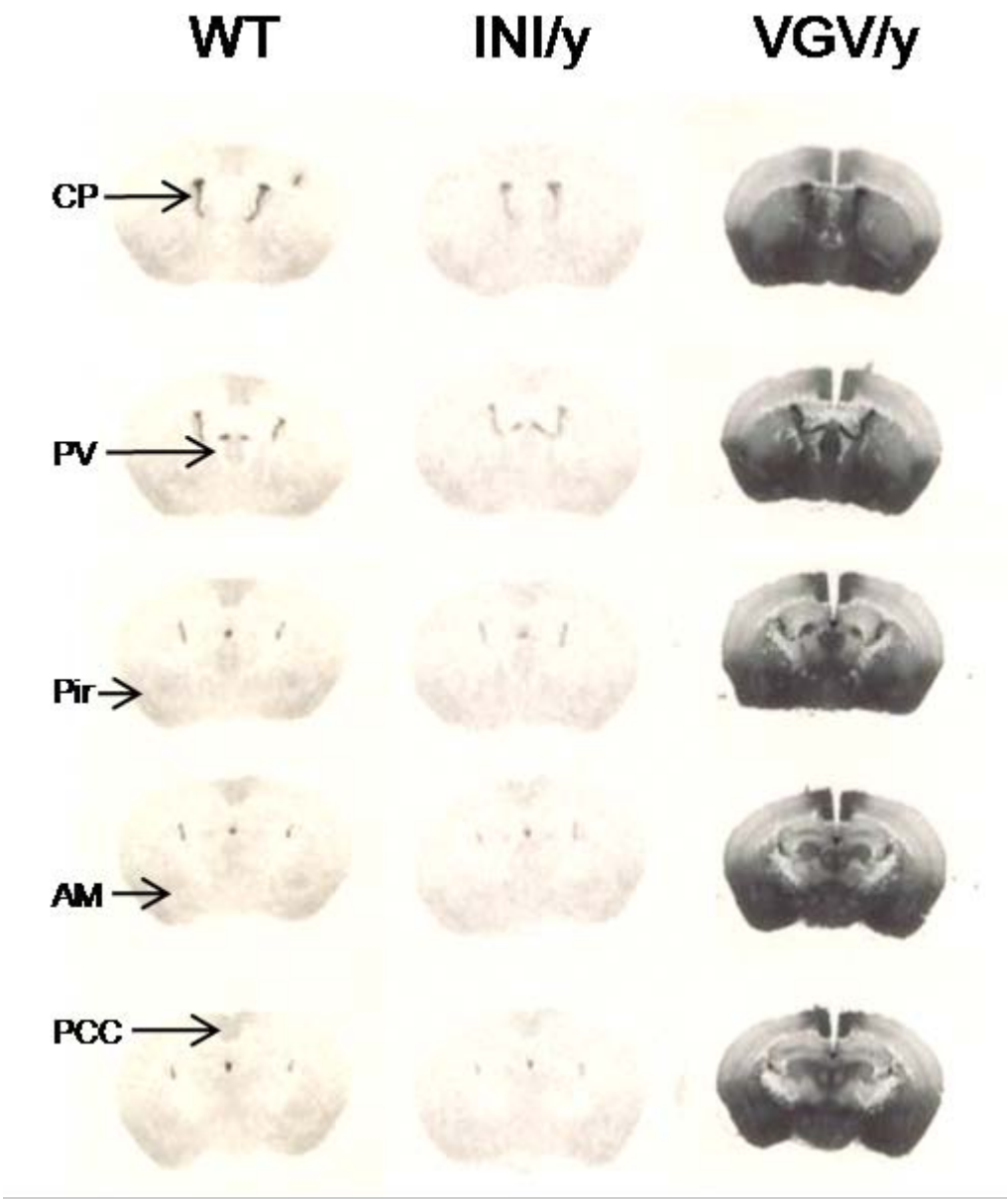

Figure 31: Regional 5-HT_{2C} Receptor Density

[^{3}H]-mesulergine autoradiographs of brain sections displaying $5HT_{2C}$ receptors in WT, INI/Y, and VGV/Y mice. Increases in $5HT_{2C}$ receptor expression in VGV/Y mice are observed in several brain structures. Decreases in $5HT_{2C}$ receptor expression in INI/Y mice are shown in the same structures. Abbreviations: CP, choroid plexus; PV, paraventricular thalamic nucleus; Pir, piriform cortex; Am, amygdala; PCC, posterior cingulate cortex. These data are courtesy of Dr. Clinton Canal

Biogenic amine levels

Given that the 5-HT_{2C} receptor has been reported to exert an inhibitory influence on DA release, we investigated if DA turnover was decreased in VGV mice and further we assessed whether the inverse agonist, SB206553, differentially altered DA turnover in VGV/Y mice as a secondary measure of receptor function. Twenty minutes after treatment with saline or 3 mg/kg of SB206553, biogenic amine levels were determined in striatum and cortex of VGV and wild-type male mice (Table 5). Dopamine turnover was measured by the ratio of DOPAC/DA and serotonin turnover was measured by the ratio 5HIAA/5HT. Although basal DA turnover was altered in VGV mice, the effect was restricted to cortex. After saline injection, the cortex of VGV mice showed reduced dopamine turnover ($p = 0.039$ for DOPAC/DA, least-squared difference post-hoc tests, (Figure 32a), which is consistent with literature implicating a 5-HT_{2C} receptor inhibitory influence on DA release (De Deurwaerdere et al., 2004).

A more striking dopamine phenotype occurred in both cortex and striatum following SB206553 challenge. Significant genotype by drug interactions were observed for the ratio DOPAC/DA ($F(1,16)=20.39$, $p = 0.004$) and for 5HIAA/5HT ($F(1,16)=19.42$, $p = 0.0004$). Similar profiles of the marginal means were observed in each model, where VGV mice showed increased turnover, whereas WT mice did not (Figure 32a & 32b).

TABLE 5. Biogenic Amine: Levels in frontal cortex and striatum after saline and SB206553 administration.

Frontal Cortex

Amine/metabolite	DOPAC		Dopamine		5-HIAA		5-HT		HVA	
Genotype	WT	VGV	WT	VGV	WT	VGV	WT	VGV	WT	VGV
Saline	7.1±1.8	10.2±1.8	31.5±10.4	68.2±11.4*	10.4±0.88	13.6±1.7	25.9±1.2	30.8±2.2	6.7±1.6	11.2±1.8
SB206553	6.6±0.8	15.6±1.7	28.9±6.7	34.2±6.3	9.1±0.7	16.0±0.8	24.5±2.4	20.0±0.9	6.0±0.7	13.9±1.3

Striatum

Amine/Metabolite	DOPAC		Dopamine		5-HIAA		5-HT		HVA	
Genotype	WT	VGV	WT	VGV	WT	VGV	WT	VGV	WT	VGV
Saline	15.3±1.3	11.8±0.9	111.9±5.6	116.6±7.5	19.4±3.1	16.9±1.8	26.6±1.6	24.8±1.4	17.1±1.2	27.5±1.5
SB206553	14.39±1.4	24.1±1.0	110.8±10.7	95.0±15.1	18.9±0.7	26.2±1.1	25.9±1.8	25.4±0.6	16.2±1.3	16.1±1.1

The results are presented as means ± SEM (n=5). * significantly different from WT, $p<0.01$. Data were analyzed with anovas

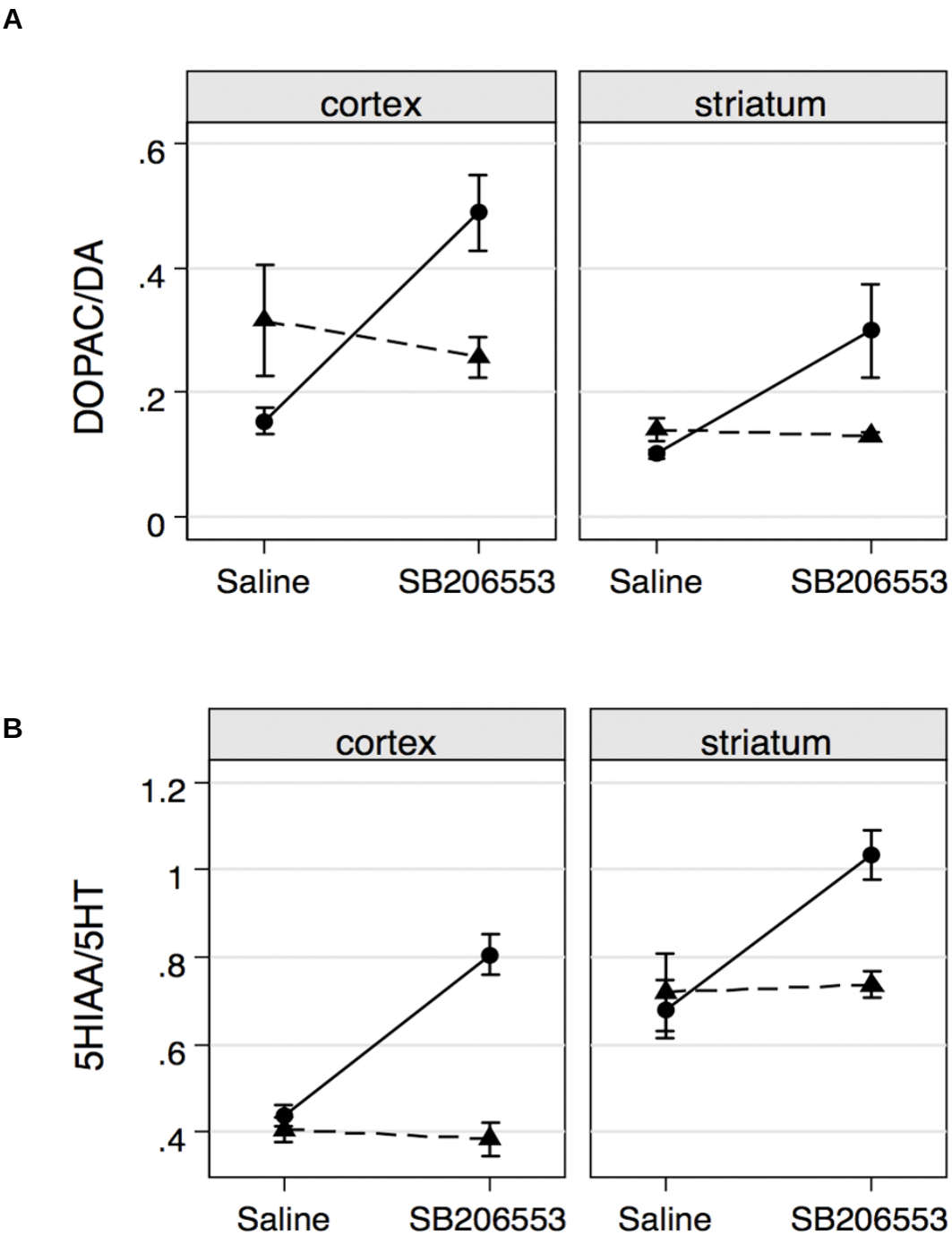

Figure 32. SB206553 increases DA and 5-HT Turnover in VGV mice

(A) Dopamine turnover (DOPAC/DA) following administration of saline or SB206553(3mg/kg) in WT (▲) and VGV (●) mice; cortex (left), striatum (right). (B) Serotonin turnover (5HIAA/5HT) after the same conditions.

Discussion

$5HT_{2C}$ receptor RNA transcripts undergo five adenosine to inosine RNA editing events, resulting in the generation of distinct amino acids in the second intracellular loop of the protein, a region critical for G-protein coupling (Ballesteros et al., 1998; Gaborik et al., 2003; Moro et al., 1993). Combinations of the five edited nucleotides predict 24 different protein isoforms (Burns et al., 1997); many isoforms display altered signaling properties in transfection experiments in cell lines. The fully-edited VGV isoform, where all five sites are edited, shows the most dramatic decrease in agonist potency, as well as silencing of receptor constitutive activity and elimination of high-affinity, Gpp(NH)p sensitive agonist binding (Niswender et al., 1999). The current set of experiments takes advantage of genetically modified mice solely expressing the VGV isoform to show for the first time, in brain, that RNA editing of the $5HT_{2C}$ receptor alters the proximal event in cell signaling, receptor-G-protein coupling.

Several techniques were used to highlight the *in vivo* properties of the $5HT_{2C\text{-}VGV}$ receptor including [^{3}H]-mesulergine saturation binding, [^{3}H]-mesulergine autoradiography, [^{125}I]-DOI saturation binding, and [^{125}I]-DOI GTP sensitive high affinity binding in membranes and in autoradiography. Nonlinear regression analysis of [^{125}I]-DOI saturation binding revealed two saturable binding sites in membranes from brains of WT mice, with the high affinity site representing nearly 10% of the total binding. In contrast, nonlinear regression

analysis of [^{125}I]-DOI saturation binding to membranes from mice expressing the VGV isoform reveals only a single, low-affinity site, even though receptor density is increased dramatically. Based on the extended ternary complex model of receptor-G-protein coupling (Samama et al., 1993), it is likely that the loss of high affinity binding reflects reduced G-protein coupling. To test this further, [^{125}I]-DOI binding was carried out in the presence and absence of a GTP analog. The addition of Gpp(NH)p reduces the binding of [^{125}I]-DOI in brain membranes from WT mice, but has no detectable effect on [^{125}I]-DOI binding in membranes from VGV/Y mice, suggesting that the G-protein coupling capacity of brain $5HT_{2C\text{-}VGV}$ receptors is markedly reduced. An unexpected finding was the marked increase in 5-HT2C receptor density throughout the brains of VGV/Y mice relative to WT, suggesting the possibility that Gq/11 protein may become rate-limiting in the case of this large increase in receptor density in VGV/Y mice. However, this is the normal receptor expression level in one brain structure, the choroid plexus, where the receptor functions at a high level (Conn et al, 1986). Early quantitative studies of Milligan (1993) show that the level of Gq protein in rat frontal cortex is 17pmol/mg protein and combined Gq/G11, 25pm/mg protein, which is 5-fold higher than receptor density in VGV/Y mice. Furthermore, we have previously shown that the levels of Gq and G11 protein are not altered in VGV/y mice (Canal et al, 2009). These results suggest that Gq/11-protein is not rate limiting in the brains of VGV/Y and that the reduction in GTP-sensitivity reflects reduced coupling capacity in VGV/Y mice.

Consistent with a reduction in G-protein coupling capacity found in membranes prepared from brains of VGV/Y mice, receptor autoradiography in intact brain slices reveals that, although total ^{3}H-mesulergine binding is increased, the proportion of GTP-sensitive high affinity [^{125}I]-DOI binding is reduced nearly 8-fold in choroid plexus of VGV/Y mice. These experiments confirm that the blunted G-protein coupling of the VGV isoform described in cell lines is reproduced in a native setting. We conclude therefore that it is valid to interpret *in vivo* increases in the degree of RNA editing of the $5HT_{2C}$ receptor as a loss of function at the cellular level. An attempt to evaluate the classical effector pathway, Gq mediated-PLC activation, was unsuccessful. Previous studies of $5HT_{2C}$ receptor-mediated PLC activation in rat brain were performed in choroid plexus (Conn et al, 1986), but this was not feasible in mice. In mouse forebrain, 5HT failed to elicit a PLC signal, in wild-type or VGV/Y mice.

Our studies also suggest that changes in the proportion of edited isoforms alter the density of $5HT_{2C}$ receptor binding sites within the brain. VGV/Y mice, solely expressing the VGV isoform, have a 25-fold increase in $5HT_{2C}$ receptor density relative to WT mice, similar to that described by Morabito et al (2007). In heterozygous VGV/X mice, the percentage of RNA isoform encoding VGV and the density of $5HT_{2C}$ receptor binding sites is approximately halfway between WT and VGV/Y mice. This gene-dose dependent increase in binding site density supports the notion that the increases are not the result of erroneous genetic manipulation. In addition, receptor autoradiography suggests that the increased expression of $5HT_{2C}$ receptor in VGV/Y mice does not reflect aberrant distribution

of the receptor. We suggest that the increased density is a compensatory response to reduced cellular function of the editing receptor. In support of this conclusion, transgenic mice solely expressing the non-edited, highly functional, $5HT_{2C\text{-}INI}$ receptor have reduced receptor density (unpublished results).

Although our pharmacological studies in brains of mice expressing the VGV isoform document reduced G-protein coupling capacity, the marked increase in $5HT_{2C}$ binding site density may mitigate this diminished cellular signal in the intact animal. To investigate the functional consequences *in vivo* of these opposing effects, we employed a behavioral assay of $5HT_{2C}$ receptor activation. $5HT_{2C}$ receptor agonists, such as MK212 have long been known to induce hypolocomotion (Gleason et al., 2001; Gleason and Shannon, 1998; Stiedl et al., 2007). In ligand binding assays, MK212 is essentially equipotent at rat 5-HT_{2C} and 5-HT_{2B} receptors (National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # NO1MH32004 (http://pdsp.med.unc.edu/), however, recent studies show that the MK212-elicited hypolocomotion is eliminated in $5HT_{2C}$ receptor null mice (Fletcher et al., 2009), confirming that this behavior is a valid measure of *in vivo* $5HT_{2C}$ receptor activation in mice. MK212 elicits a dose-dependent decrease in locomotor activity in both WT and VGV/Y mice, but the sensitivity to MK212 is dramatically increased in VGV/Y mice. This observation confirms the work of Kawahara et al (2008), and importantly shows that the marked behavioral sensitization is reproduced on another background strain. These data suggest that the large increase in receptor density overcomes the reduced G-protein coupling of the VGV receptor. Still a third variable,

receptor internalization, may play a role given the evidence that RNA editing alters the ability of the $5HT_{2C}$ receptor to interact with key regulatory molecules, G-protein receptor kinase and beta-arrestin (Marion et al, 2004).

Given emerging evidence suggesting that the 5-HT_{2C} receptor attenuates DA release and 5-HT_{2C} receptor antagonists augment the action of dopamine releasing agents such as cocaine (Alex and Pehek, 2007; De Deurwaerdere et al., 2004; Filip and Cunningham, 2003; Navailles et al., 2003), we examined DA turnover as another index of *in vivo* 5-HT_{2C} receptor function. 5-HT_{2C} receptor regulation of DA release is complex, involving direct and indirect pathways; microdialysis studies suggest that the mechanism of regulation differs in the nigrostriatal versus mesocorticolimbic DA systems (Di Matteo et al., 1999; Di Giovanni et al., 2000; see Di Matteo et al., 2008 for review). This may explain the regional difference seen in saline-treated VGV/Y mice, where DA turnover in the mutant mice is decreased in frontal cortex but not in striatum. Microdialysis experiments combined with direct microinjection would be valuable in pursuing this question. The finding that SB206553 increases DA turnover in frontal cortex and striatum of VGV/y mice is consistent with the evidence that 5-HT_{2C} receptors inhibit DA release in both areas. The ability of SB206553 to increase DA release is thought to reflect constitutive activity of the 5-HT_{2C} receptor (De Deurwaerdere et al, 2004); however, such an explanation is less plausible in VGV mice since *in vitro* data shows that this isoform is essentially devoid of constitutive activity (Berg et al., 2008a; Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000). An alternative explanation is suggested by our finding of an increased

5-HT turnover in VGV mice given SB206553; enhanced release of 5-HT onto the up-regulated 5-HT_{2C} receptors may play a role in the SB206553 modulation of DA release in VGV mice.

In summary, i*n vitro* studies in transfected cell lines have shown that RNA editing of $5HT_{2C}$ RNA transcripts profoundly alters the signaling properties of the $5HT_{2C}$ receptor protein. The current studies utilizing genetically modified mice solely expressing the most extensively edited isoform of the $5HT_{2C}$ receptor show, for the first time, that the G-protein coupling capacity in the brain is altered, i.e., expression of fully edited $5HT_{2C\text{-}VGV}$ receptor isoform within the brain blunts receptor-G-protein coupling. We further show that changes in the degree of editing significantly alters the density of receptor binding sites within the brain, suggesting that the pattern of protein isoforms, and hence the inferred overall function of the receptor protein, may not reflect the pattern of RNA isoforms. This conclusion has important implications for human studies of disease-related alterations in RNA editing of the $5HT_{2C}$ receptor, where inferences about protein function are based on the profile of RNA edited isoforms. Our studies further emphasize a critical need for a method that differentiates $5HT_{2C}$ receptor protein isoforms in brain tissue. Until such a method becomes available, clinical and laboratory investigations must continue to rely on mRNA isoform distribution to infer protein distribution and function. Our studies emphasize the need for caution in these interpretations.

Chapter IV: Conclusions and New Questions

Summary and Future Directions

We were able to establish *in vitro* and *in vivo* ligand specificity for 5-HT_{2A} and 5-HT_{2C} receptors using several techniques. This allowed us to pursue the larger goal of examining the *in vivo* consequences of 5-HT_{2C} receptor pre-mRNA editing. To accomplish this we employed genetically modified mice solely expressing the 5-$HT_{2C\text{-}VGV}$ receptor isoform. Using these mice we show for the first time *in vivo* diminished G-protein coupling in 5-$HT_{2C\text{-}VGV}$ receptors that mirrors published *in vitro* data. Our conclusions about G-protein coupling and constitutive activity are based on the assumption that the number of constitutively active 5-HT_{2C} receptors and/or 5-HT_{2C} receptors in the high-affinity state varies directly and positively with overall 5-HT_{2C} receptor density. We have not tested this assumption, such an experiment may now be possible if one were to take advantage of mice created by Kimura et al. (2009), which over-express 5-HT_{2C} in the forebrain. Basal and SB206553 mediated GTPγS binding in WT and 5-HT_{2C} over-expressing mice would allow one to determine whether the number of constitutively active 5-HT_{2C} receptors is absolute or proportional to overall density *in vivo*. Unlike the *in vitro* data, we were not able to reproduce the decrease in agonist potency in 5-$HT_{2C\text{-}VGV}$ receptors. This was due to the fact that we were unable to measure a proximal event to 5-HT_{2C} receptor activation, combined with

what appear to be compensatory changes in receptor density in all genotypes examined. We attempted to measure IP_3 formation in both WT and VGV/Y mice, and were unable to achieve an adequate signal above noise. This was especially surprising given the receptor density in the VGV/Y mice.

Implications for Human Studies

All studies examining the relationship between 5-HT_{2C} mRNA editing and various neurological disorders, such as those summarized in Table 1, use predicted mRNA isoform distribution to infer receptor protein distribution. While my work shows that this is not ideal, it is the best method currently available.

Examining the possible impact of 5-HT_{2C} mRNA editing on human neurological disorders at this point is still largely theoretical since we can only measure editing profiles directly from post-mortem brain tissue. The data from these studies is highly variable as seen in Table 1, and as a result no clear conclusions can be made concerning the role of 5-HT_{2C} receptor editing in the examined disorders. Furthermore, it is difficult to make a phenotypic extrapolation from VGV/Y mice to human beings because the mutant mice represent an extreme case where all 5-HT_{2C} receptors are edited. One would predict that a human being with such high levels of 5-HT_{2C} receptor editing would display altered behavioral patterns, particularly in response to stressors. Locomotor control might be affected as well as dopaminergic function in general. The patient might be hypoactive, with impaired sympathetic nervous function.

Even if we were able to measure 5-HT_{2C} receptor RNA isoform distribution in a living subject, it is of great importance to know the actual distribution of receptor protein when trying to determine the impact of 5-HT_{2C} receptor editing on disorders like schizophrenia and depression. We should therefore make a concerted effort to identify alternative markers of 5-HT_{2C} receptor editing, such as markers of ADAR activation, that may be present and measurable outside of the central nervous system. Without such a method, development of potential therapies targeting editing of the 5-HT_{2C} receptor will not be feasible since it would still be impossible to diagnose the degree of editing with a noninvasive procedure that patients will be willing to undergo. Clearly, this is a key challenge for the future of drug developments aimed at targeting 5-HT_{2C} receptors.

Increased 5-$HT_{2C\text{-}VGV}$ receptor density

Possible mechanisms for the increase in 5-HT_{2C} receptor density may include: erroneous genetic manipulation, increased mRNA levels, and changes in mRNA stability or translation efficiency. The possibility of erroneous genetic manipulation is unlikely since the density changes are gene dose dependent. To explore this further it is important to look at a different strain of mice. Kawahara et al. (2008) developed mice solely expressing the 5-$HT_{2C\text{-}VGV}$ receptor isoform on the C57B6 background. Since the mice were created by different methods and on a different background strain from the mice used in our studies, they represent the ideal method for exploring whether the increased 5-HT_{2C} receptor

density reported in the current work is the result of erroneous genetic manipulation or possibly a compensatory mechanism at work. The Kawahara mice show the same increases in agonist and antagonist sensitivity, however, Kawahara et al. (2008) claim to only observe an increase in cell-surface functional 5-HT_{2C} receptor density in their VGV/Y mice (Figure 35). This conclusion is based on the argument that [^{125}I]-DOI selectively labels cell surface functional receptors, an assumption that was not tested and is inconsistent with the lipid soluble nature of this amphetamine analog. Kawahara et al. (2008) also observed no significant changes in mRNA level, an observation confirmed by Morabito et al. (2009). Membrane saturation binding using the current protocol would allow for a direct comparison of density in these two mouse strains, which would provide further insight to the mechanism of the density phenotypes observed in both strains of mice. Mice expressing only the 5-$HT_{2C\text{-}INI}$ receptor show decreased receptor density whereas heterozygous VGV/X animals show 10-fold increased receptor density and at least 10-fold increased agonist potency. VGV/Y expressing only the 5-$HT_{2C\text{-}VGV}$ receptor, show an even greater increase in receptor density, (approximately 25-fold) and an increase in agonist potency equal to VGV/X mice, which may be due to floor effects, and the fact 5-HT_{2C} agonist induced hypolocomotion is not a direct effect of the receptor, but more likely the result of 5-HT_{2C} receptor inhibition of dopamine release. These phenotypes point to compensatory gene-dose dependent changes in receptor density. This in the case of the VGV/Y mice is sufficient to reverse decreases in receptor signaling at the cellular level and markedly augment function when the

entire organism is considered. Complete mRNA analysis of the VGV/X mice is necessary to confirm if there is indeed some disruption between RNA and translation to protein in these mice. Comprehensive regional analysis of the 5-HT_{2C} receptor binding site density must be completed to determine whether regional differences in 5-HT_{2C} receptor density are maintained. Several brain areas have been examined by Morabito et al, and Clinton Canal (personal communication), however comprehensive examination of the entire brain is necessary.

These strains of mice should be further examined to determine the developmental timeline of the 5-HT_{2C} receptor overexpression. A density sampling should be taken every 7 days between embryonic day 7 and postnatal day 84, such a course should allow for observation of the critical period, if any, for 5-HT_{2C} receptors in the development of these mice. It is of great importance to determine at which point during development this marked increase in receptor density occurs, as this could illuminate possible novel roles for the 5-HT_{2C} receptor during development.

Inverse agonist / antagonist induced hyperactivity

This signaling augmentation in the case of VGV/X and VGV/Y mice was not limited to agonist ligand potency. We observed a strange aggressive behavior that borders on compulsion accompanied with extreme hyperactivity, when mice were injected with a 5-HT_{2C} antagonist /inverse agonist SB206553

(Figure 33). In contrast, SB206553 appears to have a hypolocomotive effect in WT mice, especially when stereotypic movements are considered (not shown). The SB206553 induced hyperactivity cannot be directly accounted for by the increase in DA turnover reported in chapter III, as both dopamine D_1 and D_2 antagonists potentiate the effects of the 5-HT_{2C} inverse agonist SB206553 (Figure 34), although this observation more than likely involves complex signaling circuits. Dracheva et al. (2009) show increases in 5-HT_{2C} pre-mRNA editing in rats with high basal locomotor activity. These rats may be an example of natural occurrence of the same phenomena we have observed in these genetically modified mice. In order to test that hypothesis, a way of identifying and quantifying receptor protein isoforms is necessary.

Opposing locomotor effects of various ligands

We also observed unexpected effects of several ligands when VGV/Y mice were compared to WT mice including: 8-OH-DPAT (5-HT_{1A} agonist) and clonidine (adrenergic α_{2a} agonist) (Figure 36). One possible explanation for this phenomenon is that VGV/Y mice now have markedly increased 5-HT_{2C} receptor signaling where there may have been only marginal signaling before, but this is just speculation. The change in signaling stoichiometry, in VGV/Y mice especially, may have wide ranging effects on a number of neurotransmitter systems. This warrants further study into these changes with a possible site being the gabaergic interneurons which may modulate serotonin and adrenergic

signaling or vice versa. Micro dialysis studies in these mice would be of particular interest.

Mice are not rats

During the course of this work I noticed that the literature is nearly devoid of basic pharmacologic data specific to mice. The assumption is that neurotransmitters and ligands will behave in a similar manner as in rats. I found that this is not the case, M100907 for example is exquisitely selective for 5-HT_{2A} over 5-HT_{2C} in rats. In the strain of mice used in our experiments however, M100907 only showed marginal selectivity. There is a wealth of data highlighting mouse strain differences in a variety of behaviors, mRNA expression, and genetic code; it is not farfetched to imagine that these strain differences may extend to receptor protein expression, receptor protein distribution as well as receptor protein function. Some, if not most of the basic science that we take for granted and base many assumptions upon must be reconsidered for the mice that we now employ on such a widespread basis.

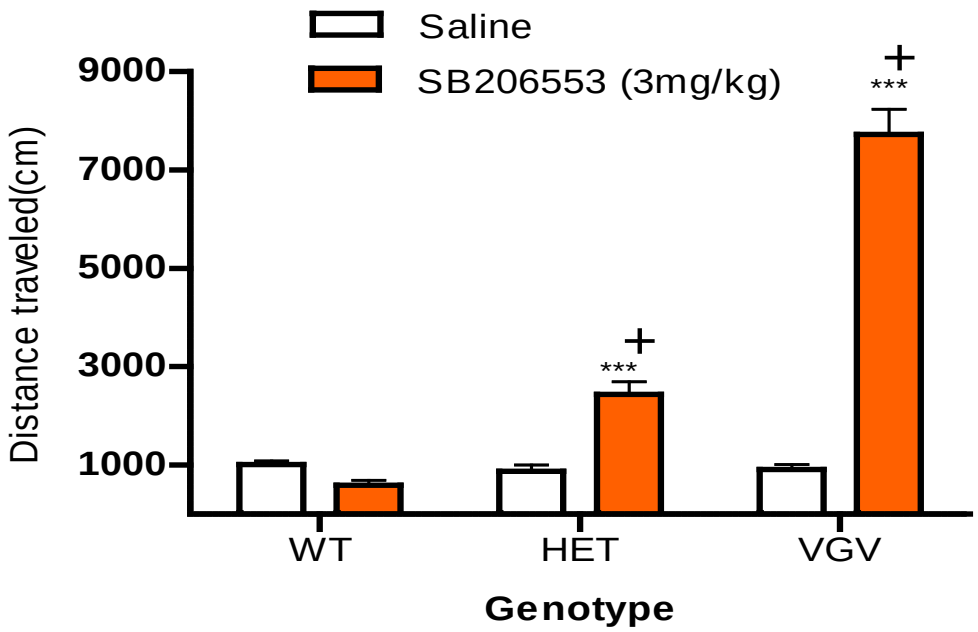

Figure 33: SB206553 Induced Hyperlocomotion

Exploratory locomotor activity of WT, HET (VGV/X), and VGV (VGV/Y) mice after single subcutaneous injection of either, saline, or 3mg/kg SB206553. *** Significantly different from saline within group, $p<0.001$. + Significantly different from other genotypes, $p<0.001$

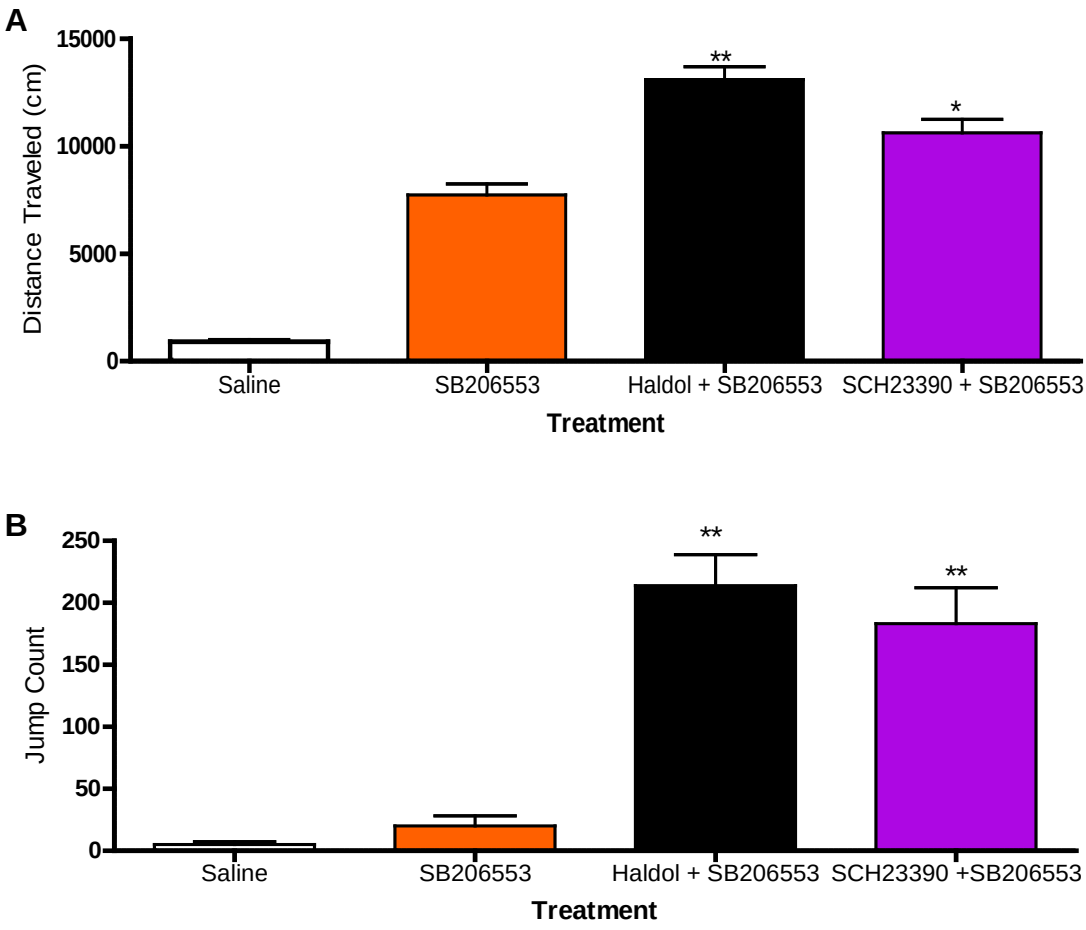

Figure 34: DA Receptor Antagonism Potentiates SB206553 Induced Hyperlocomotion

Exploratory locomotor activity (A) and jumping (B) after single subcutaneous injection of either saline or SB206553 (3mg/kg) or double subcutaneous injections of either haloperidol (1mg/kg) or SCH-23390(0.5mg/kg) followed by SB206553 (3mg/kg) 20 minutes later. ** Significantly different from SB206553,

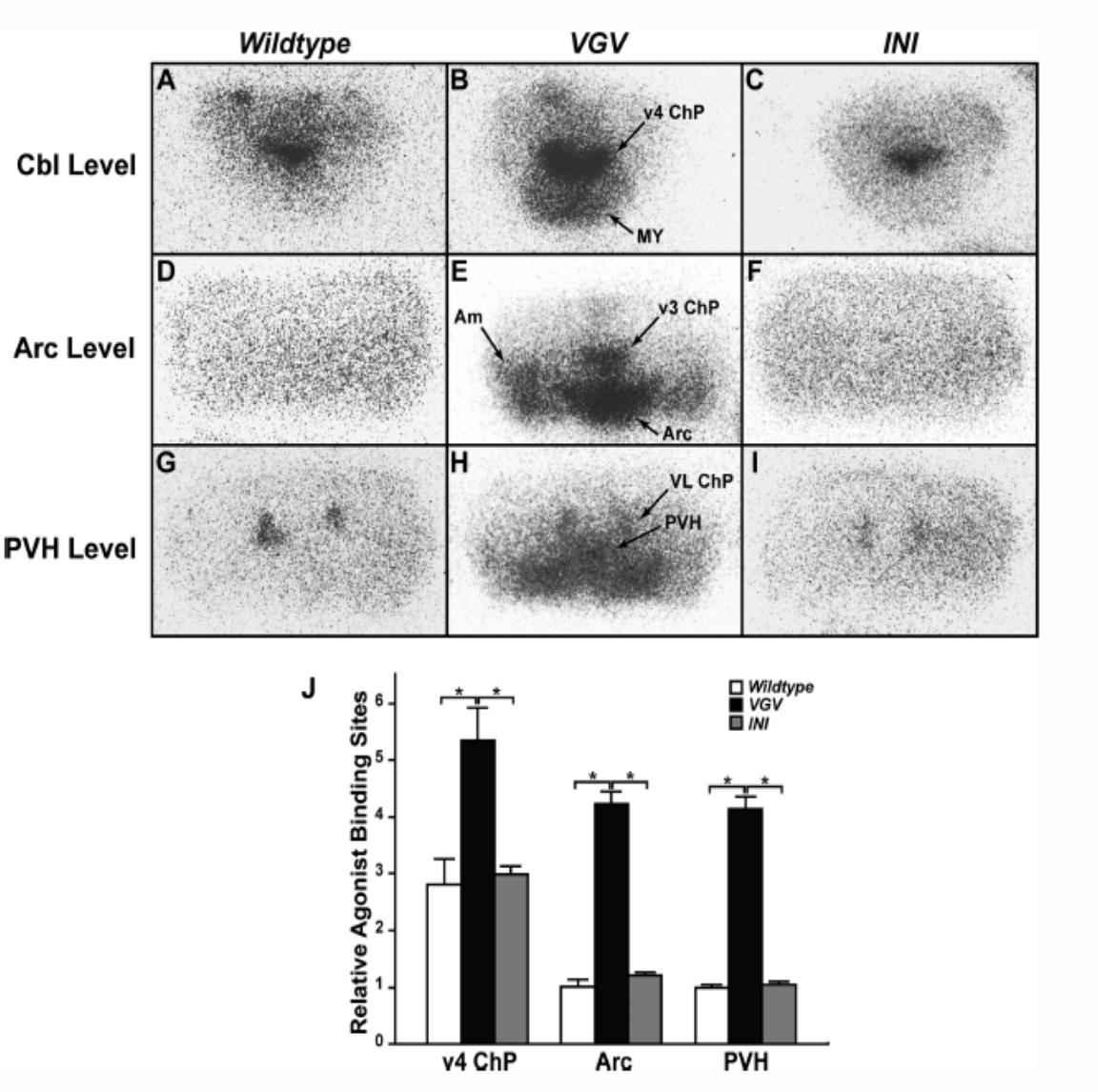

Figure 35: Increased Binding in Kawahara Mice: Autoradiography

[^{125}I]-DOI binding in various brain areas in the Kawahara VGV/Y and INI/Y mice. The authors attribute the increase in VGV/Y binding to an increase in functional receptors which our data refutes.

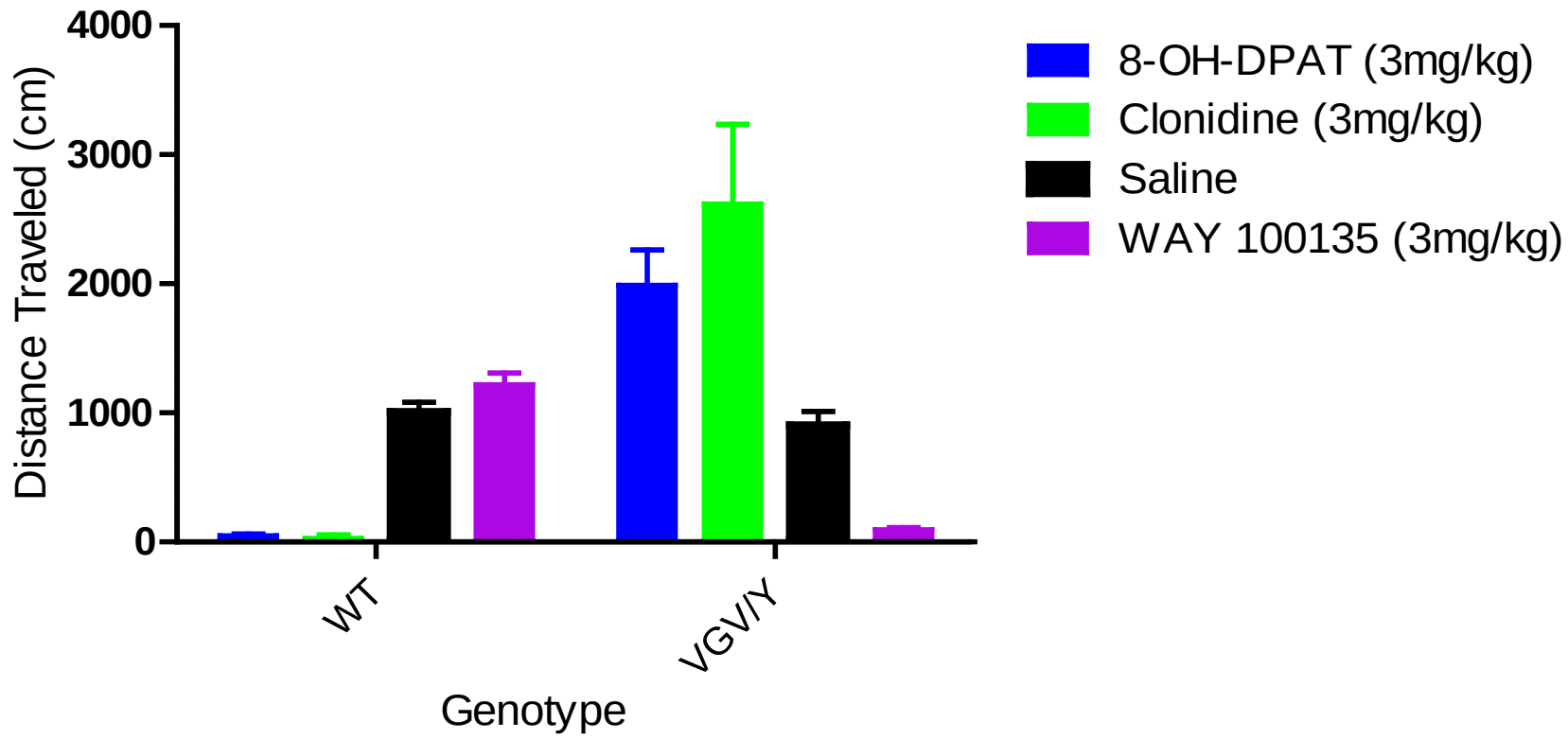

Figure 36: Opposing Effects Of Ligands In Wild Type Vs. VGV/Y Mice Changes In Locomotor Behavior In Wild Type And VGV/Y Mice In Response To Treatment With Various Drugs.

Conclusions

The current work further illuminates some of the inherent problems in present methodologies. Global modification of genes often goes far beyond the intended effects. Our knowledge of the roles of many genes during development is limited enough that we are unable to predict the effects that these modifications will have on intact organisms. Further studies should also include analyses of regional modification of genes, both in terms of developmental period and CNS localization. One such study (Kimura et al., 2009) examines phenotypic changes in mice overexpressing the 5-HT_{2C} receptor in forebrain. They find an increase in anxiety-behavior and reduced activity in a novel environment, interestingly we also have observed hypolocomotion in novel environments under basal conditions in VGV/Y mice. Similar work will allow for a clearer picture of the behavioral and biochemical effects 5-HT_{2C} receptor function and dysfunction at a regional level.

In summary, i*n vitro* studies in transfected cell lines have shown that RNA editing of $5HT_{2C}$ RNA transcripts profoundly alters the signaling properties of the $5HT_{2C}$ receptor protein. The current studies utilizing genetically modified mice solely expressing the most extensively edited isoform of the $5HT_{2C}$ receptor show, for the first time, that the G-protein coupling capacity in the brain is altered, i.e., expression of fully edited $5HT_{2C\text{-}VGV}$ receptor isoform within the brain blunts receptor-G-protein coupling. We further show that changes in the degree of

editing significantly alters the density of receptor binding sites within the brain, suggesting that the pattern of protein isoforms, and hence the inferred overall function of the receptor protein, may not reflect the pattern of RNA isoforms. This conclusion has important implications for human studies of disease-related alterations in RNA editing of the $5HT_{2C}$ receptor, where inferences about protein function are based on the profile of RNA edited isoforms. Our studies further emphasize the critical need for a method that differentiates $5HT_{2C}$ receptor protein isoforms in brain tissue.

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