ALTERED ISOFORM EXPRESSION OF THE SEROTONIN 2C RECEPTOR DISRUPTS NORMAL MATERNAL CARE

By

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LIST OF ABBREVIATIONS

5HT: serotonin, 5-hydroxytryptamine

A: adenosine

ACh: acetylcholine

ADAR: adenosine deaminase that acts on RNA

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

cAMP: cyclic adenosine monophosphate

BNST: bed nucleus of the stria terminalis

CeA: central nucleus of the amydgala

CNS: central nervous system

DA: dopamine

E: embryonic day

EDTA: Ethylenedinitrilotetraacetic acid

EPM: elevated plus maze

G: guanosine

GABA: γ-aminobutyric acid

GFAP: glial fibrillary acidic protein

GLUR2: glutamate receptor, ionotropic, AMPA 2, GRIA2

GLUR3: glutamate receptor, ionotropic, AMPA 3, GRIA3

GLUR4: glutamate receptor, ionotropic, AMPA 4, GRIA4

GLUR5: glutamate receptor, ionotropic, kainate 1, GRIK1

GLUR6: glutamate receptor, ionotropic, kainate 2, GRIK2

GPCR: g-protein coupled receptor

H&E: hematoxylin & eosin

I: inosine

KO: knock-out (mouse)

Kv1.1: potassium voltage-gated channel, Shaker-related subfamily, member 1, KCNA1

LSD: lysergic acid diethylamide

LTD: long-term depression

LTP: long-term potentiation

mCPP: *m*-chlorophenylpiperazine

NAc: nucleus accumbens

NeuN: neuronal nuclei

P: postnatal day

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PI: phosphoinositol

PLA_2: phospholipase A_2

PLC: phospholipase C

PLD: phospholipase D

PNS: peripheral nervous system

qPCR: quantitative real-time RT-PCR

RT: reverse transcription

SSRI: selective serotonin reuptake inhibitor

VTA: ventral tegmental area

WT: wild-type (mouse)

CHAPTER I

INTRODUCTION

Serotonin

The monoamine neurotransmitter serotonin (5-hydroxytryptamine; 5HT) modulates a wide variety of behaviors including appetite, locomotion, sexual behavior, nociception, sleep, drug abuse and hallucinations (Dubovsky and Thomas, 1995; Pandey et al., 1995). Serotonergic neurons are among the first neurons to be generated in mammalian development, with release of 5HT occurring before other synapses are established (Gaspar et al., 2003). 5HT-containing cell bodies and dendrites are located in the raphe nuclei of the brainstem and are contained within the B1-B9 cell groups (Dahlstrom and Fuxe, 1964). B1-B5 form the caudal division and project to the spinal cord while B6-B9 form the rostral division and project to forebrain regions (Figure 1) (Lidov and Molliver, 1982; Wallace and Lauder, 1983).

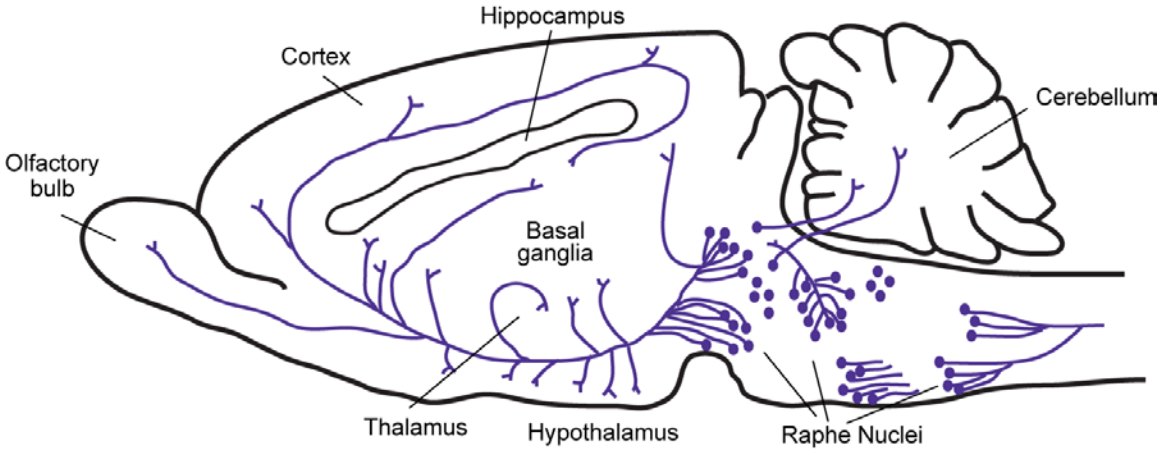

Figure 1: Major serotonergic pathways in the rodent brain. Serotonergic neurons are located in the raphe nuclei in the brainstem. Projections into the spinal cord terminate in the dorsal horn and are important in pain sensation while forward projections run into the medial forebrain bundle and terminate forebrain regions.

These neurons are formed early in development, on embryonic days (E) 10-12 in mouse, and within the first month of development in primates (Levitt and Rakic, 1982). In cultures of raphe neurons, 5HT increases axon outgrowth and may inhibit other neuronal precursors from differentiating into serotonergic neurons (De Vitry et al., 1986; Whitaker-Azmitia and Azmitia, 1989). Given the existence of multiple distinct 5HT receptor subtypes, it is thought that each receptor has a limited set of actions during specific periods in development (Gaspar et al., 2003). In developing mouse embryos, 5HT appears to act in a dose-dependent manner, regulating activation of specific 5HT receptor subtypes (Lauder et al., 2000). Since 5HT is released from nerve terminals in virtually all regions in the CNS, the serotonergic system is implicated in nearly all physiological functions and thus, perturbations of this system may be responsible for the pathogenesis of several diseases including hypertension, hormonal dysfunction, depression, anxiety, and migraine (Fink and Gothert, 2007).

5HT receptors

Molecular, physiological and pharmacological studies have provided evidence for at least fourteen distinct 5HT receptor subtypes which have been classified into seven families ($5HT_1$-$5HT_7$) based on genomic structure, amino acid sequence similarities, relative ligand binding affinities and ability to couple to specific signal transduction pathways (Hoyer et al., 1994) (Figure 2).

Each of these families, with the exception of the $5HT_3$ family, belongs to a larger family of seven transmembrane-spanning, G-protein-coupled receptors (GPCRs) with a carboxyl terminus oriented towards the cytoplasm and an amino terminus oriented extracellularly; the $5HT_3$ receptor is not a GPCR, but rather a ligand-gated ion channel. The GPCRs contain 3 extracellular domains (e1-e3) which may be glycosolated, and contain cysteine residues that may form disulfide bonds to provide structural

conformation of the G-protein (Raymond et al., 2001). Three intracellular domains (i1-i3) contain sites for phosphorylation by serine-threonine kinases as well as sites for interactions with other G-proteins (Raymond et al., 2001). Neurotransmitter binding alters the conformation of the receptor and exposes a binding site for the α-subunit of the interacting heterotrimeric G-protein complex, thus triggering a GTP-GDP exchange that leads to the dissociation of the α subunit from the βγ complex of the heterotrimer and propagating signal transduction.

Receptor Subtype	Signal Transduction Pathway	G Protein
$5HT_{1A}$ $5HT_{1B}$ $5HT_{1D}$ $5HT_{1E}$ $5HT_{1F}$	↓ adenylate cyclase	G_i/G_o
$5HT_{5A}$ $5HT_{5B}$	↓ adenylate cyclase	G_i/G_o
$5HT_{2A}$ $5HT_{2B}$ $5HT_{2C}$	↑ PLC; PI hydrolysis	G_q/G_{11}
$5HT_4$ $5HT_6$ $5HT_7$	↑ adenylate cyclase	G_s
$5HT_3$	Ligand-gated channel	—

Figure 2: 5HT receptor family. There are at least 14 distinct 5HT receptors that have been classified into 7 families based on sequence homology, ligand affinities and coupling to signal transduction pathways.

$5HT_1$ receptors

The $5HT_1$ class of receptors includes 5 members: $5HT_{1A}$, $5HT_{1B}$ (formally $5HT_{1D\beta}$), $5HT_{1D}$ (formally $5HT_{1D\alpha}$), $5HT_{1E}$ and $5HT_{1F}$ (formally $5HT_{1E\beta}$, $5HT_6$). All members of this family couple to G_i/G_o messenger systems, inhibiting adenylyl cyclase activity thereby

decreasing levels of cAMP. The genes encoding these receptors are all intronless and thus alternatively spliced isoforms do not exist. $5HT_{1A}$ receptors have the highest density in limbic areas in the brain (Lanfumey and Hamon, 2004); $5HT_{1A}$ autoreceptors are localized in cell bodies and dendrites of 5HT neurons of the dorsal and median raphe nuclei and are thought to be important for selective serotonin reuptake inhibitor (SSRI) mechanism of action (Riad et al., 2000; Sotelo et al., 1990). $5HT_{1A}$ receptors are also expressed post-synaptically where they can inhibit neuronal firing through the activation of G-protein-gated inwardly rectifying potassium (GIRK) and Ca^{2+} channels (Andrade et al., 1986; Oleskevich, 1995; Sodickson and Bean, 1998). $5HT_{1A}$ receptors are thought to play a role in neuroendocrine function and thermoregulation (Goodwin et al., 1985; Goodwin et al., 1987), sexual behavior (Ahlenius and Larsson, 1989; Maswood et al., 1998), memory (Edagawa et al., 1998), depression and anxiety (Blier et al., 1997; Parks et al., 1998) and migraine therapy (Leone et al., 1998). $5HT_{1B}$ receptors are expressed in the CNS with greatest levels of expression observed in basal ganglia and frontal cortex (Bennett-Clarke et al., 1993; Boschert et al., 1994). $5HT_{1B}$ receptors are thought to play a role in pathophysiology of obsessive-compulsive disorder, addiction and depression (Gingrich and Hen, 2001). $5HT_{1D}$ receptors are expressed at lower levels than $5HT_{1B}$ receptors and have been found in dorsal raphe, caudate and cortex as well as in the human heart (Bruinvels et al., 1994; Bruinvels et al., 1993). Historically, it has been difficult to discern their separate functions due to the lack of drugs that can specifically target $5HT_{1B}$ receptors over $5HT_{1D}$ receptors (Hoyer and Middlemiss, 1989) but $5HT_{1D}$ receptors are thought to be expressed on the terminals of 5HT neurons where they can serve as autoreceptors and also on other neuronal terminals (dopamine (DA), γ-aminobutyric acid (GABA), glutamate) where they can inhibit neurotransmitter release (Pauwels, 1997; Riad et al., 2000). $5HT_{1E}$ receptors were first discovered in binding homogenates of human frontal cortex (Leonhardt et al., 1989), but subsequent studies

have not been able to elucidate their functional role due to the lack of specific pharmacological agents. The same is also true of $5HT_{1F}$ receptors, but within the CNS, they are concentrated in raphe, hippocampus, cortex, striatum, thalamus and hypothalamus (Adham et al., 1993).

$5HT_3$ receptors

$5HT_3$ receptors (formally M) have been classified as a ligand-gated ion channel based on electrophysiological features and sequence (Boess and Martin, 1994). These receptors are found on neurons in both the CNS and PNS. Within the CNS, $5HT_3$ receptors are present in hippocampus (CA1 pyramidal cell region), dorsal motor nucleus of the solitary tract and the area postrema (Laporte et al., 1992); within the PNS, they are located on sensory neurons and pre- and post-ganglionic autonomic neurons. This receptor subclass is thought to regulate gastrointestinal motility and intestinal secretion (De Ponti and Tonini, 2001).

$5HT_4$ receptors

$5HT_4$ receptors couple to G_s to stimulate adenylyl cyclase activity and are mainly present in hypothalamus, nucleus accumbens, amygdala, basal ganglia and hippocampus (Medhurst et al., 2001; Vilaro et al., 2002). While only one subtype of this receptor is known, there are at least 10 differentially distributed, functional splice variants in humans with alternative C-terminal domains (Blondel et al., 1998; Blondel et al., 1997; Claeysen et al., 1997; Claeysen et al., 1999). Indirect evidence suggests that $5HT_4$ receptors are localized post-synaptically but may also be present on terminals of other neurons where they can regulate neurotransmitter (DA, 5HT, GABA, ACh) release and enhance synaptic transmission (Bockaert et al., 2004). Activation of $5HT_4$ receptors produces a long-lasting inhibition of voltage-gated K^+ channels, including Ca^{2+}-activated

K^+ channels (Ansanay et al., 1995; Fagni et al., 1992). $5HT_4$ receptor agonists can modulate long-term potentiation (LTP) and long-term depression (LTD) to modulate the responses to learning, memory, reward, novelty and stress (Bockaert et al., 2004; Compan et al., 2004; Kemp and Manahan-Vaughan, 2004, 2005).

$5HT_5$ receptors

$5HT_5$ receptors include two family members: $5HT_{5A}$ and $5HT_{5B}$. Only the $5HT_{5A}$ receptor is found in humans; $5HT_{5B}$ has been found only in rats and mice and is believed to be a pseudogene in humans (Erlander et al., 1993; Matthes et al., 1993; Plassat et al., 1992; Rees et al., 1994; Wisden et al., 1993). The coupling status of this receptor is unknown in native neuronal tissue but cell-culture studies have shown coupling to both G_i/G_o and $G_{q/11}$ (Noda et al., 2003). *In situ* hybridization studies have revealed the greatest level of expression in hypothalamus, hippocampus, corpus callosum and glia, but not in the peripheral tissues (Plassat et al., 1992; Rees et al., 1994). Their co-localization with glial fibrillary acidic protein (GFAP) suggests mainly astrocytic expression (Matthes et al., 1993). $5HT_5$ receptors have high affinity for lysergic acid diethylamide (LSD) and 5-carboxyamidotryptamine (Waeber et al., 1998).

$5HT_6$ receptors

$5HT_6$ receptors couple to G_s proteins and are almost exclusively expressed in the CNS with high concentrations in striatum, amygdala, nucleus accumbens, hippocampus, cortex and olfactory tubercle (Gerard et al., 1996; Grimaldi et al., 1998; Sleight et al., 1998). Low levels of $5HT_6$ receptors can be found in the adrenal gland and the stomach (Monsma et al., 1993; Ruat et al., 1993a). However, they are largely localized on GABAergic rather than 5HT neurons (Gerard et al., 1996; Sebben et al., 1994). This receptor subtype has high affinity for typical and atypical antipsychotics, including

clozapine (Meltzer et al., 2003). They may also modulate cholinergic neurons, suggesting a function in learning and memory, thus serving as a treatment target in cognitive disorders (Bourson et al., 1998; Branchek and Blackburn, 2000). Furthermore, $5HT_6$ receptors have also been implicated in psychosis, anxiety, epilepsy and mood disorders (Ohmori et al., 2001).

$5HT_7$ receptors

$5HT_7$ receptors (formally $5HT_X$) also couple to G_s proteins and are mainly found in thalamus, hypothalamus, cortex, hippocampus and amygdala (Gustafson et al., 1996; Hedlund and Sutcliffe, 2004; Thomas and Hagan, 2004). This receptor is thought to play a role in the control of circadian rhythms, as they are expressed in the suprachiasmatic nucleus of the hypothalamus (Lovenberg et al., 1993). They chiefly localize to soma and axon terminals of GABAergic neurons. Outside the CNS, $5HT_7$ receptors are also found in intra- and extra-cranial blood vessels and meningeal tissues as well as in the periphery where they mediate smooth muscle relaxation in various organs (Hedlund and Sutcliffe, 2004). Alternative splicing of this receptor can generate at least four spliced isoforms, which differ in the C-terminus, but do not appear to differ in pharmacological properties, signal transduction or tissue distribution (Ruat et al., 1993b). $5HT_7$ receptors demonstrate high affinity for atypical antipsychotic agents and may mediate some of their effects (Roth, 1994). These receptors may also be involved in learning and memory as $5HT_7$ receptor knockout mice have a decrease in synaptic plasticity and contextual fear conditioning (Thomas and Hagan, 2004).

$5HT_2$ receptor family

The $5HT_2$ class of receptors includes three members: $5HT_{2A}$ (formerly D, $5HT_2$), $5HT_{2B}$ (formerly $5HT_{2F}$) and $5HT_{2C}$ (formerly $5HT_{1C}$) (Hoyer et al., 1994). The $5HT_2$ family

preferentially couples to $G_{q/11}$, subsequently leading to the activation of phospholipase C (PLC) to increase the hydrolysis of inositol phosphates and subsequently the cytosolic calcium concentration by release from intracellular stores (Figure 3). While the $5HT_2$ family shares 46-50% overall sequence identity, each member of the $5HT_2$ family has a distinct expression pattern.

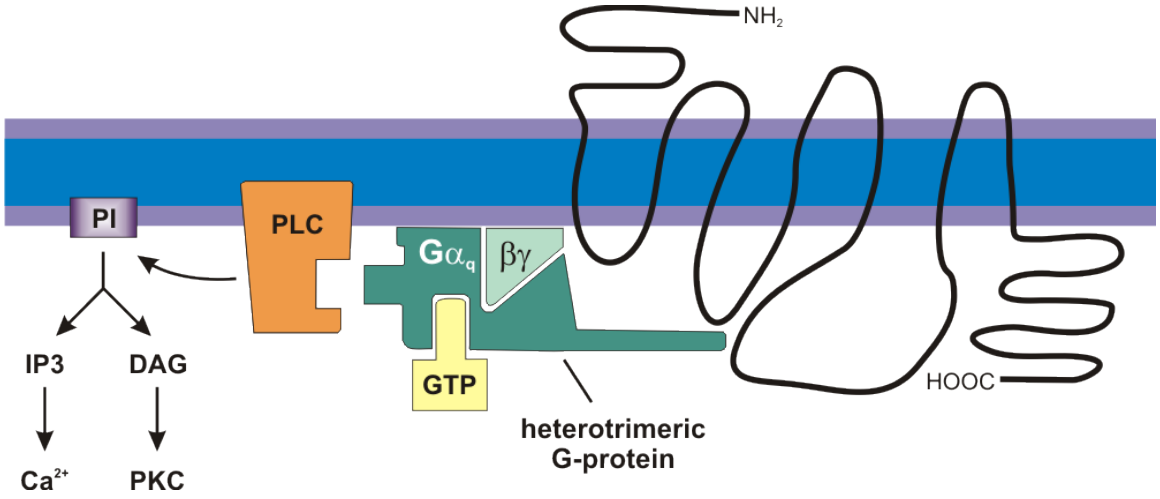

Figure 3: $5HT_2$ receptor family topology and coupling. The $5HT_2$ receptor family preferentially couples to $G_{\alpha q}$ to activate phospholipase C (PLC). PLC promotes the hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphophate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG activates protein kinase C (PKC) while IP_3 binds to its intracellular receptor to promote the intracellular calcium (Ca^{2+}) release.

$5HT_{2A}$ receptors

$5HT_{2A}$ receptors are widely distributed within peripheral and central tissues. Peripherally, they modulate contractile responses in vascular smooth muscle and play a role in platelet aggregation. Within the CNS, $5HT_{2A}$ receptors are located within cortex, olfactory tubercle, nucleus accumbens, hippocampus and basal ganglia and these receptors are thought to be responsible for regulation and stimulation of hormone secretion (ACTH, cortisol, oxytocin, prolactin) (Jakab and Goldman-Rakic, 1998). $5HT_{2A}$

receptors also activate phospholipase A2 (PLA_2) and phospholipase D (PLD) in addition to PLC (Raymond et al., 2001). Surprisingly, some antagonists can induce $5HT_{2A}$ receptor desensitization and internalization both in vivo and in vitro (Roth et al., 1991; Sanders-Bush et al., 1990). $5HT_{2A}$ receptor knockout animals have reduced inhibition in conflict anxiety paradigms without affecting fear-conditioned and depression-related behaviors (Weisstaub et al., 2006).

$5HT_{2B}$ receptors

$5HT_{2B}$ receptors are located primarily in the fundus of the stomach. Activation of $5HT_{2B}$ receptors leads to smooth muscle contraction. They are also present in human liver and kidney as well as endothelial cells of pulmonary arteries where they are responsible for vasorelaxation upon activation. Within the CNS, $5HT_{2B}$ receptor-like immunoreactivity can be seen in cerebellum, lateral septum, hypothalamus and medial amygdala (Sanden et al., 2000; Wainscott et al., 1993). $5HT_{2B}$ receptors may mediate anxiolytic properties in social interactions as well as hyperphagia and reduction in grooming frequency. $5HT_{2B}$ receptors also regulate embryonic morphogenesis; $5HT_{2B}$ receptor antagonists have been shown to interfere with cranial neural crest cell migration and induced their apoptosis. $5HT_{2B}$ receptor knockout animals lead to embryonic and neonatal lethality as a result of specific heart defects (Nebigil et al., 2000). Surviving animals have severe ventricular hypoplasia associated with impaired proliferation of myocytes (Nebigil et al., 2000; Nebigil et al., 2001).

$5HT_{2C}$ receptors

$5HT_{2C}$ receptor expression is limited to the CNS, with the highest levels of expression in choroid plexus and significant expression in hippocampus, striatum, amygdala and hypothalamus (Pasqualetti et al., 1999; Teitler and Herrick-Davis, 1994).

In addition to the activation of PLC, $5HT_{2C}$ receptors can activate PLA_2-mediated arachidonic acid (AA) release, but some agonists preferentially activate PLA_2 or PLC (Raymond et al., 2001), a property known as agonist-directed trafficking of receptor stimulus. This means that a single receptor subtype can couple with different efficacies to several downstream signaling pathways, depending on the nature of the agonist. Similar to the properties of $5HT_{2A}$ receptors, $5HT_{2C}$ receptors are down-regulated following chronic antagonist exposure (Van Oekelen et al., 2003), although some studies have suggested that only agonists and partial agonists of $5HT_{2C}$ receptors have the ability to cause receptor down-regulation (Schlag et al., 2004).

Using a yeast two-hybrid screen, the $5HT_{2C}$ receptor has been shown to interact with a novel multivalent PDZ (named after three proteins: Postsynaptic density-95, *Drosophilia* Discs large and Zonula occuludens-1) protein called multi-PDZ domain protein (MUPP1). PDZ domain proteins play important roles in the organization and/or regulation of signaling events in cells. MUPP1 contains 13 PDZ domains (Ullmer et al., 1998) and PDZ domain 10 appears to be critical for $5HT_{2C}$ receptor association (Becamel et al., 2001; Parker et al., 2003). Interaction between MUPP1 and the $5HT_{2C}$ receptor leads to a conformational change in MUPP1 (Becamel et al., 2001) and deletion of the 2C receptor PDZ recognition motif (SXV) prevents phosphorylation of the receptor and delays resensitization of receptor responses (Backstrom et al., 2000). When MUPP1 and $5HT_{2C}$ receptor cDNAs are transiently co-transfected into COS-7 cells, both proteins colocalize together and form clusters within intracellular membranes and on the cell surface; MUPP1 and $5HT_{2C}$ receptor mRNA also display overlapping patterns of expression in rat brain (Becamel et al., 2001). Further studies reveal that 5HT regulated MUPP1 interaction with $5HT_{2C}$ receptors in a dose-dependent manner and alkaline phosphatase treatment restored the interaction, suggesting that MUPP1 interactions with $5HT_{2C}$ receptors are dynamically regulated by phosphorylation (Parker et al., 2003).

$5HT_{2C}$ receptor-dependent modulation of neurotransmitter release

Acetylcholine

$5HT_{2C}$ receptors are involved in the regulation and modulation of several neurotransmitters including acetylcholine (ACh). The cholinergic system plays an important role in memory and cognition; increasing the concentration of ACh in the synaptic cleft is thought to be an important therapeutic target in Alzheimer's dementia. Using *in vivo* microdialysis in the hippocampus, Zhelyazkova-Savova *et al.* measured ACh release when rats were administered an intraperitoneal dose (IP) of *m*-chlorophenylpiperazine (mCPP) (Zhelyazkova-Savova et al., 1999). In mCPP-administered animals, ACh release in the hippocampus was increased 96% over vehicle treated animals (Zhelyazkova-Savova et al., 1999); this increase in ACh release could be prevented with an IP dose of mesulergine, a $5HT_{2A/2C}$ receptor antagonist. Increases in ACh release were only seen with systemic doses of mCPP and not local administration; these effects were also observed in the cortex, suggesting that the action of mCPP is occurring outside of the hippocampus (Zhelyazkova-Savova et al., 1999).

Dopamine

$5HT_{2C}$ receptors have been shown to control dopamine (DA) release from mesolimbic and mesocortical dopaminergic neurons (Di Giovanni et al., 1999). Following IP administration of the $5HT_{2C}$ receptor inverse agonist, SB206553, basal DA release was increased in both the nucleus accumbens (NAc, 42%) and striatum (33%). In contrast, the $5HT_{2A}$ receptor antagonist, SR46349B, or the mixed $5HT_{2A/2B/2C}$ receptor antagonist, ritanserin, failed to increase DA output in either of these regions (Di Giovanni et al., 1999). Taken together, these results suggest that $5HT_{2C}$ receptors exert a tonic inhibitory control on both mesolimbic and nigrostriatal dopaminergic pathways. Further support for this hypothesis was demonstrated by the ability of the selective $5HT_{2C}$

receptor agonist, RO 60-0175, to decrease DA release in NAc (26%) and this effect could be completely blocked by the $5HT_{2C}$ receptor antagonist, SB242084 (Di Matteo et al., 2000a; Di Matteo et al., 2000b).

However, since all of these drugs were administered systemically, the location of $5HT_{2C}$ receptors involved in these effects is unknown. $5HT_{2C}$ receptors are found in all brain regions receiving projections from mesolimbic and nigrostriatal dopaminergic neurons (Abramowski et al., 1995; Mengod et al., 1990; Molineaux et al., 1989). Double-labeled *in situ* hybridization studies revealed co-expression of $5HT_{2C}$ receptor mRNA with glutamic acid decarboxylase (GAD) mRNA, but not with tyrosine hydroxylase (TH) mRNA, indicating that $5HT_{2C}$ receptor expression is restricted to GABAergic neurons (Eberle-Wang et al., 1997).

$5HT_{2C}$ receptors mediate an indirect inhibition of DA release due to inherent constitutive activity of this receptor. Increases in DA release in both NAc and striatum were detected by microdialysis in rats systemically injected with the inverse agonist SB206553 or antagonist SB242084 (Berg et al., 2005; De Deurwaerdere et al., 2004), with observed increases of DA release much greater with SB206553. The agonist RO-0175 was able to inhibit DA release in both of these areas; SB242084 was able to counteract the inhibitory effects of RO-0175 as well as the potentiated release observed with SB206553 when co-injected (Berg et al., 2005; De Deurwaerdere et al., 2004).

To determine if DA release within the mesolimbic system was regionally-dependent, *in vivo* microdialysis experiments have been performed in the rat. Intra-ventral tegmental area (VTA) injections of antagonist (SB242084) abolished the decrease in accumbal DA outflow induced by agonist (RO-0175), but did not affect the increase in DA outflow induced by inverse agonist (SB206553). In contrast, intra-NAc injections of antagonist (SB242084) blocked both agonist (RO-0175) and inverse agonist (SB206553)-induced changes in DA outflow. However, only intra-NAc administration of

SB206553, but not intra-VTA, SB206553 alone increased basal DA outflow (Navailles et al., 2006). These studies indicate that there are anatomical differences for the constitutive activity of $5HT_{2C}$ receptors.

More recently, Navailles *et al.* (Navailles et al., 2008) have investigated the role of $5HT_{2C}$ receptors within the VTA and NAc to mediate cocaine-induced behavioral effects. Intra-VTA injections with agonist (RO-0175) at higher doses attenuated the increase in accumbal DA outflow induced by systemic administration of cocaine; intra-VTA injections of antagonist (SB242084) were unable to modify these effects. Within the NAc, local administration of agonist (RO-0175) amplified cocaine-induced DA outflow at low concentrations, while inhibiting this outflow at higher doses. Intra-NAc injections of antagonist (SB242084) resulted in opposite effects when applied at these concentrations. In the NAc, but not VTA, there exists both excitatory and inhibitory control of cocaine-induced DA outflow, suggesting a functional balance of different $5HT_{2C}$ receptor populations within these brain regions as well as within the mesolimbic DA pathway (Navailles et al., 2008).

Taken together, these studies implicate the $5HT_{2C}$ receptors in the indirect inhibition of DA release and suggest that the constitutive activity of $5HT_{2C}$ receptors helps to mediate these effects; inverse agonists at this receptor produce a more pronounced increase in basal DA release than antagonists. The inverse agonist properties of certain atypical antipsychotics (clozapine, olanzapine) may therefore be responsible for their clinically advantageous effects.

Post-transcriptional regulation of the $5HT_{2C}$ receptor

Alternative splicing

The serotonin 2C receptor primary RNA transcript can be alternatively spliced to generate at least 3 alternative splice variants that can alter its function by changing the

C-terminal region of the protein. The full length cDNA (4.8 kb) is created by splicing the predicted exon 5 splice donor to the exon 6 splice acceptor and is referred to as RNA2, encoding the full-length, fully functional 458 amino acid receptor protein (Figure 4). The shortest of these splice variants is referred to as RNA1 (or $5HT_{2C}$-tr) and results in a 95-nucleotide deletion from the 3' end region of exon 5 and creates a cDNA of approximately 4.7 kb. This deletion leads to a shift in the reading frame and a premature termination codon at nucleotide positions 840-842 to generate a protein with 172 amino acids, including 19 carboxyl-terminus amino acids that are unique (Canton et al., 1996; Xie et al., 1996). RNA1 has patterns of expression in rat brain regions overlapping with RNA2, with the choroid plexus displaying the greatest amount of RNA1 (60% of total) while most neuronal tissues contain about 20-30% expression of the RNA1 isoform (Canton et al., 1996; Xie et al., 1996). This version of the receptor displays no 5HT binding or phosphoinositol (PI) hydrolysis activity in NIH-3T3 fibroblasts (Canton et al., 1996; Xie et al., 1996). Another truncated version (RNA3 or $5HT_{2C}$R-ΔCOOH) of the receptor results from the addition of a 90-nucleotide sequence in intron 5 and splicing joins this cryptic splice donor site in intron 5 with exon 6 resulting in a cDNA of 4.9 kb. RNA3 is found in most regions of human brain, with the highest level of expression observed in spinal cord; expression can also be detected in amygdala, hippocampus and thalamus with the relative expression of RNA3 being 10-20% of that observed for RNA2 (Wang et al., 2000b). RNA3 encodes a receptor protein that is also devoid of 5HT binding or PI hydrolysis activity in NIH-3T3 and COS-7 cells (Wang et al., 2000b). Each of these alternative 5'-donor splice sites are conserved between humans, mice and rats suggesting a possible biological significance for the generation of multiple $5HT_{2C}$ receptor variants by alternative splicing.

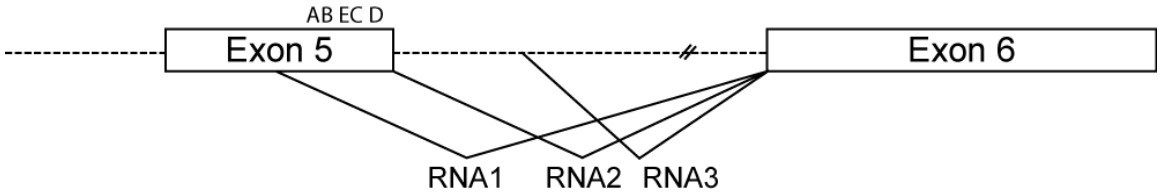

Figure 4: Alternative splicing of $5HT_{2C}$ receptor transcripts. A portion of the $5HT_{2C}$ receptor pre-mRNA containing intron 4-exon 5-intron 5-exon 6 is shown. The editing sites within exon 5 are indicated (sites A-E) and the three splicing patterns leading to RNA1, RNA2 and RNA3 are presented. Introns are represented by dashed lines while exons are represented by rectangles.

RNA editing

Sequence comparisons of rat, mouse and human $5HT_{2C}$R genomic DNA with their corresponding cDNAs revealed as many as five adenosine-to-guanosine (A-to-G) discrepancies (Burns et al., 1997; Niswender et al., 1998; Wang et al., 2000b) in the $5HT_{2C}$ receptor transcript. A-to-G discrepancies between genomic and cDNA sequences are indicative of adenosine-to-inosine (A-to-I) editing events in which specific adenosine moieties are post-transcriptionally converted to inosine by hydrolytic deamination (Polson et al., 1991). These five editing sites (termed sites A-E) were predicted to alter amino acids 157, 159 and 161, within the putative second intracellular loop of the rat and mouse receptors, to generate as many as thirty-two different mRNA transcripts encoding twenty-four distinct protein isoforms (Figure 5). Sequence analysis of cDNAs isolated from dissected rat and human brains have predicted the region-specific expression of as many as 15 major $5HT_{2C}$ receptor isoforms encoded by twenty-three distinct RNA species (Burns et al., 1997; Niswender et al., 1999) and analyses of $5HT_{2C}$ receptor expression in dissected mouse brain have revealed a similar pattern of edited RNA isoform expression (see Chapter 2, Table 1). Since the location of the editing sites is downstream of the proximal RNA1 splice site, the RNA1 isoform cannot be edited while RNA2 and RNA3 are capable of undergoing these A-to-I modifications.

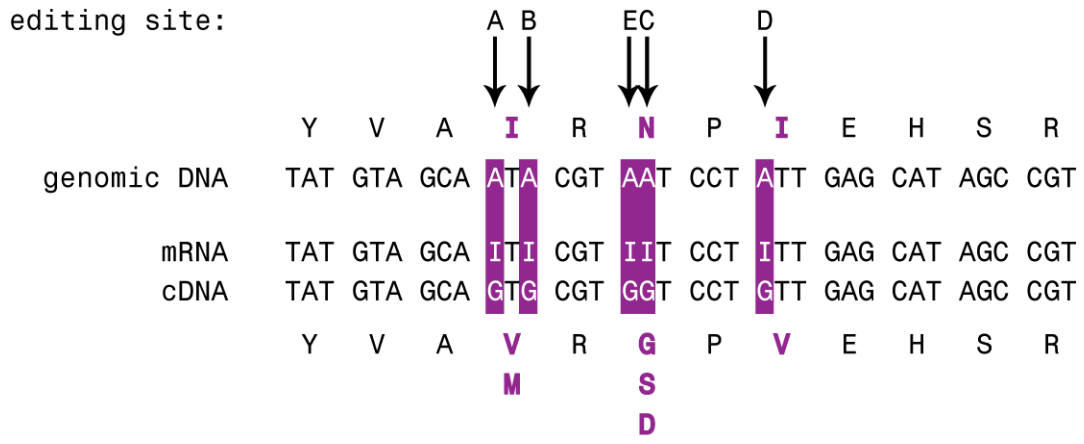

Figure 5: Nucleotide and predicted amino acid sequence alignment between mouse $5HT_{2C}$ receptor genomic DNA, mRNA and cDNA. The positions of the 5 editing sites within the nucleotide sequence are indicated by arrows. Conversion at the various positions results in the amino acid codon changes indicated; for example, an A to G substitution at the A site converts an isoleucine codon to a valine codon.

The second intracellular loop of GPCRs plays an important role in the coupling of G-proteins, especially for receptors linked to $G_{\alpha q}$ (Pin et al., 1994; Wong et al., 1990). Recent studies have shown that RNA editing alters the selectivity of $5HT_{2C}R$:G-protein coupling, where the non-edited isoform (INI) can couple to $G_{\alpha q}$, $G_{\alpha 13}$ and $G_{\alpha 15}$, while more extensively edited isoforms (VSV and VGV) lose the ability to couple to $G_{\alpha 13}$ and $G_{\alpha 15}$. The fully edited isoform (VGV), however, gains the ability to couple to $G_{\alpha 11}$ (Figure 6) (Price et al., 2001). Comparisons of receptor function between INI and VSV and VGV $5HT_{2C}R$ isoforms revealed that all three isoforms are coupled to the activation of phosphoinositide (PI) hydrolysis. Analysis of 5HT dose-response curves in transfected NIH-3T3 cells demonstrated that serotonergic agonists were 15- and 40-fold less potent when acting through the edited VSV and VGV receptor isoforms, respectively, suggesting that edited isoforms couple less efficiently to the intracellular signaling machinery (Figure 7) (Burns et al., 1997; Niswender et al., 1998). In addition to the PLC-PI pathway, the $5HT_{2C}$ receptor also couples to the phospholipase A_2-arachidonic acid

(PLA_2-AA) pathway. While serotonin was found to increase AA release as well as IP accumulation for each edited isoform examined, the potency for 5HT to stimulate AA release was less for the edited VSV and VGV receptors when compared to the INI isoform (Berg et al., 2001).

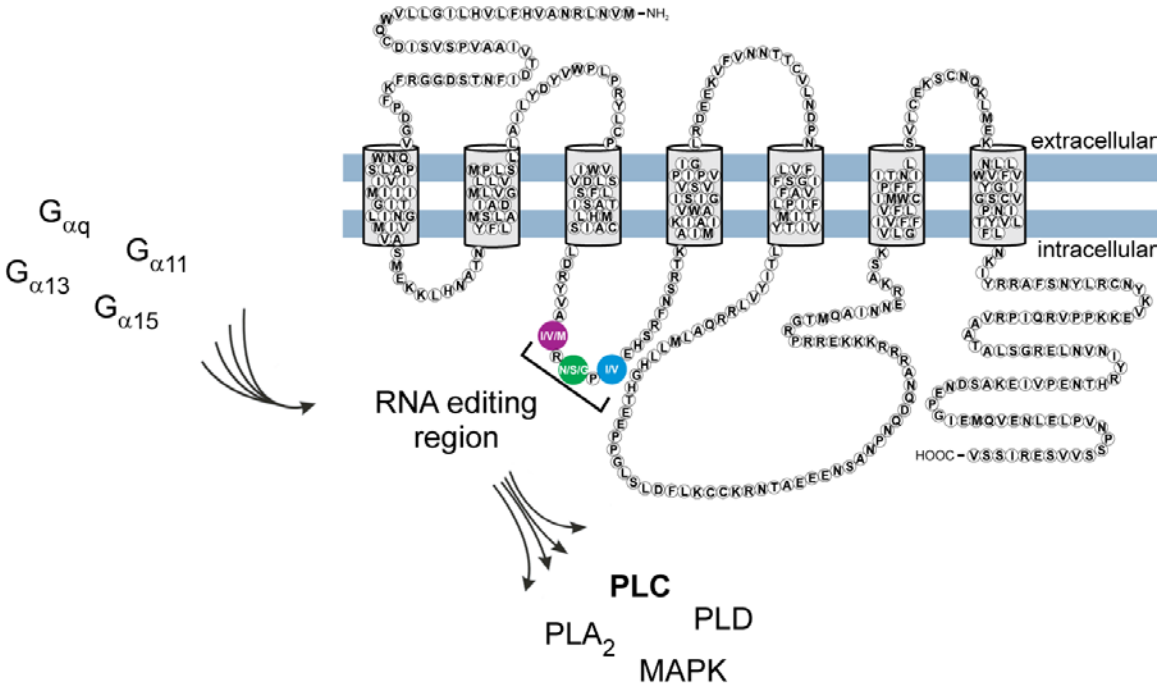

Figure 6: RNA editing of $5HT_{2C}$ receptor transcripts. A schematic diagram of the predicted topology, primary amino acid sequence and editing sites within the mouse serotonin ($5HT_{2C}$) receptor is presented. Amino acids altered by the editing of 5-HT_{2C} pre-mRNA transcripts are indicated in purple, green and blue and represent sites 157, 159 and 161, respectively. The second intracellular loop is proposed to interact with a variety of heterotrimeric G-proteins to activate the PLC, PLA_2, PLD and MAPK effector systems.

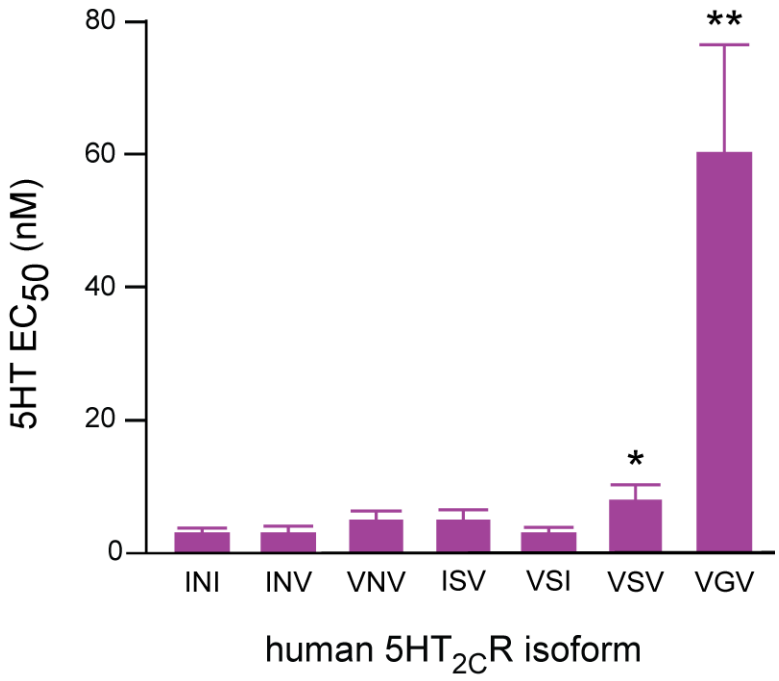

Figure 7: 5HT potency to activate phosphoinositide hydrolysis in cultured cells transiently expressing human edited $5HT_{2C}$ receptors. Human isoforms were transiently expressed in NIH-3T3 cells and the EC_{50} of 5HT activation was determined. 5HT is 15- and 40-fold less potent when acting through edited VSV and VGV receptor isoforms, respectively. Values represent the mean±SEM; *: $p<0.05$, **:$p<0.01$. Data from Niswender et al., 1999.

Studies with $5HT_{2C}$ receptor have indicated that this receptor displays constitutive activity and therefore does not fit the simple ternary complex model for GPCRs (Niswender et al., 1999). This property of the $5HT_{2C}$ receptor was first identified when it was observed that inverse agonists such as clozapine were able to reduce the basal level of PI hydrolysis, indicating that the receptor is able to spontaneously isomerize into the active receptor state (R*), thereby activating G-proteins in the absence of agonist (Barker et al., 1994; Grotewiel and Sanders-Bush, 1999). Competition binding studies using human isoforms of the $5HT_{2C}$ receptor revealed a guanine nucleotide-sensitive high-affinity state, only observed for the INI receptor, providing direct evidence that RNA editing generates $5HT_{2C}$ receptor isoforms that differ in G-protein coupling efficiency

(Figure 8) (Niswender et al., 1999), affecting both basal activity and ligand potency (Herrick-Davis et al., 1999).

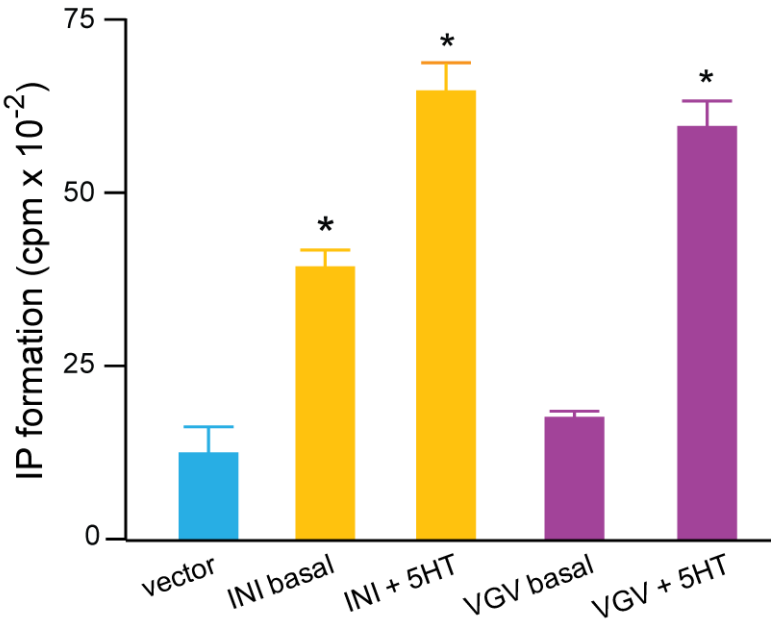

Figure 8: Constitutive activity of transiently expressed $5HT_{2C}^{INI}$ and $5HT_{2C}^{VGV}$ receptors. cDNAs for edited receptors were transiently expressed in COS-7 cells and [^{3}H] inositol monophosphate formation was measured under basal and stimulated conditions. Values represent mean±SEM; *: $p<0.05$. Data from Niswender et al., 1999.

RNA editing of the $5HT_{2C}$ receptor may also play a role in receptor desensitization and trafficking of edited receptor isoforms. Using HEK293 cells, Marion *et al.* (Marion et al., 2004) have shown decreased surface receptor expression of isoforms with the greatest constitutive activity, namely the INI isoform. The VGV isoform is expressed at the cell surface under basal conditions and is rapidly internalized into intracellular vesicles in the presence of a $5HT_{2C}$ receptor agonist. In contrast, the INI isoform was predominately localized to intracellular vesicles and stimulation with 5HT did not significantly alter this distribution. Treatment with the $5HT_{2C}$ receptor inverse agonist, SB206553, however did result in a translocation of the INI isoform to the plasma membrane surface. Similar to other GPCRs, $5HT_{2C}$ receptor isoforms are internalized in

a βarrestin2 (βarr2)-dependent manner, but the distribution of edited receptor isoforms is dependent on the constitutive activity of the receptor with the VGV isoform requiring 5HT stimulation to traffic βarr2 to the surface.

RNA editing has also been suggested to play a role in differential responses to therapeutic agents. Lysergic acid diethylamide (LSD) behaves as a partial or nearly full agonist when acting through the INI isoform, but for the VGV isoform, LSD had an attenuated ability to activate the PI pathway when compared to serotonin (Backstrom et al., 1999) and actually blocks 5HT signaling (Niswender et al., 2001). Clozapine, an atypical antipsychotic used in the treatment of schizophrenia, has been shown to behave as an inverse agonist at the $5HT_{2C}$ receptor. Inverse agonists have been demonstrated to have higher affinity for the inactive R form as compared to the active R* conformation of the receptor. Further studies have indicated that clozapine displays a significantly greater affinity for VGV as compared to the INI isoform (Fitzgerald et al., 1999). Clozapine and the typical neuroleptic, loxapine, decrease basal PI hydrolysis when acting through the INI isoform, but do not have this effect on the VGV isoform (Niswender et al., 2001).

Tetracyclic antidepressants, such as mirtazapine and mianserin, have been shown to suppress constitutive activity at recombinant and native $5HT_{2C}$ receptors. In HEK293 cells transiently co-transfected with $5HT_{2C}R^{INI}$ and G_q, Chanrion *et al.* (Chanrion et al., 2007) have shown that mirtazapine decreased basal IP production and therefore constitutive activity in a concentration-dependent manner; this effect is also observed for mianserin but its maximal effects are less pronounced. The decrease in constitutive activity can be abolished by treatment with the $5HT_{2C}$ receptor antagonist, SB242084. Prolonged treatment with these tetracyclic antidepressants also increases the cell-surface expression of $5HT_{2C}$ receptor isoforms, as well as increase 5HT-induced IP production. In studies of cultured cortical neurons pre-treated with mirtazapine, 5HT

induced transient Ca^{2+} release. Taken together, these results suggest that inverse agonists may be more beneficial therapeutically than antagonists in abolishing the activity of overactive (less edited) $5HT_{2C}$ receptor isoforms.

These data suggest that RNA editing could play a role in the pathophysiology of schizophrenia and/or depression. Recent studies have observed alterations in $5HT_{2C}$ receptor editing are observed in patients diagnosed with schizophrenia (Sodhi et al., 2001), and suicide victims with a history of major depression (Gurevich et al., 2002b; Niswender et al., 2001), suggesting that editing of the $5HT_{2C}$ receptor may be involved in neuropsychiatric disorders and the maintenance of appropriate serotonergic tone.

RNA editing may also be regulated by sustained changes in serotonergic neurotransmission. Chronic administration of fluoxetine, an SSRI, to mice leads to changes in RNA editing at the E, C and D sites; E and C site editing increases while D site editing decreases in these treated animals (Gurevich et al., 2002a). These changes are opposite to the editing frequencies observed in brains of patients with major depression, suggesting that chronic fluoxetine treatment can prevent depression-specific changes in $5HT_{2C}$ receptor mRNA editing (Gurevich et al., 2002a). In attempts to more fully define the role of serotonergic neurotransmission in RNA editing, Gurevich *et al.* depleted mice of 5HT with treatment of para-chlorophenylalanine (pCPA), a potent irreversible inhibitor of tryptophan hydroxylase. pCPA treated animals had decreased expression of more fully edited (and less active isoforms) (Gurevich et al., 2002a). Conversely, animals treated with a $5HT_{2A/2C}$ receptor agonist, (+/-)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (DOI), increases the expression of these more edited isoforms (Gurevich et al., 2002a). Neither treatment altered $5HT_{2C}$ receptor mRNA levels, indicating that RNA editing of $5HT_{2C}$ receptor transcripts is the primary mechanism by which $5HT_{2C}$ receptors can maintain their signaling properties in response to changes in serotonergic neurotransmission.

Small nucleolar RNAs and their role in $5HT_{2C}$ receptor function

Small nucleolar RNAs (snoRNAs) belong to a class of non-coding RNAs that are localized to the nucleolus and function mainly as guide RNAs to direct site-specific nucleoside modifications of ribosomal RNA (rRNA) and small nuclear RNA (snRNA) (Cavaille et al., 1996; Filipowicz and Pogacic, 2002). Most mammalian snoRNAs are expressed ubiquitously, but some are expressed exclusively in the brain (Cavaille et al., 2000; Huttenhofer et al., 2001; Rogelj and Giese, 2004). Two classes of snoRNAs, H/ACA box and C/D box, each have brain specific family members and are named based on their targets and conserved sequence motifs.

H/ACA box snoRNAs contain two conserved sequence motifs: a box H (ANANNA) nucleotide sequence and an ACA sequence at their 3' end. Their main function is to direct pseudouridylation, the conversion of uridine to psuedouridine, with the conversion site determined by the formation of a snoRNA-rRNA hybrid (Balakin 1996). C/D box snoRNAs have two conserved terminal sequence motifs, box C (RUGAUGA) and box D (CUGA), with a matching set of internal sequence motifs C' and D'; C/D box snoRNAs direct the 2'-*O*-methylation of the ribose moiety in the nucleotide five bases upstream of the D (or D') box (Cavaille et al., 1996; Kiss-Laszlo et al., 1996).

Seven brain-specific snoRNAs have been cloned from mice; MBII-12, MBII-48, MBII-49, MBII-52, MBII-78 and MBII-85 belong to the C/D box class while MBI-36 belongs to the H/ACA box class. MBII-13 and MBII-78 are encoded by single copy genes, while the remaining brain-specific snoRNAs are found in multiple copies within the same locus (Huttenhofer et al., 2001). In humans, HBII-13, HBII-52 and HBII-85 are mapped to the 15q11-q13 region, a region implicated in Prader-Willi Syndrome; HBI-36 maps to intron 2 of the $5HT_{2C}$ receptor.

The expression of MBI-36, MBII-48, MBII-52 and MBII-85 is not uniform within the brain; their expression is most prominent in hippocampus and amygdala with MBI-36

expressed almost exclusively in the choroid plexus while MBII-52 is absent in the choroid plexus (Cavaille et al., 2000). MBII-52 (as well as the human ortholog HBII-52) is unique in that its potential target is known; it exhibits an 18-nucleotide complementarity to the $5HT_{2C}$ receptor in the region of the RNA editing sites (Cavaille et al., 2000) and is predicted to 2'-*O*-methylate the ribose moiety for the adenosine at the edited C-site. Methylation at this position has been shown to decrease the rate of ADAR-mediated deamination and therefore MBII-52 is predicted to represent a negative regulator of RNA editing at the C-site (Cavaille et al., 2000). To address a potential role for MBII-52 in the regulation of RNA editing, Vitali *et al.* (Vitali et al., 2005) revealed that expression of MBII-52 was able to decrease editing at the C-site (~50%) without affecting D-site editing (Vitali et al., 2005).

In addition to MBII-52's role in the regulation of RNA editing, Kishore *et al.* (Kishore and Stamm, 2006) also showed that MBII-52 regulates alternative splicing of the $5HT_{2C}$ receptor by binding to a silencing element within exon 5b. Additionally, MBII-52 promotes the formation of non-edited isoforms by masking a silencer, though this process is editing independent; this silencing element can be weakened by RNA editing at the C, D and E sites (Kishore and Stamm, 2006), suggesting that the snoRNA MBII-52, and its human ortholog HBII-52, provide another level of $5HT_{2C}$ receptor regulation for RNA editing and splicing.

$5HT_{2C}$ receptor and behavioral implications

$5HT_{2C}$ receptors have been implicated in a variety of human neuropsychiatric and behavioral disorders, including depression, dysthymia, obsessive-compulsive disorder, anxiety, schizophrenia and sleep disturbances (Dubovsky and Thomas, 1995; Frank et al., 2002; Julius, 1991; Masellis et al., 1998; Pandey et al., 1995; Teitler and Herrick-Davis, 1994). Mice lacking the $5HT_{2C}$ receptor were generated by introducing a series of

premature stop codons within exon 6, leading to a truncated version of the receptor (Tecott et al., 1995). Initial characterization of these animals found that they were more susceptible to spontaneous epileptic seizures, which resulted in lethality in some animals, especially if the seizure reached the tonic-clonic stage (Tecott et al., 1995). In addition, $5HT_{2c}$ receptor knockout (KO) mice have a 13% increase in body mass versus their wild-type littermates, with significantly larger fat stores (48%) due to hyperphagia (Tecott et al., 1995). This chronic hyperphagia leads to a maturity-onset obesity with increases in plasma insulin and leptin resembling Type 2 diabetes (Nonogaki et al., 1998).

An examination of long-term potentiation (LTP) within the hippocampus revealed a selective impairment of perforant path LTP with matching deficits in behavioral assays of dentate gyrus function (Tecott et al., 1998); $5HT_{2C}$ receptor KO animals failed to demonstrate preference for the platform-trained quadrant in the Morris Water Maze suggesting that $5HT_{2C}$ receptors may play a role in synaptic plasticity within the hippocampus (Tecott et al., 1998). Additionally, $5HT_{2C}$ receptor KO animals display deficits in sleep; KO mice had less non-REM sleep with increased and longer bouts of waking as well as enhanced responses to sleep deprivation suggesting that $5HT_{2C}$ receptors are involved in sleep regulation (Frank et al., 2002).

To investigate the potential role of $5HT_{2C}$ receptor and the regulation of cocaine responses, KO animals were used in several behavioral paradigms (Rocha et al., 2002). Baseline locomotor responses revealed increased activity in KO animals, though habituation did occur over a 4 day period. KO animals also displayed enhanced locomotor responses to cocaine with significantly increased amounts of DA release in the NAc following cocaine administration. Using self-administration studies, KO animals made significantly more lever presses, pressing the active lever twice as many times as WT animals to obtain significantly larger number of cocaine injections. These studies

implicate $5HT_{2C}$ receptors in the psychostimulant and reinforcing effects of cocaine through activation of mesolimbic DA transmission (Rocha et al., 2002).

In studies with the hypothalamic-pituitary-adrenal (HPA) axis, $5HT_{2C}$ receptors have been determined to be necessary for 5HT-induced HPA axis activation (Heisler et al., 2007a). HPA axis activation is modulated by many inputs converging on the paraventricular nucleus of hypothalamus (PVH), where corticotrophin-releasing hormone (CRH) is synthesized (Spiess et al., 1981). Within the PVH, high levels of expression of $5HT_{2C}$ receptor were found and approximately one-half of these receptors overlapped with the distribution of CRH neurons. The high affinity $5HT_{2C}$ receptor agonist, mCPP, is able to depolarize PVH neurons and the $5HT_{2C}$ receptor antagonist, RS102221, can block this effect; $5HT_{2C}$ receptor KO mice do not display increases of CRH release or CRH mRNA in response to mCPP, suggesting that 5HT enhances the activity of PVH CRH neurons via activation of $5HT_{2C}$ receptors. Furthermore, $5HT_{2C}$ receptor KO animals exhibit blunted CRH and corticosterone (CORT) release after 5HT-induced HPA axis stimulation, demonstrating the involvement of $5HT_{2C}$ receptors in HPA axis function (Heisler et al., 2007b).

$5HT_{2C}$ receptor KO animals also demonstrate decreased anxiety in several behavioral paradigms (elevated zero maze, open field, response to novel object, mirrored chamber) (Heisler et al., 2007b). To investigate the possible mechanisms through which anxiety-related behaviors are altered in KO animals, c-fos iummunoreactivity (FOS-IR), a marker of neuronal activation, was used following exposure to anxiety stimuli; KO animals were found to have selective reductions of FOS-IR in the bed nucleus of stria terminalis (BNST) and the central nucleus of the amygdala (CeA). These brain regions are rich in CRH neurons (Swanson et al., 1983) and KO animals were also found to have a blunted response in extended amygdala CRH neuronal activation in response to anxious stimuli (Heisler et al., 2007b). Taken together,

these data suggest that $5HT_{2C}$ receptor-mediated modulation of limbic CRH is important in the regulation of anxiety-related behaviors.

To determine if expression of other serotonin family receptors or the serotonin transporter (SERT) are altered in response to genetic ablation of the $5HT_{2C}$ receptor, Lopez-Gimenez *et al.* (Lopez-Gimenez et al., 2002) performed autoradiographic analysis for members within the 5HT family in $5HT_{2C}$ receptor KO animals. As expected, differences between WT and KO animals were found using radioligands that label the $5HT_{2C}$ receptor. KO animals had an absence of labeling in the choroid plexus and decreased labeling in other brain regions. However, no significant alterations were found with the remaining radiolabeled ligands in any brain region, indicating that the mouse 5HT system does not exhibit any obvious compensatory regulation of SERT or other 5HT receptor subtypes in the absence of $5HT_{2C}$ receptors. This is somewhat surprising, given that animals lacking SERT display alterations in $5HT_{1A}$ receptor (Fabre et al., 2000; Li et al., 2000), $5HT_{1B}$ receptor (Fabre et al., 2000), $5HT_{2A}$ receptor (Rioux et al., 1999) and $5HT_{2C}$ receptor (Li et al., 2003) mRNA levels and/or function, indicating that the ablation of other 5HT family members, or at least SERT, leads to adaptive compensatory alterations.

Specific Aims

The monoamine neurotransmitter serotonin (5-hydroxytryptamine; 5HT) elicits a wide array of physiological effects by binding to distinct receptor subtypes encoded by at least fourteen separate genes. RNA transcripts encoding the 2C-subtype of the serotonin receptor ($5HT_{2C}R$) are modified by a novel processing event, referred to as RNA editing, in which up to five specific adenosine residues can be converted to inosine, thereby altering the encoded amino acid residues at positions 157, 159 and 161 of the receptor to generate as many as twenty-four $5HT_{2C}$ receptor protein isoforms. These

modified receptors are expressed in a region-specific manner in the central nervous system (CNS) and demonstrate differences in their efficacy to interact with specific G-proteins. *We hypothesize that RNA editing of the $5HT_{2C}$ receptor serves to enhance genetic diversity by generating multiple protein isoforms from a single genomic locus and that these isoforms, each possessing distinct expression patterns and functional properties, serve unique physiological roles in the CNS.*

To address this hypothesis, the following specific aims were proposed:

I. To evaluate the developmental regulation of $5HT_{2C}$ receptor expression and RNA editing patterns. To characterize potential changes in RNA editing during development, male mice at various developmental stages (embryonic days (E) 15 and 19, postnatal days (P) 0, 21, adult) were collected and RNA was prepared from whole brain. RNA editing of the $5HT_{2C}$ receptor, as well as other substrates that undergo A-to-I conversion was quantified. Expression levels for ADAR1, ADAR2 and $5HT_{2C}$ receptor mRNA levels were quantified using real-time RT-PCR and the developmental patterns of expression were determined.

II. To generate a mutant strain of mouse solely expressing the non-edited (INI) isoform of the $5HT_{2C}$ receptor. To further examine the physiologic relevance of multiple, functionally-distinct $5HT_{2C}$ receptor isoforms, mice capable of expressing only the non-edited (INI) isoform were generated via targeted gene modification in embryonic stem cells. Mutant mice were assessed for potential alterations in the level of $5HT_{2C}$ receptor mRNA expression, alterations in the patterns of $5HT_{2C}$ receptor splicing and changes in $5HT_{2C}$ receptor protein levels.

III. To characterize phenotypic alterations in INI-mutant mice. Previous studies in heterologous cell systems have revealed differences in the efficacy of receptor:G-protein coupling between edited $5HT_{2C}$ receptor isoforms, yet the physiological relevance of this differential coupling has yet to be examined in a whole animal model system. Since the INI isoform of the $5HT_{2C}$ receptor exhibits the greatest basal activity and agonist potency resulting from enhanced G-protein coupling (Berg et al., 2001; Herrick-Davis et al., 1999), it is hypothesized that mutant mice bearing the INI allele will show phenotypic alterations resulting from enhanced signaling capacities compared to wild-type animals.

CHAPTER II

DEVELOPMENTAL REGULATION OF RNA EDITING

Introduction

RNA editing is a prevalent post-transcriptional processing event that can involve the substitution or deletion of RNA sequences or the modification of specific nucleoside bases. These sequence alterations serve to generate genomic diversity in the transcriptome by producing multiple protein isoforms from a single genomic locus (Gott and Emeson, 2000). In this process, adenosine residues undergo a site-selective hydrolytic deamination to inosine (A-to-I editing) in pre- and mature mRNA transcripts that is catalyzed by a family of double-stranded RNA-specific adenosine deaminases referred to as adenosine deaminases that act on RNA (ADARs) (Bass et al., 1997; Gott and Emeson, 2000).

The mammalian ADAR family has two catalytically active family members, ADAR1 and ADAR2 (Bass, 2002). Each of these enzymes contains multiple copies of a double-stranded RNA-binding motif that are required for the binding of ADARs to their target transcript(s) and an adenosine deaminase domain near the carboxyl terminus that is required for catalytic activity (Figure 9) (Hough and Bass, 1994; Kim et al., 1994a; Kim et al., 1994b; Maas et al., 1996; O'Connell and Keller, 1994; O'Connell et al., 1995). ADAR1 and ADAR2 have been shown to edit all of the identified mammalian adenosine to inosine editing sites, including transcripts encoding multiple subunits of the AMPA (GLUR2, Q/R and R/G sites; GLUR3, R/G site; GLUR4, R/G site) and kainate (GLUR5, Q/R site; GLUR6, Q/R, I/V and Y/C sites) subtypes of ionotropic glutamate receptors, the 2C-subtype of serotonin receptor ($5HT_{2C}$, sites A-E), the α3 subunit of the $GABA_A$

receptor (Gabra3, I/M site) and a voltage-gated potassium channel (Kv1.1, I/M site) (Bhalla et al., 2004; Rueter and Emeson, 1998; Rueter et al., 1999; Rula et al., 2008).

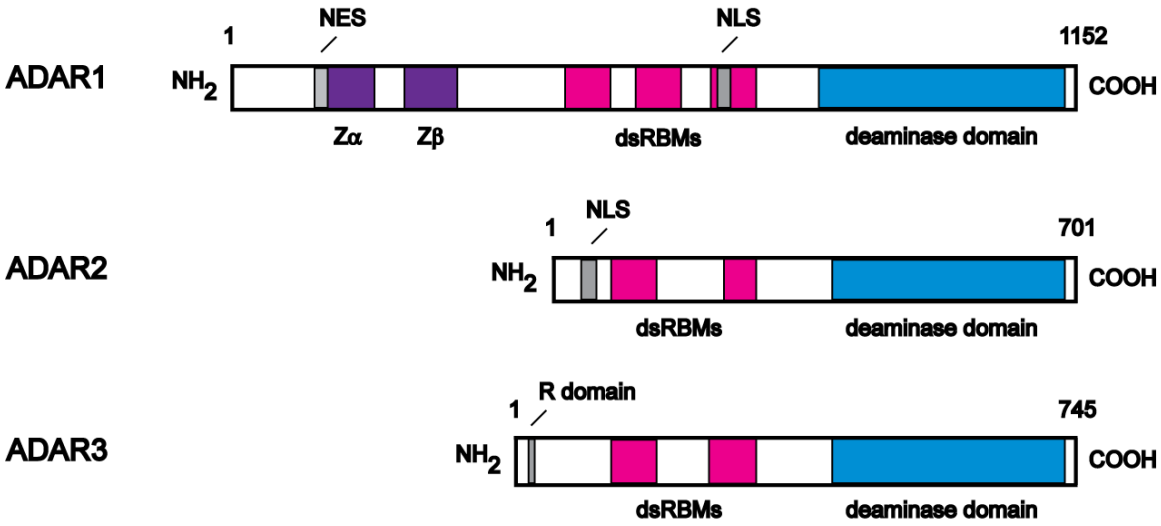

Figure 9: ADAR functional domains. A schematic representation of the ADAR1, ADAR2 and ADAR3 proteins is shown. Their relative size in amino acids, as well as the location of the putative nuclear localization (NLS) and export (NES) signals, arginine-rich (R) domain, Z-DNA binding domains (Zα and Zβ), double-stranded RNA binding motifs (dsRBMs) and the adenosine deaminase domain are presented.

RNA editing can alter the amino acid coding potential of the mRNA and thereby alter the function of the encoded protein product. Such changes include modulation of Ca^{2+} permeability (GLUR2, GLUR5, GLUR6) (Egebjerg and Heinemann, 1993; Kohler et al., 1993; Sailer et al., 1999; Sommer et al., 1991), alteration of recovery from receptor desensitization (GLUR2, GLUR3, GLUR4) (Lomeli et al., 1994), channel inactivation (Kv1.1) (Bhalla et al., 2004), and modulation of constitutive activity and G-protein coupling efficacy ($5HT_{2C}$ receptor) (Burns et al., 1997; Niswender et al., 1999; Niswender et al., 1998). In addition to alterations in coding potential, RNA editing can also affect the structure, stability, translation efficiency and splicing patterns of edited transcripts (Bass, 2002; Gott and Emeson, 2000). Moreover, environmental factors, such as early life stress and antidepressant drug administration, have been shown to alter the editing

patterns of ADAR substrates (Bhansali et al., 2007; Gurevich et al., 2002a). Alterations in RNA editing patterns for the $5HT_{2C}$ receptor have been observed in numerous human neuropsychiatric diseases, including schizophrenia, depression and suicide (Gurevich et al., 2002b; Niswender et al., 2001; Sodhi et al., 2001), whereas a decrease in editing at the Q/R site of GLUR2 mRNAs has been associated with the selective death of spinal motor neurons in sporadic amyotrophic lateral sclerosis (Kawahara et al., 2004; Kawahara et al., 2006).

Both ADAR1 and ADAR2 are known to be highly expressed in the brain, yet limited studies have been performed to characterize region-specific patterns of expression during development. One such study used *in situ* hybridization analyses to determine the developmental pattern of ADAR2 expression in the rat (Paupard et al., 2000). This study reported the earliest expression of ADAR2 in the ventral thalamus during late embryonic development, while at birth expression was still restricted to thalamic nuclei. ADAR2 expression was detected more broadly and near peak levels by postnatal day (P)21 and leveled off into adulthood with the greatest hybridization signal detected in hippocampus, olfactory bulb and thalamus. This restricted pattern of ADAR2 expression during development was quite surprising given the broad expression pattern for GLUR2 transcripts in the central nervous system and previous observations regarding the high efficiency (>99%) by which the Q/R site is edited within whole brain-derived GLUR2 mRNAs during development (Sommer et al., 1991). Recent findings by the GENSAT Consortium (http://www.gensat.org/index.html) and the Allen Brain Atlas (http://www.brainatlas.org/aba) have suggested a more widespread distribution of the ADAR enzymes.

Developmental and regional mapping of ADAR1 in the brain have not been reported, and homozygous ADAR1-null animals die at E11.5-12.5 (Hartner et al., 2004; Wang et al., 2004); the phenotype is characterized by slowed development, an altered

fetal liver structure and impaired hematopoiesis, as well as widespread apoptosis in heart, liver and vertebra. Examination of the neuroepithelium did not display any abnormalities (Hartner et al., 2004) raising questions regarding what role(s) ADAR1 may play, if any, during neurological development.

There is limited data describing not only developmental regulation of ADAR enzymes, but also of editing patterns for ADAR substrates, particularly the $5HT_{2C}$ receptor. Characterization of $5HT_{2C}$ receptor expression in developing mice is limited to a single survey demonstrating receptor immunoreactivity as early as E10-10.5 in the mesenchyme underlying the mesencephalic flexure of the brain (Lauder et al., 2000). Immunohistochemical as well as Northern and western blotting analyses performed during rat brain development have been more extensive, indicating that $5HT_{2C}$ receptor mRNA levels increase 5-fold between E17 and P27 and reach peak levels by P13 (Roth et al., 1991); however, $5HT_{2C}$ receptor protein levels demonstrate a more attenuated developmental profile, increasing only 2-fold during the same time period (Roth et al., 1991). In sharp contrast to the $5HT_{2C}$ receptor is the developmental expression of the $5HT_{2A}$ receptor, which shows a 54% amino acid homology to the $5HT_{2C}$ receptor, with considerably sequence similarity in the putative transmembrane regions. $5HT_{2A}$ receptor mRNA levels increase 13-fold between E17 and P27, peaking around P5, while protein levels increase by 8-fold between E17 and P13 and then decline (Roth et al., 1991). Monoclonal antibodies generated against $5HT_{2C}$ receptor have been used to examine $5HT_{2C}$ receptor levels in different brain regions (cortex, hippocampus, brainstem, cerebellum, thalamus and others). Expression levels of the $5HT_{2C}$ receptor in cerebral cortex, cerebellum and other tissues was expressed at a high level by P3 and increased only slightly during further postnatal development. In brainstem, $5HT_{2C}$ receptors were also highly expressed by P3, they continued to increase slightly until P14 and then declined at P21; in the hippocampus, $5HT_{2C}$ receptors were detected at low levels by P3

and their expression increased throughout subsequent development (Li et al., 2004). Transient expression of $5HT_{2C}$ receptors in layer IV of rat primary sensory cortex and thalamus during P7 to P28 further supported a potential involvement of the $5HT_{2C}$ receptor in neuronal plasticity.

Thus, our current study examined the temporal and spatial regulation of ADAR1 and ADAR2 in mouse forebrain, as well as developmental regulation of RNA editing in multiple ADAR substrates. Our data suggest that ADAR1 and ADAR2 are expressed broadly across nearly all brain regions by P0. Furthermore, these enzymes appear to be expressed in neurons, but not in glial cells, in the mouse forebrain, supporting a potential role for RNA editing in neuronal development across a wide variety of forebrain regions.

Experimental procedures

Animals

129S6/SvEvTac (Taconic, Hudson, NY) male mice from the following developmental stages: E15, E19, P0, P21, P100 (plug day=E0, n=6) were used for this study. To determine the gender of embryonic and early postnatal day animals, a PCR-based strategy was developed to detect the genes encoding *Smcy* and its X-chromosome homolog *Smcx*. Both Y and X chromosome homologs are widely expressed in all male tissues and SMCX escapes X-inactivation in females (Agulnik et al., 1994). PCR amplification using primers (sense: 5'-CCGCTGCCAAATTCTTTGG-3', antisense: 5'-TGAAGCTTTTGGCTTTGAG-3') designed to amplify both X- and Y- copies generated amplicons of 290 and 330 bp, respectively (Jimenez et al., 2003).

Immunohistochemistry

At E15, dams were deeply anesthetized with sodium pentobarbital and embryos were obtained by Caesarean section. Whole embryonic heads were immersion-fixed in

4% paraformaldehyde. For postnatal ages, mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. Brains were removed and postfixed overnight at 4°C. All brains were immersed in a series of sucrose (10%, 20%, 30%) solutions prior to sectioning for immunohistochemistry. Coronal sections were cut on a freezing microtome at 40 µm, or on a cryostat (20 µm, E15 only), and stained using modifications of previously published protocols (Stanwood et al., 2005; Stanwood et al., 2001). Briefly, sections were treated with 0.1 M glycine (pH 7.4) to reduce nonspecific labeling, blocked in 4% nonfat dried milk, 0.2% Triton-X 100 in PBS (pH 7.4) and incubated at 4°C for 3 days with antibodies directed against ADAR1 [guinea pig polyclonal; 1:1000, (George et al., 2005)] and ADAR2 [sheep polyclonal; 1:500, (Rueter et al., 1999; Sansam et al., 2003)], respectively. Following five washes in blocking solution at room temperature, sections were incubated with biotinylated anti-mouse, anti-rabbit, anti-sheep or anti-guinea pig IgG (Jackson, 1:1000) for 60 min. Avidin-biotin amplification (Vectastain ABC Standard, Vector Laboratories, Burlingame, CA) and 3-3'-diaminobenzidine reactions were used to visualize labeled proteins. Sections from different ages were processed in parallel to minimize variability across developmental time points. Adjacent sections were stained with 0.5% cresyl violet to aid in anatomical identification of regions of interest. Negative controls, in which primary antibodies were omitted, revealed no specific labeling. Background labeling of blood vessels was observed in the E15-derived samples.

Immunofluorescent techniques were used for colocalization analyses where mouse monoclonal antibodies directed against either NeuN (Chemicon MAB377; 1:100), glial fibrillary acidic protein (Chemicon MAB360; 1:250), or S100β (DakoCytomation Z0311, 1:4000) were used in combination with guinea pig anti-ADAR1 and sheep anti-ADAR2 antisera; appropriate Cy2- and Cy3-labeled secondary antibodies were utilized (1:1000, Jackson). Sections were mounted onto gelatin/poly-L-lysine-coated slides,

dehydrated, coverslipped with DPX (EMS, Hatfield PA) and imaged using an Axioplan II microscope and Axiocam HR (Carl Zeiss, Thornwood, NY). Images were digitally captured using Axiovision software (version 4.1) and exported as TIFF files. Images were merged in Adobe Photoshop where brightness was adjusted, as necessary. Fields within the cerebral cortex and hippocampus were collected for cell counting in a subset of the adult animals (n=3-4 animals per immunohistochemistry condition).

RNA characterization

At each developmental age, mice ($n \geq 6$) were rapidly sacrificed and whole brain or brain regions (adult only: choroid plexus, cortex, hippocampus, hypothalamus, olfactory bulb, striatum) was immediately frozen in liquid nitrogen. RNA was isolated and purified using a PerfectPure RNA tissue kit (5 PRIME, Gaithersburg, MD) according to the manufacturer's instructions.

For isolation of specific brain region, dissections were performed as described in Glowinski and Iversen (Glowinski and Iversen, 1966). Briefly, a coronal cut was made on the ventral surface of the brain at the level of the optic chiasm (Bregma ~-0.3 mm). The region generated from this first cut was used to harvest cortex and striatum; olfactory bulb was removed and striatum was separated from cortex with corpus callosum and lateral ventricles defining the boundaries. The residual white matter was removed and remaining tissue was defined as cortex. Choroid plexus was removed from both lateral ventricles and the third ventricle. In the remaining tissue sample, the hippocampus was removed and then a second coronal cut was made in front of the cerebellum (Bregma ~3.2 mm). Hypothalamus was separated from thalamus using the anterior commissure and mammillary bodies as reference boundaries.

To determine the $5HT_{2C}$ receptor editing pattern during development, a Pyrosequencing™ (Biotage AB, Uppsala, Sweden) strategy was utilized with individual

cDNA clones at each age, as described (Sodhi et al., 2005). Briefly, first strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega, Madison, WI) and then amplified using specific primers (sense: 5'-ATT AGAATTCTATTTGTGCCCCGTCTGG-3', antisense: 5'-ATCAAAGCTTGACGGCGTAG GACGTAG-3') to amplify the region of the $5HT_{2C}$ receptor containing the editing sites as well as flanking sequence. Following RT-PCR amplification, amplicons were purified and subcloned into a pGEM-Easy T vector (Promega, Madison, WI). Individual clones were selected (n≥50 for each animal, total≥300) and PCR amplification of a single bacterial colony was performed using a biotinylated antisense primer (5'-CGAATTGAAACGGCTATGCT-3'). PCR products were then incubated with streptavidin sepharose beads (GE Healthcare, Piscataway, NJ) and this mixture was transferred to a Multiscreen PCR filter plate (Millipore, Billerica, MA). The double-stranded PCR product was denatured and the biotinylated antisense strand remained bound to the filter. The antisense strand was annealed to a sequencing primer (5'-ATATCGCTGGATCGGTATGTAG-3') that terminated before the editing sites. This assay was developed for a 96-well plate format and the plate and a cartridge containing substrates [adenosine 5' phosphosulfate (APS) and luciferin], enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and dNTPs was loaded into the Pyrosequencer. Computer software automatically dispensed each of the cartridge components, beginning with substrates and enzymes. At each cycle, a dNTP was added to the reaction; DNA polymerase catalyzed addition of the base if it was complementary and each incorporation resulted in the release of inorganic pyrophosphate (PPi). ATP sulfurylase converted PPi to ATP using APS as an adenosine donor; ATP then catalyzed the luciferase-mediated conversion of luciferin to oxyluciferin to generate light. This visible light was detected by a charge couple device (CCD) camera and the software represented this light as a peak on a pyrogram™.

To determine the extent of site-selective editing for other ADAR substrates, first strand cDNA was synthesized from 1µg of total RNA using 10U AMV RT (Promega, Madison, WI) in a 10µl reaction containing 5µM random hexamers (Applied Biosystems, Foster City, CA), 1mM dNTPs (New England Biolabs, Ipswich, MA) and 10U RNasin (Promega, Madison, WI), under the following conditions: 25°C for 10 minutes, 42°C for 1hr and 95°C for 5 minutes followed by PCR amplification using Ampli-Taq DNA polymerase (Applied Biosystems, Foster City, CA), and assessed by a modified primer-extension analysis (Rueter et al., 1999). The extension products were resolved by denaturing polyacrylamide gel electrophoresis and quantified using phosphorimager analyses (GE Healthcare, Piscataway, NJ) as previously described (Feng et al., 2006).

To quantify levels of mRNA expression, first-strand cDNA was synthesized as described above and subjected to TaqMan real-time PCR analysis (Applied Biosystems, Foster City, CA). All primers and probes used for real-time PCR analysis were products of Assay-On-Demand from Applied Biosystems (ADAR1, assay ID Mm00508001_m1; ADAR2, assay ID Mm00504621_m1; $5HT_{2C}R$, assay ID Mm00434127_m1). Eukaryotic 18S rRNA (product no. 4319413E; Applied Biosystems, Foster City, CA) was included in each multiplex PCR as an internal control. Real-time PCR and subsequent analysis were performed with an ABI Prism 7900HT sequence detection system (SDS v2.3; Applied Biosystems, Foster City, CA). Quantitation of target gene expression in all samples was normalized to 18S rRNA expression by the equation $C_{T(\text{target})} - C_{T(18S)} = \Delta C_T$, where C_T is the threshold cycle number. The mean ΔC_T value of samples from each tissue for all wild-type animals was determined and used as a reference point for the samples from the same tissues for mice. Differences between wild-type mice and each developmental time point, including individual variation, were calculated by the equation $\Delta C_{T(\text{individual age})} - \Delta C_{T(\text{mean wild-type})} = \Delta\Delta C_T$. Changes in target gene expression (*n*-fold) in each sample were

calculated by $2^{-(\Delta\Delta CT)}$ from which the means and standard errors of the mean (SEM) were derived.

Statistical Analyses

All statistical analyses were performed using GraphPadPRISM (GraphPad Software, Inc., La Jolla, CA), unless otherwise indicated. Values are reported as mean ± SEM, and *P* values of <0.05 were considered significant.

Results

ADAR1 and ADAR2 Transcripts

ADAR1 and ADAR2 mRNA levels were determined over a developmental time course in whole mouse brain using quantitative real-time RT-PCR (qPCR). Overall, the steady-state level of ADAR1 and ADAR2 transcripts increased gradually during development. At E15, expression of each of these mRNAs was relatively low, and expression continued to increase for each ADAR-encoding mRNA species through adulthood (P100; Figure 10).

To further assess the expression of ADAR1 and ADAR2 transcripts in dissected brain regions from adult mice, we again used qPCR to quantify steady-state mRNA levels in frontal cortex, hippocampus, hypothalamus, olfactory bulb, striatum, thalamus and choroid plexus; values from dissected brain regions were normalized to whole brain expression levels (Figure 11). ADAR1 displayed relatively low levels of expression in the choroid plexus, frontal cortex and hippocampus ($p<0.001$ for each region as compared to whole brain; Figure 11A). In contrast, ADAR1 appeared to be relatively enriched in the hypothalamus and thalamus (Figure 11A), while ADAR1 levels in the olfactory bulb and striatum were comparable to those from whole brain.

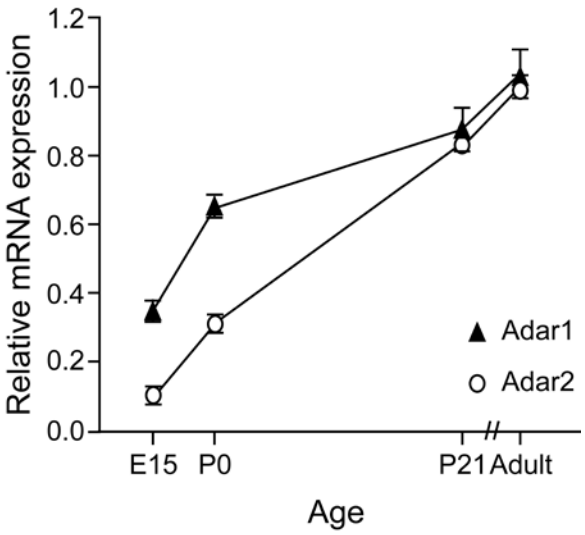

Figure 10: Temporal regulation of ADAR1 and ADAR2 expression. ADAR1 and ADAR2 mRNA expression in whole mouse brain over a developmental time course was determined (mean ± SEM) by qPCR. Expression levels of each ADAR were normalized to the adult mRNA level and varied significantly in developing mouse brain by one-way ANOVA ($p<0.001$). Dunnett's post-hoc tests revealed that each age differed significantly from adult ADAR levels ($p<0.01$).

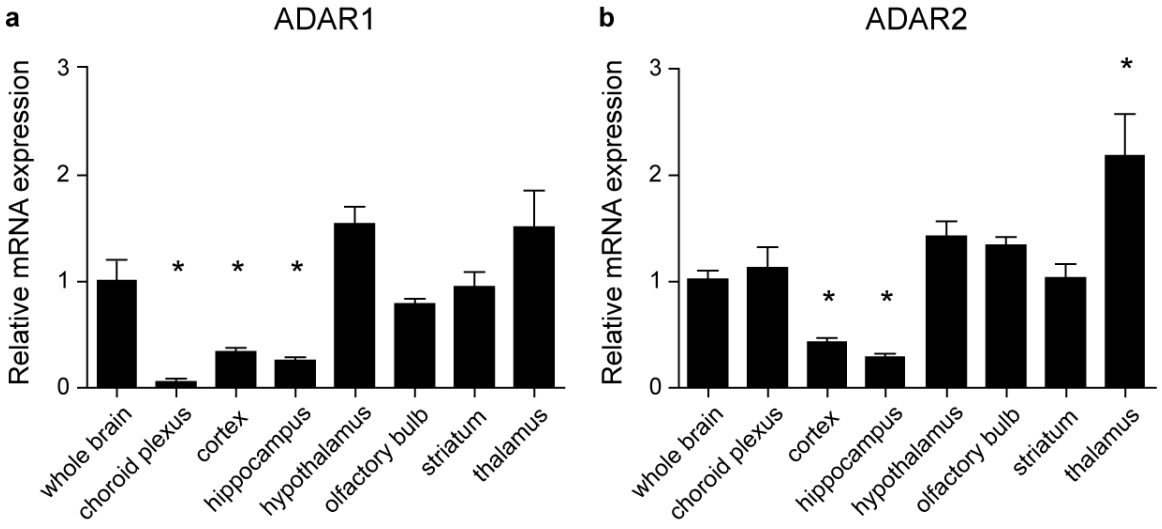

Figure 11: Region-specific expression of ADARs in the adult mouse brain. The relative expression of ADAR1 **(a)** and ADAR2 **(b)** mRNA was quantified by qPCR in mouse whole brain, and in dissected brain regions. For each region, values are expressed as mean ± SEM and normalized to the expression value observed in whole brain samples. **a)** Choroid plexus, cortex and hippocampus ADAR1 mRNA levels are significantly different that whole brain mRNA (individual t-test, Bonferroni post-hoc analyses). **b)** Cortical, hippocampal and thalamic ADAR2 transcripts are significantly different than whole brain transcripts (individual t-test, Bonferroni post-hoc analyses), *: $p<0.001$.

The general enrichment pattern for ADAR2 in most brain regions was similar to that for ADAR1, with the lowest relative expression levels observed in frontal cortex and hippocampus (Figure 11B). ADAR2 mRNA expression was the highest in the thalamus, with a two-fold increase relative to whole brain (Figure 11B). ADAR2 expression in the choroid plexus, hypothalamus, olfactory bulb and striatum was comparable to that of whole brain.

ADAR1 and ADAR2 Protein

To examine the spatial pattern of ADAR1 and ADAR2 protein expression, we used an immunohistochemical strategy with previously characterized ADAR-specific antisera (George et al., 2005; Rueter et al., 1999; Sansam et al., 2003). In adult mice, expression of both ADAR1 and ADAR2 protein was detected throughout the forebrain (Figures 12 and 13). ADAR1 labeling was prominent in the nuclei of cells contained within nearly all grey matter brain regions, including the cerebral cortex (Figure 12B and 12C), striatum (Figure 12C), hippocampus (Figure 12D), thalamus (Figure 12E), hypothalamus (Figure 12F) and amygdala (Figure 12G).

Even more intense labeling was observed using antisera derived against ADAR2 (Figure 13) in nearly all forebrain regions examined. Qualitatively, both ADAR1- and ADAR2-immunoreactivity appeared to be restricted to neurons, but expressed ubiquitously across neuronal subtypes. Colocalization studies using markers of neurons (NeuN) and astrocytes (GFAP, S100β) confirmed these findings [Figures 14 (*low power*) & 15 (*high power*)].

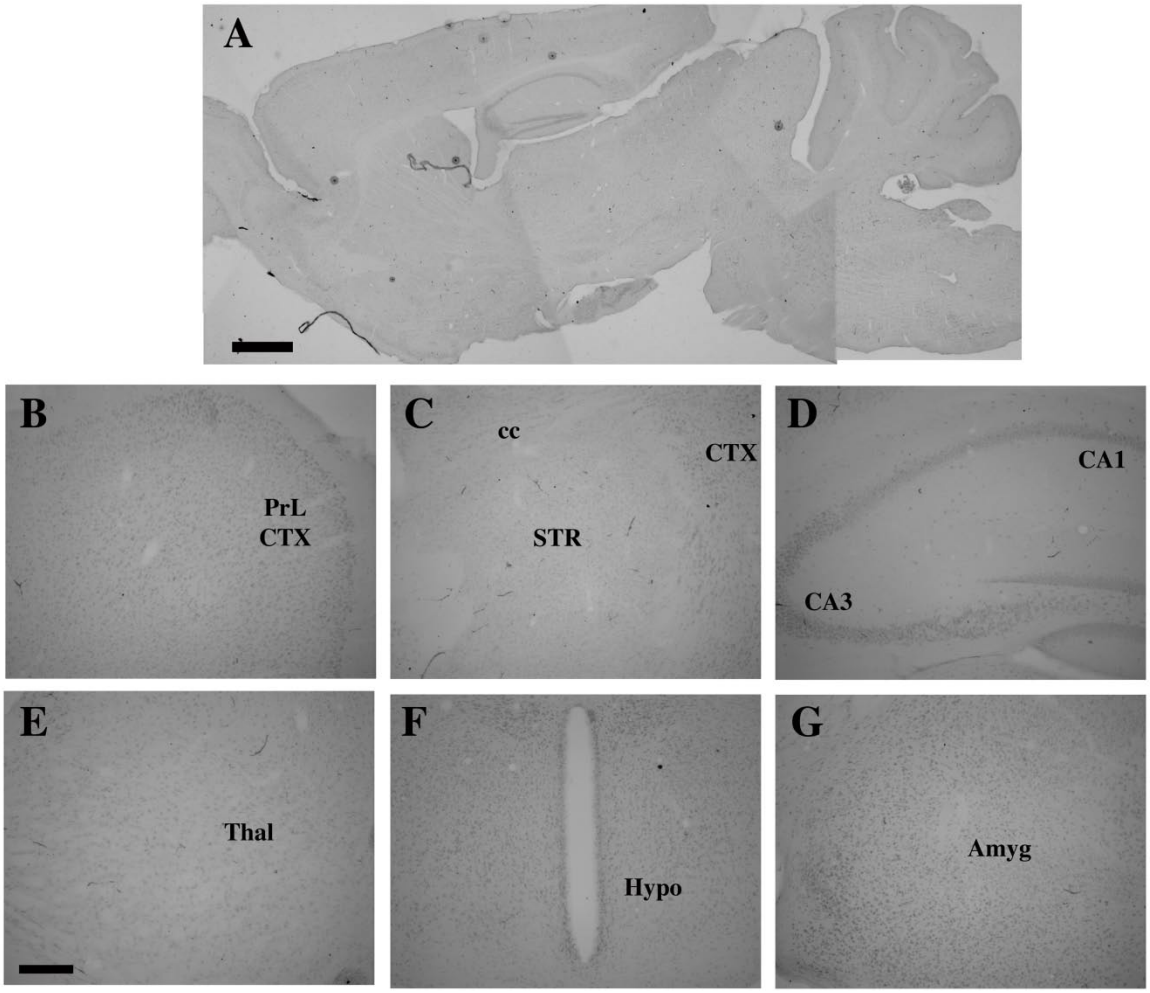

Figure 12: ADAR1 protein expression in the adult mouse forebrain. Sagittal **(a)** and coronal **(b-g)** sections of adult (P100) mouse brain were stained with specific anti-ADAR1 antisera; note the widespread expression in cell nuclei in most brain regions. Scale bars = 500 μm **(a)** and 200 μm **(b-g)**. Abbreviations: PrL CTX = prelimbic cortex; cc = corpus callosum; STR = striatum; CA1 = CA1 subregion of hippocampus; CA3 = CA3 subregion of hippocampus; HYPO = hypothalamus. The sagittal section was reconstructed as overlays from images captured at higher power in Adobe Photoshop, thus resulting in different apparent levels of background on different areas of the section.

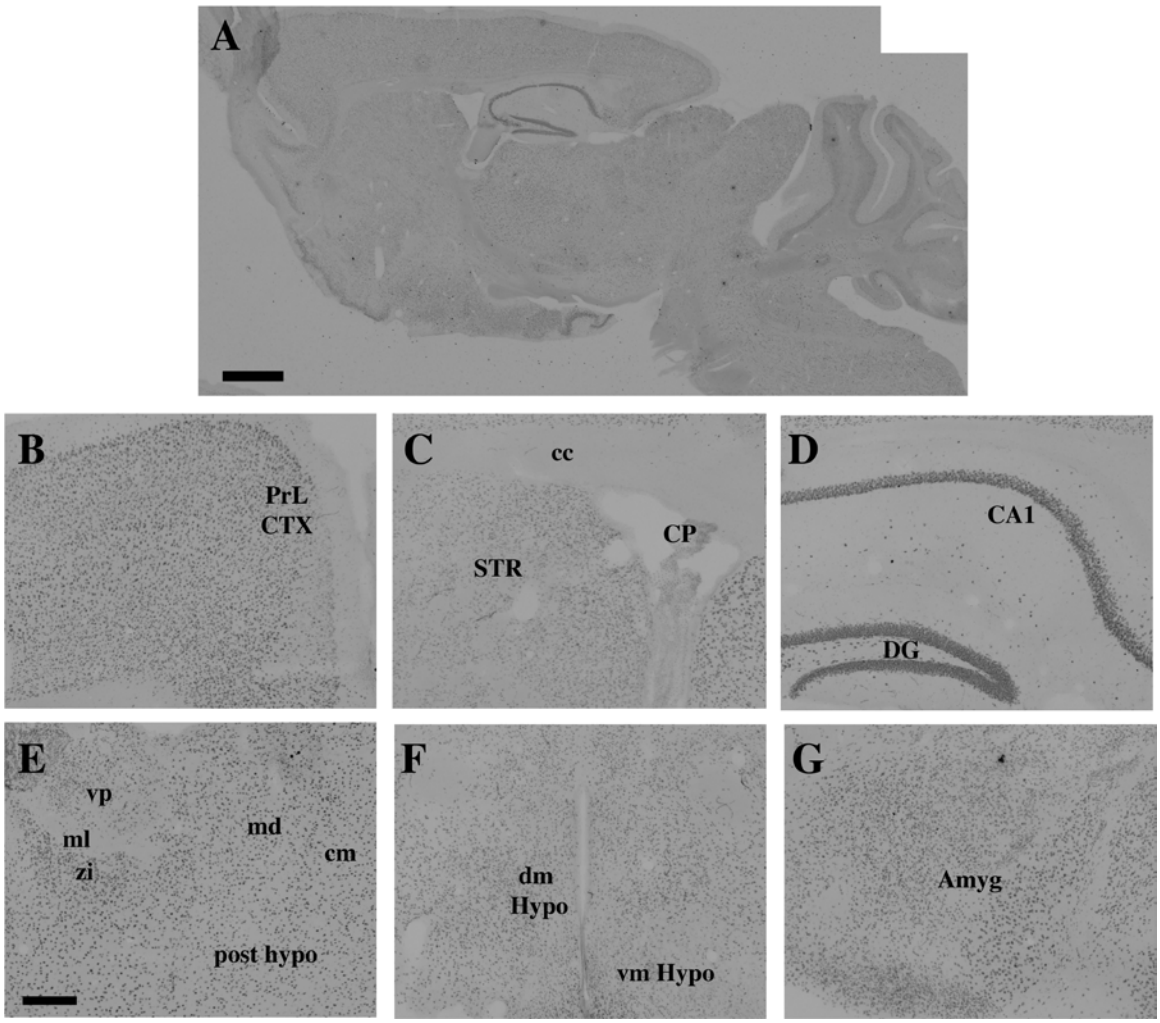

Figure 13: ADAR2 protein expression in the adult mouse forebrain. Sagittal **(a)** and coronal **(b-g)** sections of adult (P100) mouse brain were stained with specific anti-ADAR2 antisera; note the widespread expression in cell nuclei in most brain regions. Scale bars = 500 μm **(a)** and 200 μm **(b-g)**. Abbreviations: PrL CTX = prelimbic cortex; cc = corpus callosum; STR = striatum; CP = choroid plexus; CA1 = CA1 subregion of hippocampus; DG = dentate gyrus of hippocampus; zi = zona incerta; ml = medial lemniscus; md = mediodorsal thalamic nucleus; vp = ventroposterior thalamic nucleus; dm Hypo = dorsomedial hypothalamus; vm Hypo = ventromedial hypothalamus; post Hypo = posterior hypothalamic nucleus; Amyg = amygdala. The sagittal section was reconstructed as overlays from images captured at higher power in Adobe Photoshop, thus resulting in different apparent levels of background on different areas of the section.

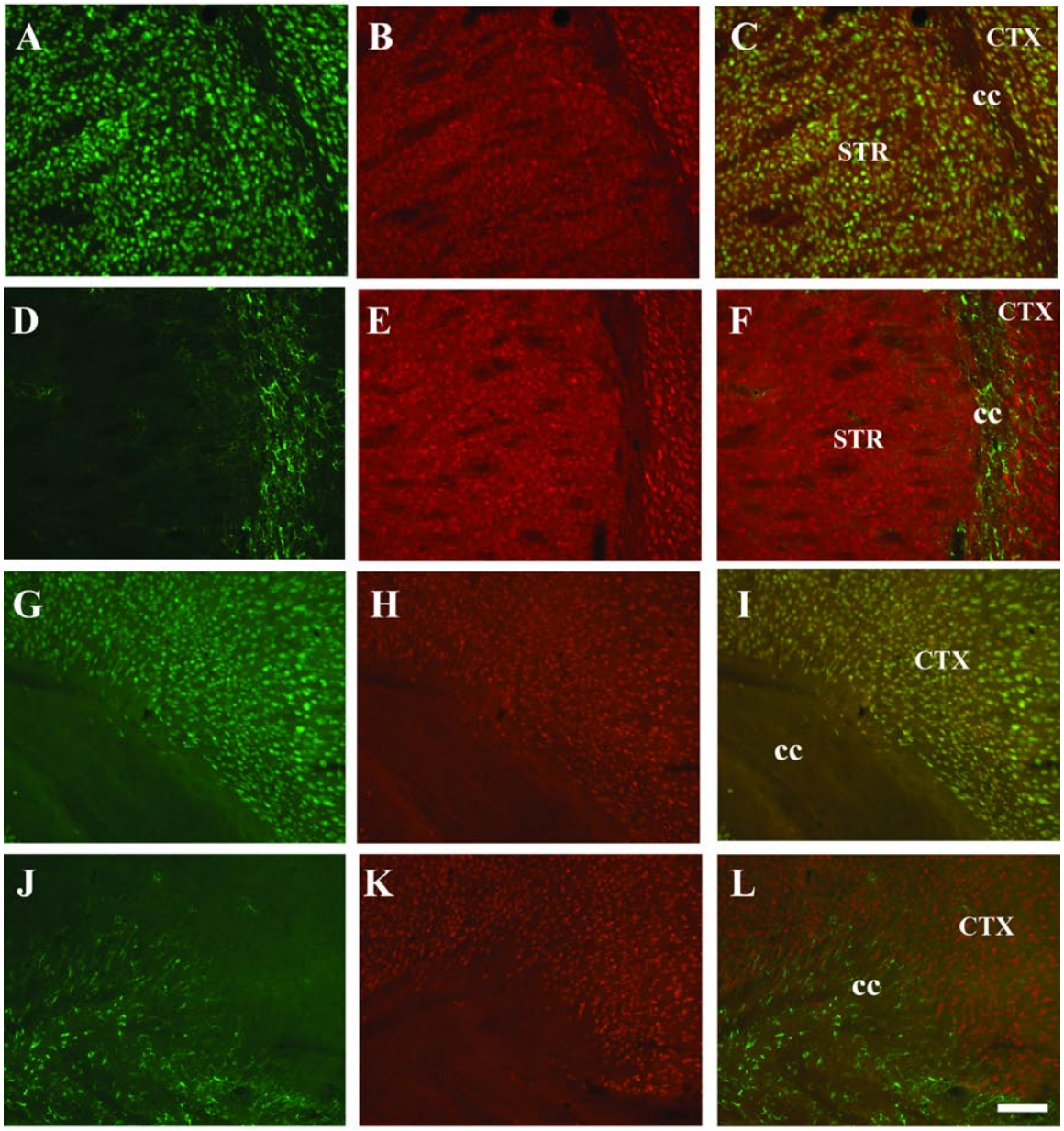

Figure 14: ADAR1 and ADAR2 protein colocalizes with NeuN, but not GFAP in the adult mouse forebrain. Coronal sections of adult (P100) mouse brain were stained with specific antibodies against NeuN **(a, g)**, GFAP **(d, j)**, ADAR1 **(b, e)**, and ADAR2 **(h, k)**. Overlayed images **(c, f, i, l)** demonstrate colocalization of ADAR1 and ADAR2 with NeuN, but not GFAP. Scale bar = 200 μm. Abbreviations: CTX = cortex; cc = corpus callosum; STR = striatum.

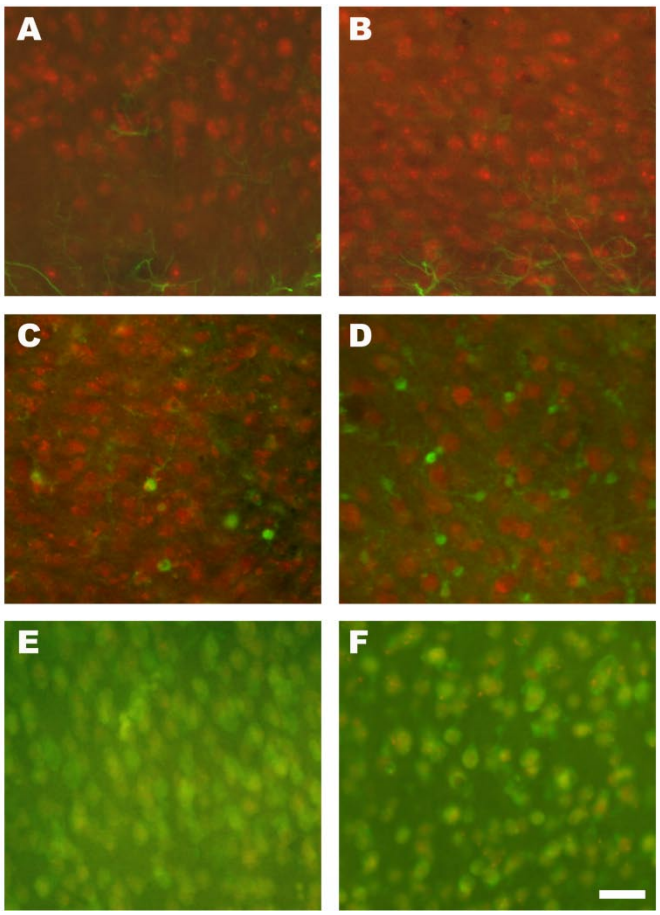

Figure 15: ADAR1 and ADAR2 protein is expressed in neurons but not glial cells. Coronal sections of adult (P100) mouse brain were double labeled with specific antibodies against GFAP (green - **a, b**), S100β (green - **c, d**) or NeuN (green - **e, f**), as well as ADAR1 (red - **a, c, e**) or ADAR2 (red - **b, d, f**). High power photomicrographs demonstrate prominent colocalization of ADAR1 and ADAR2 with NeuN, but not GFAP or S100β. Scale bar = 25 μm.

We also quantified the extent of colocalization in two brain regions, the cerebral cortex and hippocampus. The vast majority of ADAR1- and ADAR2-immunoreactive cells were positive for NeuN (97.6% ± 1.58% and 99.0% ± 0.333%, respectively). In contrast, very few ADAR1- and ADAR2-immunoreactive cells were positive for GFAP (0.513% ± 0.230% and 0.550% ± 0.268%, respectively) or S100β (1.32% ± 0.178% and 3.02% ± 1.41%, respectively). Similar results were obtained in the hippocampus, with NeuN detected in the majority of ADAR1- and ADAR2-immunoreactive cells (96.9% ±0.515% and 92.6% ± 2.03%, respectively), but very little co-expression of ADAR1 or

ADAR2 with GFAP (0% ± 0% and 1.67% ± 1.67%, respectively) or S100β (3.67% ± 1.41% and 6.00% ± 1.79%, respectively).

We then assessed ADAR1 and ADAR2 protein expression developmentally, examining labeling patterns at E15, P0 and P21. At E15, the earliest stage examined, no specific immunoreactivity for ADAR2 was detected within the forebrain (Figure 16 B, D, F). ADAR1-immunoreactive cells were seen only occasionally, with no clear delineation of cell type (Figure 16A, C, E).

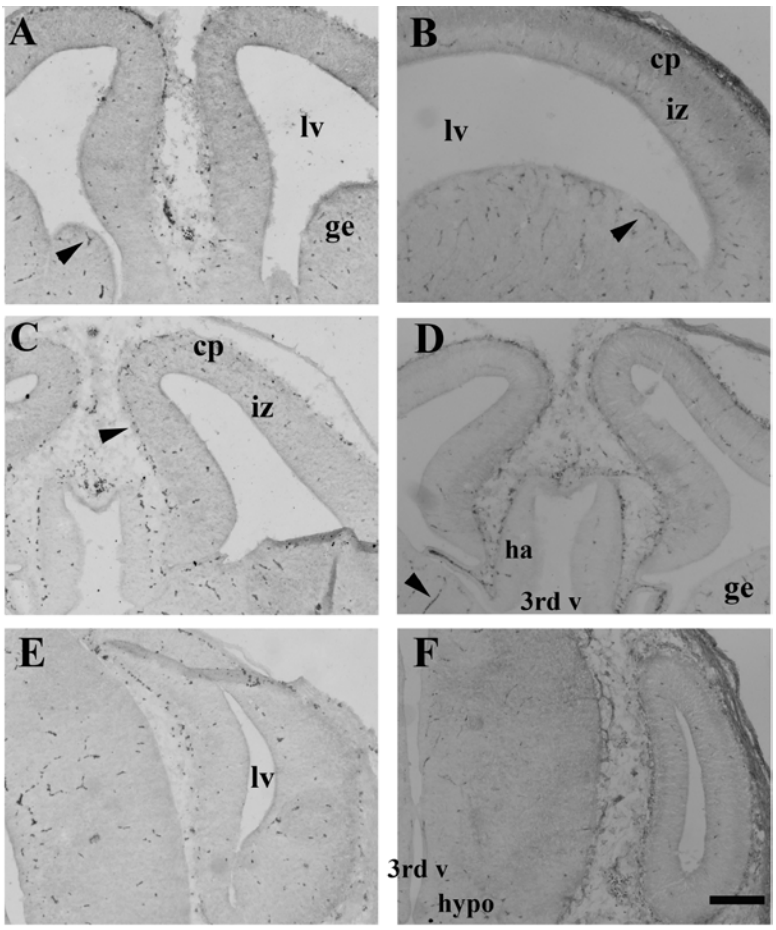

Figure 16: Paucity of ADAR1 or ADAR2 protein expression in E15 mouse forebrain. Coronal sections of E15 mouse brain were stained with specific anti-ADAR1 **(a, c, e)** and anti-ADAR2 **(b, d, f)** antisera, but no specific cellular labeling was observed. Arrowheads indicate labeling of blood vessels rather than specific cellular labeling. Scale bar = 200 µm. Abbreviations: lv = lateral ventricle; ge = ganglion eminence; cp = cortical plate; iz = intermediate zone of cortex; ha = habenula; 3rd v = third ventricle; hypo = hypothalamus.

By P0, robust expression of both ADAR1 and ADAR2 protein was detected (Figures 17 and 18). For example, ADAR1 was observed within superficial and deep layers of the still developing cerebral cortex (Figure 17A), hippocampus (Figure 17C), thalamus (Figure 17D) and hypothalamus (Figure 17D). Labeling in the striatum was limited, however (Figure 17B). Nearly identical patterns of ADAR2 protein expression were observed at P0 (Figure 18). Co-localization studies again revealed expression of each editing enzyme with NeuN, but not GFAP (Figure 19). Expression patterns nearly indistinguishable from the adult were observed at P21 (Figures 20 and 21), consistent with qPCR analyses of ADAR1 and ADAR2 transcripts (Figure 10).

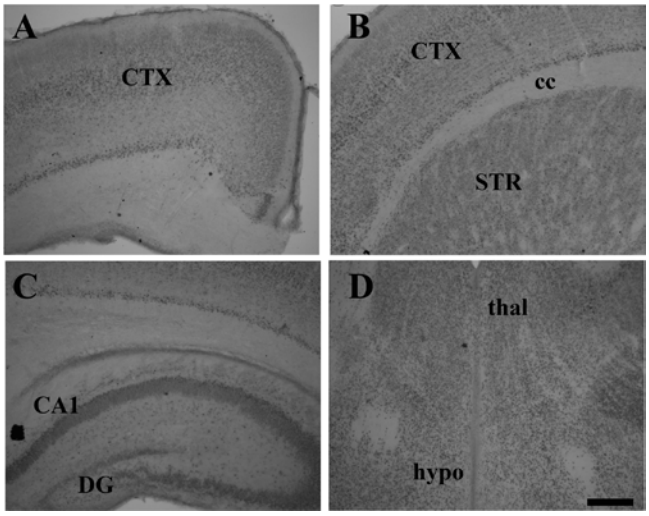

Figure 17: ADAR1 protein expression in mouse brain at P0. Coronal sections of P0 mouse brain were stained for ADAR1; note the widespread expression in deep and superficial layers of the still developing cerebral cortex **(a, b)**, hippocampus **(c)**, thalamus **(d)**, and hypothalamus **(d)**. Labeling in the striatum is fairly low **(b)**. Scale bar = 200 μm. Abbreviations: CTX = cortex; cc = corpus callosum; STR = striatum; CA1 = CA1 subregion of hippocampus; DG = dentate gyrus of hippocampus; thal = thalamus; hypo = hypothalamus.

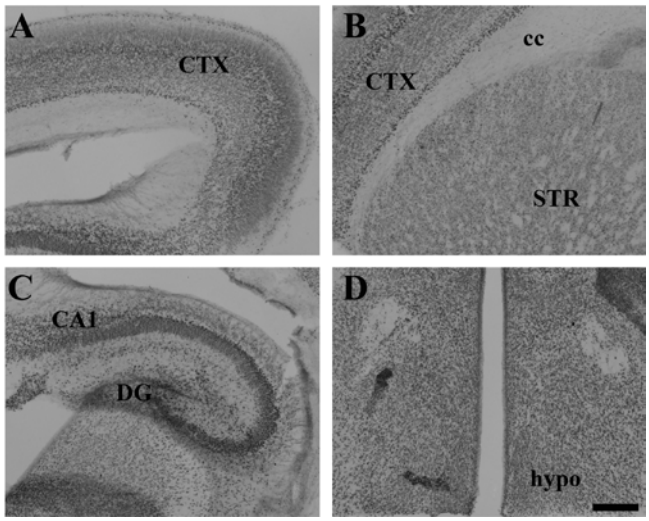

Figure 18: ADAR2 protein expression in mouse brain at P0. Coronal sections of P0 mouse brain were stained for ADAR2. Similar to ADAR1, widespread expression in deep and superficial layers of the still developing cerebral cortex **(a, b)**, hippocampus **(c)**, thalamus **(d)**, and hypothalamus **(d)** is readily observed. Labeling in the striatum is fairly low **(b)**. Scale bar = 200 μm. Abbreviations: CTX = cortex; cc = corpus callosum; STR = striatum; CA1 = CA1 subregion of hippocampus; DG = dentate gyrus of hippocampus; hypo = hypothalamus.

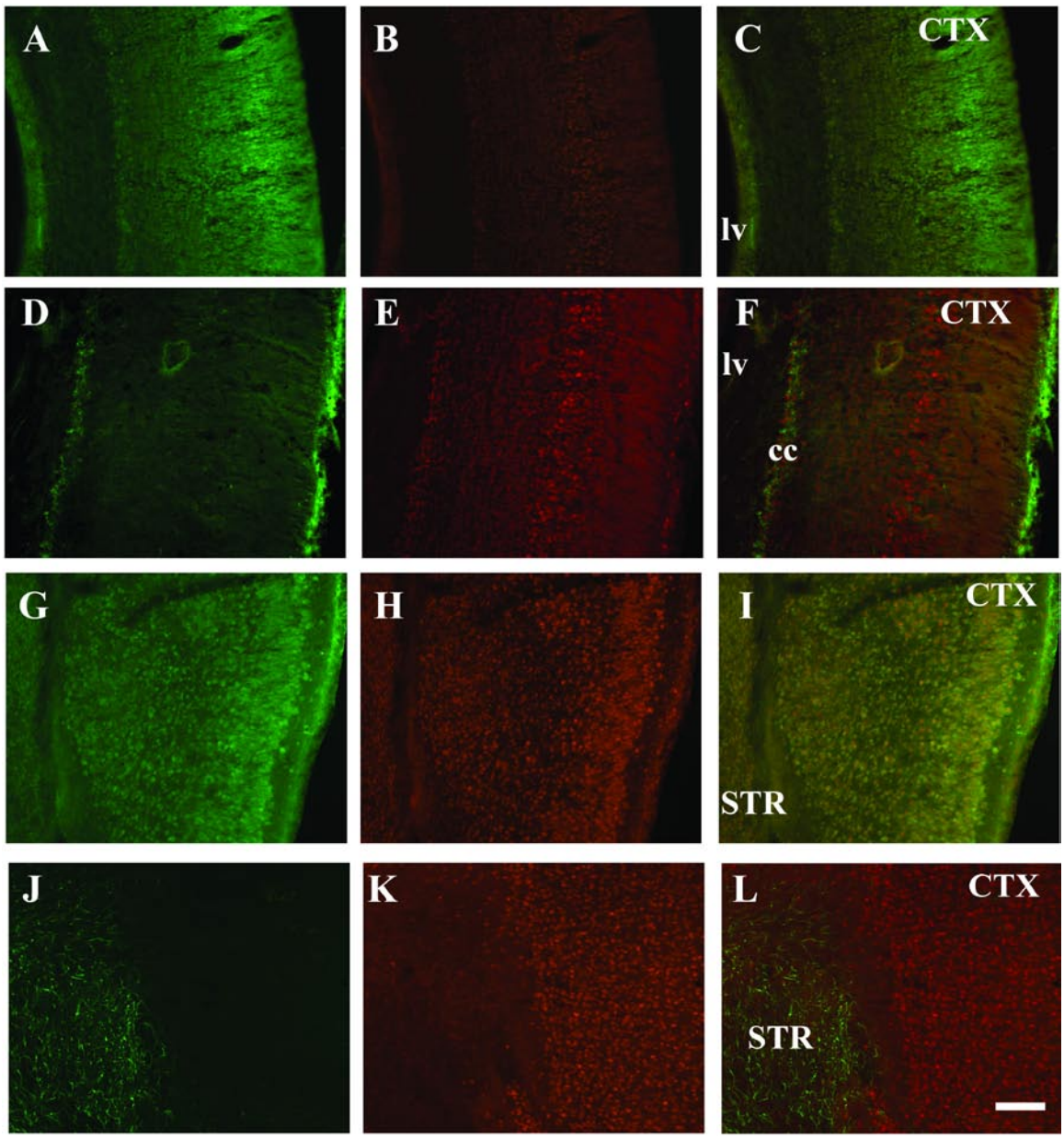

Figure 19: ADAR1 and ADAR2 protein colocalize with NeuN, but not GFAP in P0 forebrain. Coronal sections of P0 mouse brain were stained with specific antibodies against NeuN **(a, g)**, GFAP **(d, j)**, ADAR1 **(b, e)**, and ADAR2 **(h, k)**. As in the adult, overlayed images **(c, f, i, l)** of P0 tissue demonstrate prominent colocalization of ADAR1 and ADAR2 with NeuN, but not GFAP. Scale bar = 200 μm. Abbreviations: CTX = cortex; cc = corpus callosum; lv = lateral ventricle; STR = striatum.

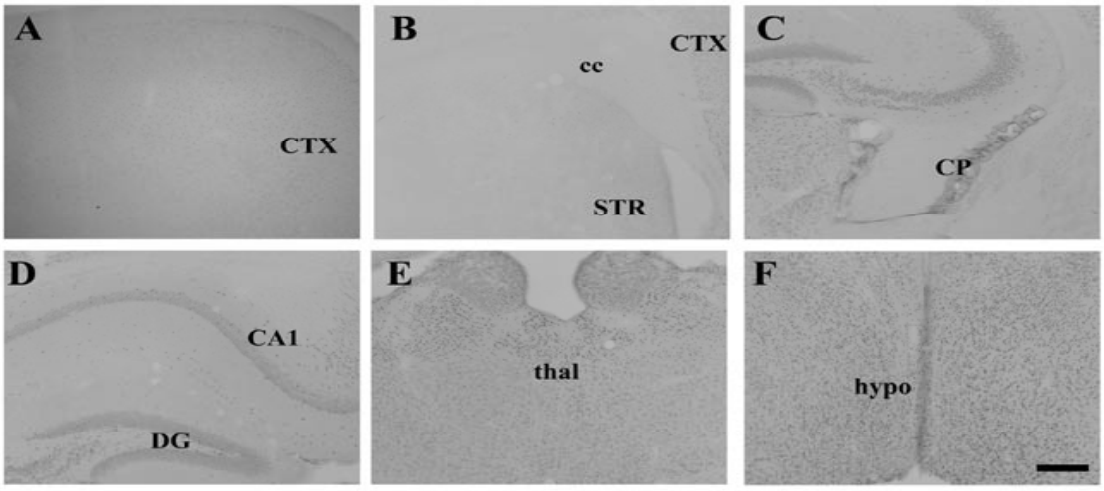

Figure 20: ADAR1 protein expression in mouse brain at P21. Coronal sections of P21 mouse brain were stained for ADAR1, revealing widespread expression similar to that observed in the adult. Abbreviations: CTX = cortex; cc = corpus callosum; STR = striatum; CP = choroid plexus; CA1 = CA1 subregion of hippocampus; DG = dentate gyrus of hippocampus; thal = thalamus; hypo = hypothalamus; Scale bar = 200 μm.

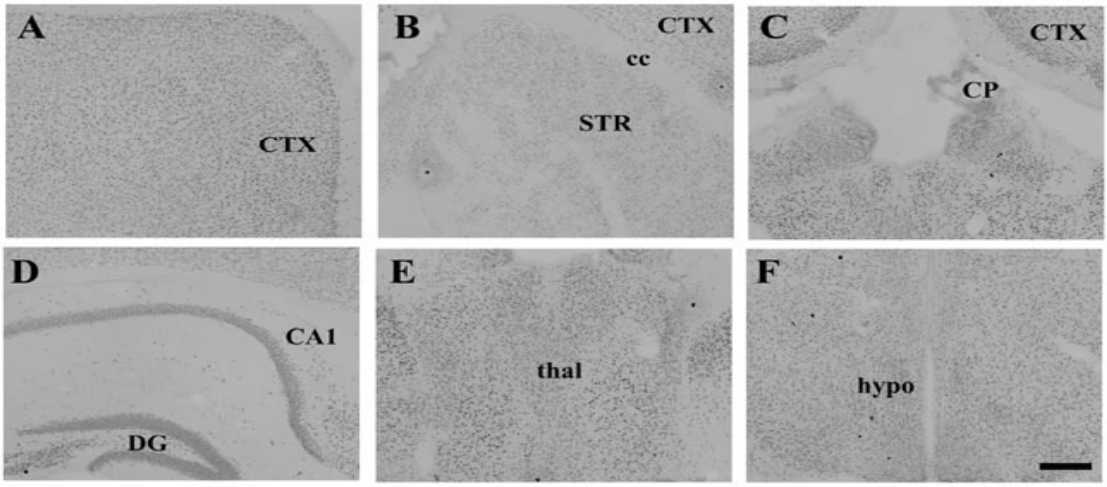

Figure 21: ADAR2 protein expression in mouse brain at P21. Coronal sections of P21 mouse brain were stained for ADAR2, revealing widespread expression similar to that observed in the adult. Abbreviations: CTX = cortex; cc = corpus callosum; STR = striatum; CP = choroid plexus; CA1 = CA1 subregion of hippocampus; DG = dentate gyrus of hippocampus; thal = thalamus; hypo = hypothalamus; Scale bar = 200 μm.

ADAR1 and ADAR2 Substrate RNAs

We next determined the editing patterns for several known ADAR1 and ADAR2 substrates in RNA isolated from whole brain extracts at specific developmental time points (Figure 22). The $5HT_{2C}$ receptor contains five editing sites (termed sites A-E) that can alter amino acids 157, 159 and 161, within the putative second intracellular loop of the receptor (Burns et al., 1997; Niswender et al., 1998). These edited isoforms have distinct functional properties affecting constitutive activity and the efficacy and specificity of receptor coupling to G proteins (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999). When we examined the extent of RNA editing at each of the five individual sites within mouse $5HT_{2C}$ receptor transcripts, most appeared to follow similar developmental patterns of editing. With the exception of the D site, the other edited sites display low levels (~10-15%) of editing at E15 and reach adult levels of editing by P0 (Figure 22A). The D site, however, was already edited in 50% of the transcripts at E15 and increased at P21, when it reached adult levels (~75%). Editing at the D site changes a genomically-encoded isoleucine codon (AUU) to a valine codon (IUU), yet this amino acid alteration has little effect on the signaling properties of the $5HT_{2C}$ receptor (Niswender et al., 1999).

The Q/R site within GLUR2 transcripts is edited to near completion by E15 and maintains this level of editing throughout adulthood (Figure 22B), as previously described (Burnashev et al., 1992). Other substrates, including the GLUR2 R/G site, the GLUR5 Q/R site and the ADAR2 auto-editing site (-1 site) demonstrated modest editing at E15, with increases at P0 and P21, at which point the adult pattern of editing was achieved (Figure 22B). Interestingly, significant editing of the Kv1.1 I/V site appears to occur sometime after P0, substantially later than the other transcripts studied (Figure 22B).

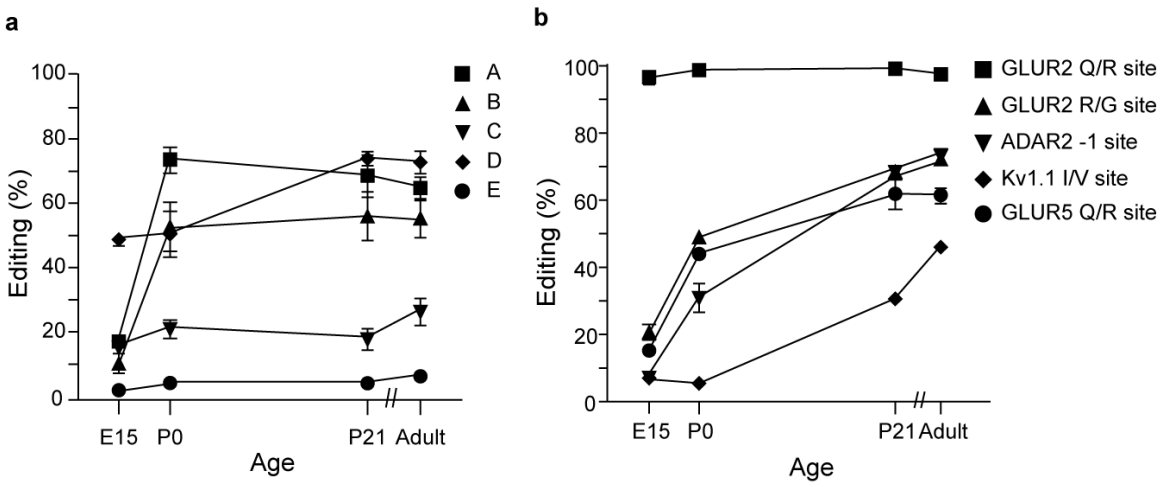

Figure 22: Developmental alterations in editing efficiency for multiple ADAR substrates. a) The extent of RNA editing for the five $5HT_{2C}$ receptor editing sites (A-E) was determined by Pyrosequencing analysis from mouse whole brain transcripts isolated over a developmental time course. With exception of the D site, each site displayed low levels of editing at E15 that gradually and significantly increased to maximal levels by P0 ($p<0.01$), with very little increase observed between P0 and adulthood. **b)** The extent of RNA editing for several other ADAR substrates was determined by primer-extension analysis in mouse whole brain transcripts isolated over a developmental time course. With exception of the GluR-2 Q/R site, which was maximally edited at the earliest age examined, each substrate displayed low levels of editing at E15 that gradually and significantly increased to maximal levels in adult animals ($p<0.01$).

Analysis of the $5HT_{2C}$ receptor distribution in mRNA isolated from whole brain or dissected brain regions (choroid plexus, cortex, hippocampus, hypothalamus, olfactory bulb, striatum) in adult animals revealed that the pattern of RNA editing in the mouse was similar to that previously observed in the rat, where the VNV isoform of the receptor represented the major $5HT_{2C}$ species in whole brain and most brain regions examined (Table 1). The choroid plexus remained a clear exception to this pattern however, with the major $5HT_{2C}$ transcripts expressed in this tissue encoding the less edited INV and non-edited INI receptor isoforms.

Amino Acid Isoform	RNA Isoforms	WB	CP	Cx	Hi	Hy	OB	Str
INI		8	36	8	13	7	15	16
MNI	B	0	2	0	0	0	0	0
ISI	C	0	2	0	0	1	0	0
INV	D	10	40	19	12	9	4	6
IDI	E	0	0	0	2	0	0	0
ISV	CD	2	10	5	2	0	0	0
IDV	ED	0	3	0	0	0	0	0
VNI	AB A	8	0	16	15	10	12	10
VNV	ABD AD	38	3	38	38	46	44	32
VDI	ABE BE	0	0	0	0	3	0	0
VSI	ABC AC	6	0	3	2	6	6	10
VGI	ABCE ACE	0	0	0	0	0	0	2
VDV	ABDE ADE	2	3	0	2	3	2	2
VSV	ABCD ACD	27	0	11	13	15	15	18
VGV	ABCDE ACDE	0	0	0	0	3	2	4

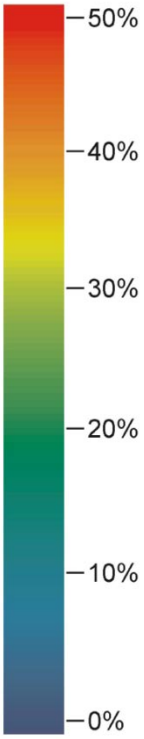

Table 1: Quantitative analysis of region-specific $5HT_{2C}$ receptor isoform expression in adult mouse brain. RNAs from whole mouse brain (WB), choroid plexus (CP), cortex (Cx), hippocampus (Hi), hypothalamus (Hy), olfactory bulb (OB) and striatum (Str) was amplified by RT-PCR, subcloned into a prokaryotic vector (pGEM-T) and individual cDNA isolates (>100 from each brain region) were analyzed by Pyrosequencing™. $5HT_{2C}$ RNA isoforms, and the corresponding amino acid variants predicted by them, are indicated; numbers represent the percentage of cDNA isolates encoding each amino acid isoform and are color-coded according to the scale at the right.

Discussion

Using multiple experimental strategies, we have examined the spatiotemporal expression patterns for ADAR1 and ADAR2 in the mouse forebrain. Quantitative RT-PCR analyses of ADAR1 and ADAR2 mRNA detected very low expression at E15, but robust and widespread expression by P0. ADAR1 and ADAR2 mRNA expression each appeared to increase steadily throughout development, with peak expression observed in adulthood. In adult animals, we examined ADAR1 and ADAR2 levels in whole brain,

as well as specific brain regions, to determine whether these editing enzymes demonstrated a preferential pattern of expression. ADAR1 and ADAR2 mRNA appear to be expressed at somewhat lower levels in the cortex and hippocampus, with message levels ~50% lower than that observed in whole brain. ADAR1 transcripts are most highly expressed in the hypothalamus and thalamus. ADAR2 also has higher expression in the thalamus than in other brain regions. High levels of RNA editing for several ADAR2-specific substrates have been observed in the thalamus including the Q/R site of the GLUR5 subunit in rat (90%) (Paschen et al., 1994), the D site of the $5HT_{2C}$ receptor in human (85%) (Wang et al., 2000b) and the I/M site of the $\alpha 3$ subunit of the $GABA_A$ receptor (90%) (Rula et al., 2008), suggesting that increased expression of ADAR2 in the thalamus can lead to maximal levels of RNA editing.

A previous study of ADAR2 expression using *in situ* hybridization with oligonucleotide probes (Paupard et al., 2000) concluded that the ADAR2 transcript is not present at birth and appears gradually over the first week of life in a region-dependent manner. In contrast, our present study using quantitative RT-PCR revealed that ADAR2 is widely distributed in mouse brain at birth and is expressed in all brain regions examined where editing substrates are expressed. Our data agree with the previous study (Paupard et al., 2000) in that expression was highest in the thalamus, suggesting that differences in assay sensitivity may have contributed to the observed differences between these studies. Like ADAR2, ADAR1 also shows widespread expression in the mouse brain and also suggests an extensive overlap between expression of editing substrates and the ADAR proteins. The Allen Brain Atlas and GENSAT databases present images of ADAR expression that are largely in agreement with our study, demonstrating that ADARs are widely distributed throughout the mouse forebrain by birth, with the possible exception of the striatum. The functional relevance of abundant

expression of both enzymes during development is unknown, but may relate to intrinsic differences in their substrate specificity.

Further, we used double-label immunohistochemistry to determine the patterns of expression for ADAR1 and ADAR2 in neuronal and glial cells. Co-labeling studies performed with GFAP or NeuN indicated coexpression of ADAR1 and ADAR2 with NeuN. Only very limited ADAR1 or ADAR2 labeling was observed in GFAP- or S100β-positive cells, indicating that ADARs are not expressed in most glial cells in the mouse forebrain. Although we did not use a direct marker of oligodendrocytes, we did not observe ADAR1 or ADAR2 labeling in any white matter tracts (Figures. 12, 13, 17, 18, 20, 21), suggesting that these ADAR proteins may be largely restricted to neurons in the mouse forebrain. In contrast, low levels of ADAR transcripts and edited glutamate receptor subunits have been previously reported in white matter from humans (Kawahara et al., 2003). Thus, there may be pertinent species differences.

Similar to previous reports (Burnashev et al., 1992; Paschen et al., 1997), we observed extensive editing of glutamate receptor transcripts prenatally. Data generated from knockout animals, single cell RT-PCR studies and *in vitro* editing activity assays have shown that the Q/R site is preferentially edited by ADAR2 (Higuchi et al., 2000; Melcher et al., 1996b). Our study reveals Q/R site editing to be greater than 99% by E15 when ADAR2 mRNA is quite low and ADAR2 protein is barely detectable. Additionally, we find that the D-site of the $5HT_{2C}$ receptor, a site that is preferentially edited by ADAR2 (Burns et al., 1997; Niswender et al., 1998), is edited in 50% of the transcripts by E15, highlighting another discordant relationship between ADAR2 expression and RNA editing levels. It is possible that the Q/R site of GLUR2 and D-site of the $5HT_{2C}$ receptor are kinetically favored and do not require abundant ADAR2 protein for efficient deamination, yet previous *in vitro* competition analyses revealed that the $5HT_{2C}$ receptor duplex is particularly weak in its ability to compete for ADAR2-mediated editing (Dawson

et al., 2004). Alternatively, ADAR2 expression could be selectively increased in neurons expressing GLUR2 and $5HT_{2C}$ receptor mRNAs; yet given the broad expression of AMPA receptors across neuronal subpopulations in the central nervous system (Geiger et al., 1995), it is unclear whether spatial patterns of A-to-I conversion in the brain reflect region-specific differences in ADAR expression. By contrast, the major $5HT_{2C}$ receptor mRNA isoforms in the choroid plexus are either non-edited or edited solely at the D-site to encode isoleucine, asparagine and isoleucine (INI) or isoleucine, asparagine and valine (INV) in the second intracellular loop of the receptor, respectively (Table 1), a pattern consistent with low levels of ADAR1 and increased levels of ADAR2 in the adult rat and mouse brains (Burns et al., 1997)). While no catalytic activity for ADAR3 has been observed with known ADAR substrates or synthetic dsRNAs (Chen et al., 2000; Melcher et al., 1996a), it is possible that this protein may also be involved in the region-specific regulation of editing patterns given its robust and restricted expression in the brain (Chen et al., 2000). Previous studies have suggested that ADAR3 may interfere with the activities of ADAR1 and ADAR2 by acting as a competitive inhibitor, thus decreasing the editing of specific substrates in those regions where it is preferentially expressed (e.g. thalamus) (Chen et al., 2000).

We have demonstrated that ADARs are expressed at low levels in fetal brain, and increase gradually over time. ADAR1 mRNA has its lowest expression in choroid plexus, cortex and hippocampus, while ADAR2 mRNA is least expressed in cortex and hippocampus, with increased expression in the thalamus. We have also reported complete ADAR colocalization with NeuN, indicating that ADAR expression is restricted to neuronal populations. ADAR1 and ADAR2 expression do not necessarily correlate with the extent of editing activity for identified substrates, raising questions regarding the regulatory mechanisms that may be necessary to maintain normal spatiotemporal patterns of editing in the mammalian brain. While *in vitro* studies of A-to-I editing have

not demonstrated a requirement for any proteins other than ADARs for the site-selective editing of specific RNA substrates, the discordance between ADAR expression levels and editing patterns in mouse brain regions suggests that additional regulatory factors may be involved in the formation of protein-RNA complexes to modulate site-selective A-to-I conversion.

CHAPTER III

GENERATION OF $5HT_{2C}R^{INI}$ MICE

Introduction

The serotonin 2C receptor ($5HT_{2C}R$) has been implicated in a variety of human neuropsychiatric and behavioral disorders, including depression, dysthymia, obsessive-compulsive disorder, anxiety, schizophrenia and sleep disturbances (Dubovsky and Thomas, 1995; Frank et al., 2002; Julius, 1991; Masellis et al., 1998; Pandey et al., 1995; Teitler and Herrick-Davis, 1994). $5HT_{2C}$ receptor expression is limited to the central nervous system (CNS), with the highest levels of expression localized in choroid plexus and significant expression also found in hippocampus, striatum, amygdala and hypothalamus (Pasqualetti et al., 1999; Teitler and Herrick-Davis, 1994). Mutant mice lacking $5HT_{2C}$ receptor expression display a variety of physiologic and behavioral deficits including leptin-independent hyperphagia and type 2 diabetes, death from spontaneous and audiogenic seizures, an increased sensitivity to the psychostimulant and reinforcing properties of cocaine and learning deficits resulting from hippocampal dysfunction (Brennan et al., 1997; Frank et al., 2002; Heisler et al., 1998; Nonogaki et al., 1998; Rocha et al., 2002; Tecott et al., 1998; Tecott et al., 1995).

Sequence comparisons of rat, mouse and human $5HT_{2C}$ receptor genomic DNA with their corresponding cDNAs revealed as many as five adenosine-to-guanosine (A-to-G) discrepancies (Burns et al., 1997; Niswender et al., 1998; Wang et al., 2000b) indicative of adenosine-to-inosine (A-to-I) editing events in which specific adenosine moieties are post-transcriptionally converted to inosine by hydrolytic deamination (Polson et al., 1991). These five editing sites (termed sites A-E) are predicted to alter the genomically encoded amino acids (isoleucine, asparagine and isoleucine; INI) at

positions 157, 159 and 161 in the second intracellular loop of the receptor (Burns et al., 1997; Wang et al., 2000b). Editing of $5HT_{2C}$ receptor transcripts can generate as many as thirty-two different mRNA transcripts encoding up to twenty-four receptor isoforms that not only have distinct functional properties affecting constitutive activity and receptor:G-protein coupling in heterologous expression systems, but also differ in their pattern of CNS expression (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999).

The second intracellular loop of G-protein coupled receptors plays an important role in the coupling of G-proteins, especially for receptors linked to $G_{\alpha q}$ (Pin et al., 1994; Wong et al., 1990). Analysis of 5HT dose-response curves in transfected NIH-3T3 cells demonstrated that serotonergic agonists were 15- and 40-fold less potent when acting through the edited VSV and VGV receptor isoforms, respectively, suggesting that edited isoforms couple less efficiently to the intracellular signaling machinery (Burns et al., 1997; Niswender et al., 1998). Studies with $5HT_{2C}$ receptor have indicated that this receptor displays constitutive activity (Niswender et al., 1999) and competition binding studies using human isoforms of the $5HT_{2C}$ receptor reveal that the INI isoform displays the greatest levels of constitutive activity. More recent studies have identified altered editing in patients diagnosed with schizophrenia (Sodhi et al., 2001), in suicide victims with a history of major depression (Gurevich et al., 2002b; Niswender et al., 2001) and in response to antidepressant treatment (Gurevich et al., 2002a), suggesting that editing may be involved in affective disorders and the maintenance of appropriate serotonergic neurotransmission.

Given that RNA editing generates $5HT_{2C}$ receptor isoforms that differ in G-protein coupling efficiency (Niswender et al., 1999), affecting both basal activity and ligand potency (Herrick-Davis et al., 1999), we sought to determine the physiologic relevance of $5HT_{2C}$ receptor editing *in vivo* by generating a mutant strain of mouse that solely

expresses the non-edited (INI) isoform of this receptor ($5HT_{2C}R^{INI}$). This chapter will focus on the biochemical alterations of $5HT_{2C}R^{INI}$ mutant mice, including alterations in $5HT_{2C}$ receptor mRNA and protein expression as well as changes in alternative splice patterns and DA levels in cortex and striatum. These changes highlight the importance and requirement for $5HT_{2C}$ receptor RNA editing for normal brain function.

Experimental Procedures

Generation of DNA constructs

Targeting vector

A genomic fragment containing a segment of the mouse $5HT_{2C}$ receptor was isolated from a 129X1/SvJ bacterial artificial chromosome (BAC) library (Genome Systems, Inc., St. Louis, MO) and a 7.4 kilobase (kb) *Avr* II fragment containing exon 5 and a portion of the flanking introns was subcloned into modified pBluescript KSII- (pBKSII-, Strategene, La Jolla, CA) and was further characterized by detailed restriction and Southern blotting analysis, intron/exon boundary mapping and limited nucleotide sequence determinations (Figure 23).

A 360 base pair (bp) *Acc* I flanked restriction fragment was isolated from the original 7.4 kb fragment and subcloned into a pBKSII- vector in which the *Acc* I site had been eliminated (Acc-/pBKSII-); this *Acc* I fragment, containing the editing sites in exon 5 and sequences from the 5'-end of intron 5, was used as a template for oligonucleotide-directed site mutagenesis to introduce eleven point mutations into the editing-site complementary sequence (ECS), a region within intron 5 that is required for A-to-I editing of $5HT_{2C}$ receptor transcripts. Mutations within this region disrupt the RNA duplex required for editing to take place while maintaining the coding potential of the gene.

Verification of INI mutations

To confirm that the introduced mutations resulted in the loss of $5HT_{2C}$ receptor editing prior to the generation of genetically-modified mice, minigene constructs containing the entire duplex region were generated via PCR (sense: 5'-TATTTGTGCCCCGTCTGG-3' and antisense: 5'-ATATAAGCTTGACAACCGATCAAAC GCATGTTACCAGTCGACGTCTGTAGGTTGTT-3') from mouse genomic or INI-mutant DNA to create wild-type and INI minigenes, respectively. These PCR amplicons were subcloned into the eukaryotic expression vector pRC/CMV (Invitrogen, Carlsbad, CA). Each minigene (5µg) was then transiently co-transfected with ADAR1 or ADAR2 (20µg), into human embryonic kidney (HEK293) cells using Ca-PO_4 coprecipitation. Crude nuclear extracts were prepared from transfected cells and immunoblotted using an anti-ADAR1 (1:1000) or anti-ADAR2 antibody (1:1000) with histone deacetylase 2 (HDAC2, 1:500) (Zeng et al., 1998) serving as an internal control for protein loading.

RNA was harvested from transfected cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and cDNA was synthesized using an intron 5 specific primer containing a unique 5'-extension (5'-CGGC CAATCGTACGTACCTCCGACAACCGATCAAACGCATGTTACCAGTCGAC-3'). Following cDNA synthesis, PCR was performed using a primer complementary to the unique extension (5'-CGGCCAATCGTACGTACCTCC-3') and a sense primer (5'-TTTTGAATTCTATTTGTGCCCCGTCTGGATTTCAC-3') to amplify $5HT_{2C}$ receptor transcripts. cDNA isolates were subcloned into pGEM (Promega, Madison, WI) and >100 cDNA clones were subjected to DNA sequence analysis to confirm that introduction of the mutations completely abolished editing while wild-type transfectants were efficiently edited by ADAR1 or ADAR2.

Once verified, the 7.4 kb *Avr* II fragment and the 360 bp fragment containing the INI-mutation were digested with *Acc* I. Digested products were purified and ligated

together to generate an *Avr* II fragment containing the INI-mutations. A selection cassette encoding the phosphoglycerate kinase (PGK) promoter-driven herpes simplex virus thymidine kinase (TK) was inserted outside the region of homology as a negative selectable marker. To insert the loxP-flanked neomycin phosphotransferase, under control of the PGK promoter, the insert was digested using *Bam* HI and *Sal* I from a previously generated targeting vector for ADAR2 ΔECS mice (Feng et al., 2006). This product was ligated into pBKSII-, digested with *Bam* HI, blunted using the large fragment of DNA polymerase I (Klenow, NEB) and then digested with *Xho* I while the *Avr* II fragment was digested with *Hpa* I and *Xho* I; digested products were purified and ligated to generate a 9.3 kb fragment.

Production of Mutant Mice

The Vanderbilt Transgenic Mouse/ES Cell Shared Resource assisted in the targeting of the mouse embryonic stem (ES) cells and the generation of chimeric mice harboring the targeted allele. The targeting vector was linearized using a *Not* I restriction site and 129S6/SvEvTac-derived mouse ES cells were electroporated. The ES cells were then selected with G418 and gancyclovir. To confirm homologous recombination in isolated ES cell clones, PCR analysis was performed using the Expand Long Template PCR kit (Roche, USA) with primers designed outside the region of homology (sense, 5'-CTGAGTGCATTGGAAAAGAGATCC-3'; antisense 5'-CCATATATCAGGATGCAGTCT TGTCA-3'); correctly targeted ES cell clones resulted in the amplification of a 9.3 kb PCR fragment due to the introduction of the 1.9 kb PGK/neomycin cassette. Two correctly targeted ES cell clones were expanded and subsequently microinjected into C57BL/6J blastocysts which were transferred to the uterus of pseudopregnant foster mothers to produce chimeric mice.

Male offspring demonstrating >70% agouti coat color chimerism were mated with female wild-type 129S6/SvEvTac mice and offspring from this mating were screened for germ-line transmission of the modified $5HT_{2C}$ receptor allele by long PCR analysis. Animals heterozygous for the mutant $5HT_{2C}$ receptor allele were mated with transgenic mice expressing Cre recombinase under the control of the mouse protamine 1 promoter (Prm-Cre 129S4/SvJae, The Jackson Laboratories, Bar Harbor, ME) (O'Gorman et al., 1997). Progeny were screened by PCR to select for mice that had successfully excised the selectable marker; sequence analyses were also performed to verify locus integrity, presence of the introduced mutations and loss of PGK/neomycin cassette. Following removal of the marker, mice were backcrossed one additional generation to wild-type 129S6/SvEvTac mice and assessed for loss of the Prm-Cre transgene using a PCR-based strategy. Heterozygous animals that successfully eliminated the Prm-Cre transgene were then mated to generate animals homozygous for the mutant $5HT_{2C}$ receptor allele.

Given the location of the $5HT_{2C}$ receptor gene on the mouse X chromosome, two breeding strategies were employed to generate all possible combinations of wild-type and INI-mutant animals; wild-type males (+/Y) were mated with heterozygous mutant females (INI/+) and hemizygous mutant males (INI/Y) were mated with heterozygous mutant females (INI/+). Resultant offspring did not differ from expected Mendelian distribution, indicating that the INI mutation does not result in embryonic or early-postnatal lethality.

Animals were housed in a humidity- and temperature controlled room on a 12-h light, 12-h dark cycle with *ad libitum* access to water and standard laboratory chow (rodent chow 5001; Ralston Purina Co., St. Louis, MO).

Analyses of genetically modified INI-mice

RNA editing

For editing studies, total cellular RNA was isolated from adult mouse whole brain as described previously in chapter 2. The extent of editing at all five sites (A, B, C, D and E) was evaluated by fluorescent dideoxynucleotide based sequencing of cDNA amplified from mouse brain $5HT_{2C}$ receptor pre-mRNA. As an additional approach to further examine the editing profile of $5HT_{2C}$ receptor transcripts in wild-type and mutant animals, a 293 nucleotide (nt) segment extending from exon 5 through exon 6, containing the edited region of mature $5HT_{2C}$ receptor transcripts, was amplified by RT-PCR from whole brain and the PCR reaction product was subcloned into a prokaryotic cloning vector (pGEM Easy-T vector; Promega, Madison, WI). Once transformed into bacteria, 10 individual cDNA isolates from animals of each genotype were selected and sequenced.

RNA expression and splicing

To examine potential alterations in the level of $5HT_{2C}$ receptor RNA expression, wild-type and INI-mutant mice were collected during a developmental time course at the following ages: E15, P0, P14, P21 and adult (~P100). As described previously (chapter 2), whole brain and dissected brain regions (adult animals only) were harvested, total RNA was isolated and a quantitative real-time RT-PCR strategy was utilized using the following set of Taqman probes from Applied Biosystems Assays-on-Demand (ADAR1, assay ID Mm00508001_m1; ADAR2, assay ID Mm00504621_m1; $5HT_{2C}$R, assay ID Mm00434127_m1; $5HT_{1B}$R, assay ID Mm00439377_s1, $5HT_{2A}$R, assay ID Mm00555764_m1; SLC6A4, assay ID Mm00439391_m1) and eukaryotic 18S rRNA (product no. 4319413E) was included in each multiplex PCR as an internal control. Real-time PCR and subsequent analysis were performed with an ABI Prism 7900HT

sequence detection system (SDS v2.3; Applied Biosystems, Foster City, CA); all reactions were run in triplicate and quantified as described previously (chapter 2).

To measure relative expression of $5HT_{2C}$ receptor mRNA splice variants in RNAs isolated from whole brain in wild-type and INI/Y mice, first-strand cDNA was synthesized as described previously (chapter 2) and then amplified with a 6-carboxyfluorescein-labeled sense primer (5'-TATTTGTGCCCCGTCTGG-3') and a nonlabeled antisense primer (5'-ATCAAAGCTTGACGGCGTAGGACGTAG-3'). Resultant amplicons corresponding to alternatively spliced $5HT_{2C}$ receptor variants were separated on a 2.5% agarose gel electrophoresis and fluorescence was quantified using a Typhoon 9400 phosphorimager (Amersham Biosciences, Piscataway, NJ) with ImageQuant TL software.

Protein expression

Receptor protein levels in dissected brain regions (cortex, hippocampus and striatum) were determined by radioligand binding from adult wild-type and INI-mutant animals. Cortex, hippocampus and striatum were harvested (n=10/assay/genotype, total of 3 assays performed) and snap-frozen on dry ice in 24-well plates and stored at -80°C until the assay could be performed. The tissue was placed directly in 2 ml of cold standard binding buffer (SBB: 50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 1 mM EDTA), homogenized at ~15,000 rpm for 7 seconds and then pelleted at 25,000 x g for 10 minutes. The supernatant was removed, 1 ml of SBB (for 1 pellet) or 2 ml of SBB (for 2 pellets) was added, and the pellet was briefly homogenized and centrifuged in a cold microcentrifuge at top speed for 5 minutes. After re-pelleting, the supernatant was removed and the remaining pellet was placed on dry ice and stored at -80°C until ready for use. An 8-point saturation curve was performed in duplicate using a [^{3}H] non-specific $5HT_{2C}$ receptor antagonist, [N^6-methyl-^{3}H]-mesulergine (concentrations: 0.25, 0.5, 1.0,

1.5, 2.0, 4.0, 6.0, and 8.0 nM), in the presence of 100 nM spiperone ($5HT_{2A}R$ antagonist), and 3 µM ritanserin to define non-specific binding. Assays were incubated for 1.5 hours in SBB at room temperature, in a 96-well format, reactions were harvested by vacuum filtration through glass filters washed 3 times in ice cold 50 mM Tris, pH 7.7 and measured by liquid scintillation spectrometry. Receptor density (B_{max}) and dissociation constant (K_D) were determined by computer-assisted nonlinear regression analyses of saturation binding isotherms (assuming ligand depletion) using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Biogenic Amine Analysis

In collaboration with the Vanderbilt Neurochemistry Core, biogenic amine studies were performed in wild-type and INI-mutant mice. Cortex, hippocampus and striatum (n=6/genotype) were harvested from adult mice and snap-frozen on dry ice in 1.5 ml eppendorf tubes. Tissue was homogenized in 0.1 M trichloroacetic acid (TCA) containing 10 mM sodium acetate, 0.1 mM EDTA, 1µM isoproterenol (to serve as an internal standard), 10.5% methanol (pH 3.8) and spun in a microfuge at 10,000 x g for 20 minutes. The supernatant was removed and stored at -80°C until the assay was performed and the pellet was saved for protein content using the BCA Protein Assay kit (Pierce, Rockford, IL). Supernatants were used to quantify the biogenic amines, norepinephrine (NE), dopamine (DA) and serotonin (5HT) and their metabolites, 3-methoxytyramine (3MT), 3, 4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindolacetic acid (5HIAA) and homovanillic acid (HVA) by a specific HPLC assay utilizing an Antec Decade (oxidation: 0.5) electrochemical detector. 20 µl of each supernatant were injected onto a C^{18} HPLC column (Phenomenex Nucleosil 5µ); biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1 M TCA and solvent was delivered at

0.7 ml/min using a Waters 515 HPLC pump. HPLC control and data acquisition were managed by Millennium[32] software (Waters Corporation, Milford, MA).

Results

Generation of mutant mice solely expressing the non-edited (INI) isoform of the $5HT_{2C}$ receptor

To confirm that introduction of eleven point mutations in intron 5 resulted in the sole expression of the INI-encoding isoform of the $5HT_{2C}$ receptor transcript, whole brain RNA was isolated from INI/Y mice, $5HT_{2C}$ receptor pre-mRNA was amplified by RT-PCR and subjected to DNA sequence analysis. At the editing sites, double A and G peaks are observed in WT animals indicating the presence of both nucleotides, however only single A peaks are observed in INI/Y animals, revealing that only the INI isoform was expressed (Figure 23D).

RNA characterization

To examine potential alterations in $5HT_{2C}$ expression in $5HT_{2C}R^{INI}$ mutant mice, we quantified the level of mature $5HT_{2C}$ transcripts in RNA isolated from whole brain or dissected brain regions in INI/Y animals and their wild-type (+/Y) littermates. Results from this analysis revealed a 30-40% decrease in steady-state $5HT_{2C}$ receptor mRNA in INI/Y mice as compared to +/Y animals (Figure 24, $p<0.01$, $n=12$). As contributions to this decrease in whole brain mRNA could result from larger decreases in specific brain regions versus a uniform decrease in whole brain, we investigated mRNA levels from adult animals in 3 brain regions where $5HT_{2C}$ receptor mRNA is highly expressed in WT animal: cortex, hippocampus and striatum. In each of these brain regions, INI/Y mice demonstrated a 30% reduction in $5HT_{2C}$ receptor transcripts when compared to their WT littermates, suggesting a global change in $5HT_{2C}$ receptor mRNA levels (Figure 24, $p<0.05$, $n=10$).

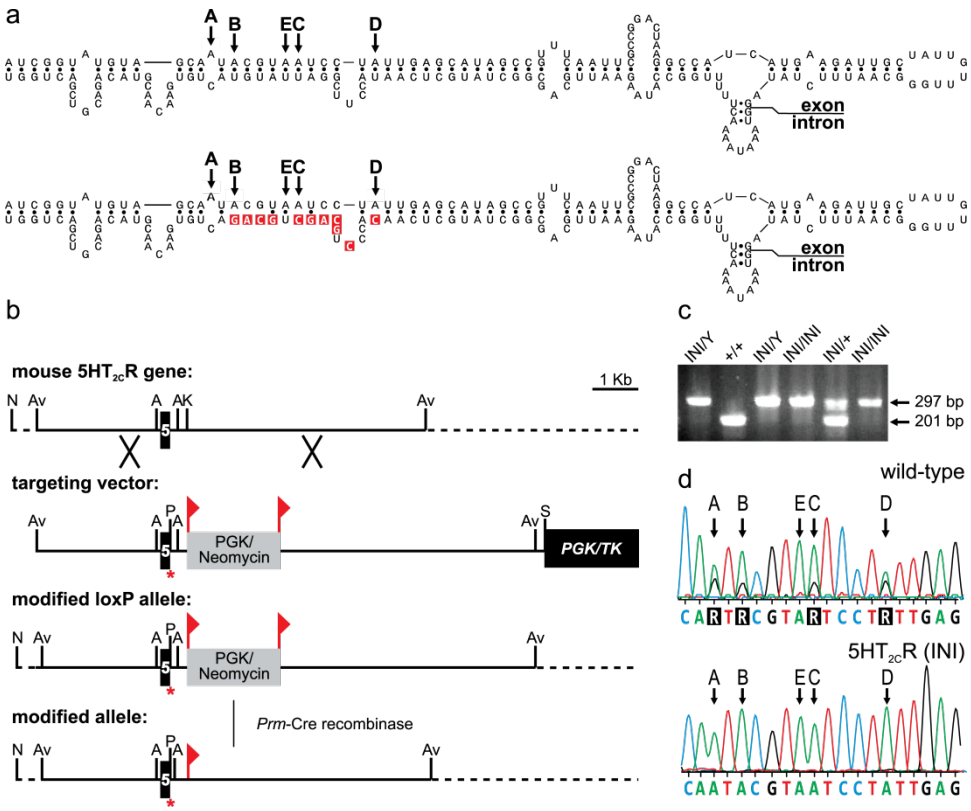

Figure 23: Targeting strategy and generation of $5HT_{2C}R^{INI}$ mice. a) The predicted secondary structure for wild-type and INI-mutant pre-mRNAs in the proximal region of intron 5 is shown. The positions of the five editing sites (A-E) are indicated and site-specific nucleotide alterations in intron 5, introduced to disrupt the proposed RNA duplex, are indicated in red lettering. **b)** Schematic diagram and abbreviated restriction map of the mouse $5HT_{2C}$ receptor gene before and after targeted gene modification; the location of exon 5, the loxP sites (►) flanking the PGK/neomycin cassette outside the region of homology, the appropriate position of the introduced mutations (*) and the sequences outside the region of homology (dotted line) are indicated; A, *Acc* I; Av, *Avr* II; K, *Kpn* I; N, *Not* I; P, *Pst* I; S, *Sfi* I. **c)** Genotype analysis of progeny from a $5HT_{2C}R^{INI}$ hemizygous male x heterozygous female breeding; migration positions of PCR amplicons corresponding to the wild-type (201 bp) or mutant (297 bp) $5HT_{2C}$ receptor alleles and animal genotypes are indicated. **d)** Sequence electropherogram traces of $5HT_{2C}$ receptor-derived RT-PCR products generated from wild-type and $5HT_{2C}R^{INI}$ hemizygous male mice. The positions of the five editing sites and the corresponding nucleotide sequences are indicated; R, purine.

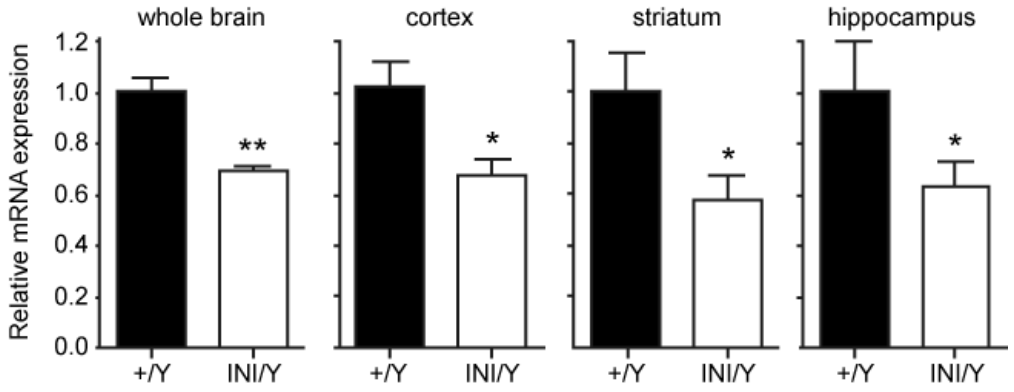

Figure 24: INI-mutant mice display decreased mRNA in whole brain and brain regions. Quantitative RT-PCR analyses of $5HT_{2C}$ receptor mRNA in adult whole brain (n=12) as well as dissected brain regions (n=10) in +/Y and INI/Y mice (mean ± SEM). *: p<0.05, **: p<0.01.

To further investigate if this decreased mRNA expression was differentially regulated during development in INI-mutant animals, we examined total $5HT_{2C}$ receptor mRNA levels in whole brain of animals isolated at several ages (E15, P0, P14 and P21) animals versus their wild-type littermates. At each developmental timepoint, $5HT_{2C}$ receptor mRNA was decreased on average ~30% in whole brain from INI/Y animals versus their wild-type littermates (Figure 25).

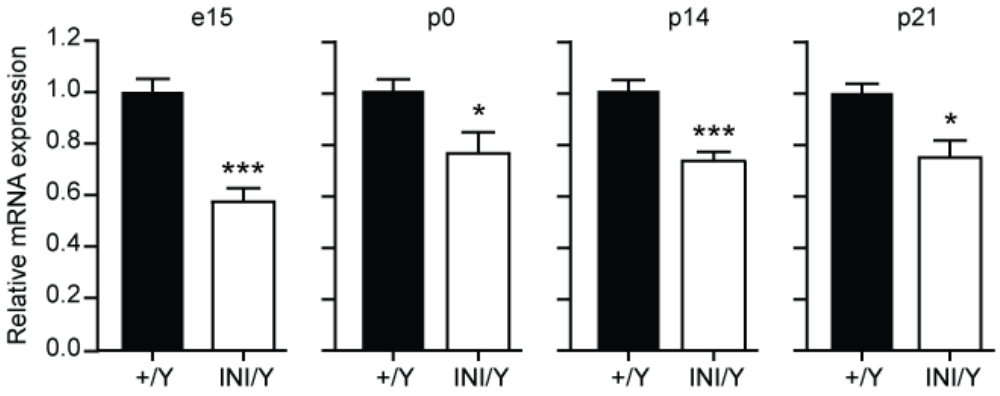

Figure 25: INI-mutant mice display decreased mRNA in whole brain during development. Quantitative RT-PCR analyses of $5HT_{2C}$ receptor mRNA in developing whole brain (n=6) in +/Y and INI/Y mice (mean ± SEM). *: p<0.05, ***: p<0.001

In addition to the observed decreases in $5HT_{2C}$ mRNA in INI/Y animals, we also quantified mRNA levels for the editing enzymes (ADAR1 and ADAR2) and several related genes in serotonergic pathways ($5HT_{1B}R$, $5HT_{2A}R$, SLC6A4) using qPCR. In whole brain samples isolated from adult mice, no differences were found in the expression of any of these genes when comparing INI/Y animals and their WT littermates (Figure 26).

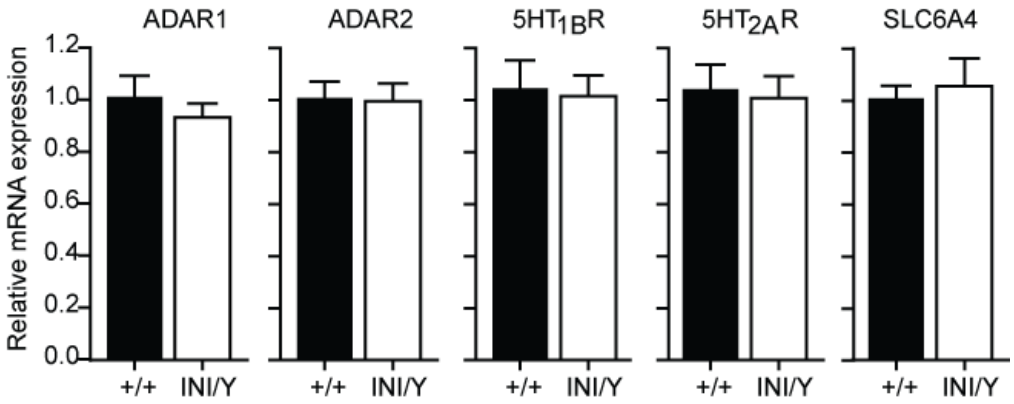

Figure 26: INI-mutant mice do not display alterations in mRNA levels for editing enzymes or related serotonergic genes. Quantitative RT-PCR analyses of ADAR1 and ADAR2 mRNA, as well as related genes in serotonergic pathways, $5HT_{1B}R$, $5HT_{2A}R$ and SLC6A4 in adult whole brain (n=6) in +/Y and INI/Y mice (mean ± SEM).

In addition to RNA editing, previous studies have shown the generation of multiple $5HT_{2C}$ receptor isoforms through the use of alternative 5'-splice sites in exon 5 and intron 5 (Canton et al., 1996; Wang et al., 2000b; Xie et al., 1996). Use of either the proximal (RNA1) or distal (RNA3) splice donor sites results in the generation of mature mRNAs encoding truncated receptor proteins resulting from premature translation termination, whereas use of the intermediate splice donor (RNA2) produces an mRNA species encoding the full-length, functional receptor protein (Canton et al., 1996; Wang et al., 2000b; Xie et al., 1996). To quantify the relative abundance of $5HT_{2C}$ receptor mRNA splice variants in $5HT_{2C}R^{INI}$ mutant mice, we developed a quantitative RT-PCR based strategy utilizing 6-carboxyfluorescein-labeled (6-FAM) sense PCR primer. In

whole brain RNA samples isolated from adult animals, mutant mice (INI/Y) displayed a 25% reduction in the relative expression of RNA2 and a concomitant increase in RNA1 compared to their wild-type littermates, while no significant change in the relative level of RNA3 was observed (Figure 22). To determine if alternative splicing of $5HT_{2C}$ receptor mRNA was developmentally regulated in $5HT_{2C}R^{INI}$ animals, RT-PCR was performed using whole brain RNA from P0 mice. While relative expression of each of the three splice isoforms at P0 was different than that observed in adult, the magnitude of difference between wild-type and INI/Y animals was the same (Figure 27).

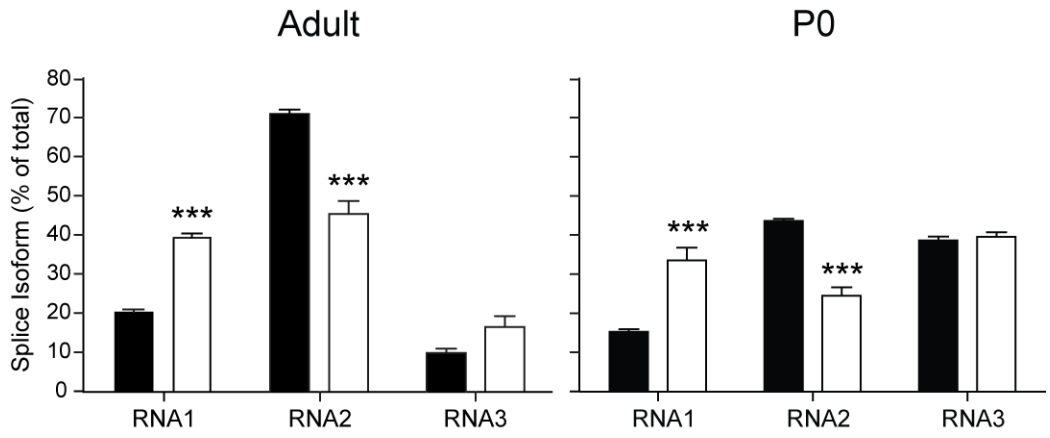

Figure 22: Altered patterns of alternative splicing in $5HT_{2C}R^{INI}$ mice. Quantification of relative mRNA expression for alternatively spliced $5HT_{2C}$ variants in adult and P0 +/Y (black bars) and INI/Y (white bars) mice. The relative level of each mRNA isoform is expressed as a percentage of total $5HT_{2C}$ receptor transcripts (n = 6, mean ± SEM). ***: $p<0.001$.

Protein expression

Given the altered mRNA expression and splicing patterns observed in INI/Y animals, we assessed whether the INI mutation would also result in changes in total $5HT_{2C}$ receptor protein expression using radioligand binding assays in cortex, hippocampus and striatum from adult +/Y and INI/Y male mice and +/+, INI/+ and INI/INI females. As predicted by the observed decreased in mRNA expression, $5HT_{2C}R^{INI}$ mice displayed a genotype-dependent reduction in total $5HT_{2C}$ receptor protein expression in

all brain regions examined (Figure 28). In cortex, hemi- and homozygous mutant mice displayed a 50-65% reduction in $5HT_{2C}$ receptor protein as compared to their wild-type littermates, while INI/+ mutants demonstrated a reduction of about 25%. Similarly, protein expression was also reduced in the hippocampus and striatum, yet the effects were more pronounced. Analysis of $5HT_{2C}$ receptor protein in hippocampal samples revealed a 65 and 75% decrease in INI/INI and INI/Y mice, respectively, while INI/+ mice showed a 40% reduction in $5HT_{2C}$ receptor protein as compared to wild-type littermates. In striatum, there was a 60-70% reduction in $5HT_{2C}$ receptor protein in INI/Y and INI/INI mice, with little to no change (~5%) observed in INI/+ mutant animals.

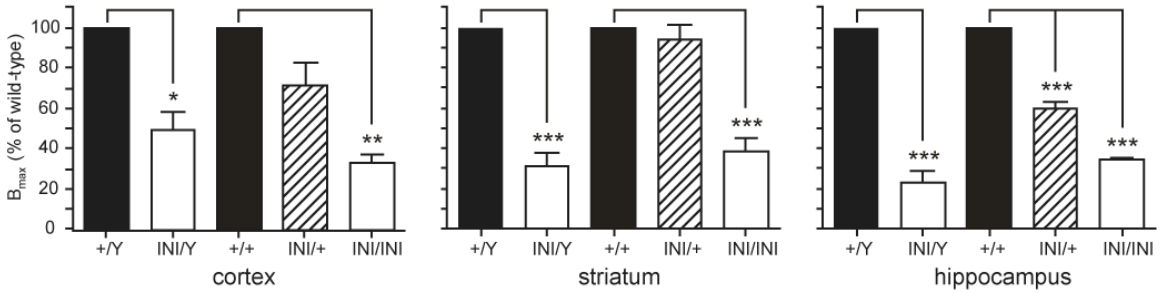

Figure 28: $5HT_{2C}R^{INI}$ mice show decreased $5HT_{2C}$ receptor protein. Summary of saturation binding analyses for $5HT_{2C}$ receptor expression in dissected cortex, striatum and hippocampus for adult wild-type (+/Y and +/+) and mutant (INI/Y, INI/+, INI/INI) mice are presented. Maximal binding (B_{max}) is expressed as a percentage of wild-type values for each gender (n = 3 groups of 10 pooled brain regions, mean ± SEM) *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Biogenic Amine Analysis

In collaboration with the Vanderbilt Neurochemistry Core, we measured the levels of the biogenic amines (NE, 5HT, DA) and their metabolites (3MT, DOPAC, HVA, 5HIAA) in wild-type and $5HT_{2C}R^{INI}$ adult animals in cortex, hippocampus and striatum. We observed minor region-specific changes in biogenic amine levels with increased levels of DA in INI/Y mice as compared to their wild-type littermates in striatum ($p<0.05$, 2-way ANOVA) (Figure 29). We also observed increased DA levels in INI/INI mice as

compared to wild-type littermates in cortex ($p<0.05$, 2-way ANOVA) (Figure 29). No other significant differences were seen with additional amines in the remaining brain regions analyzed.

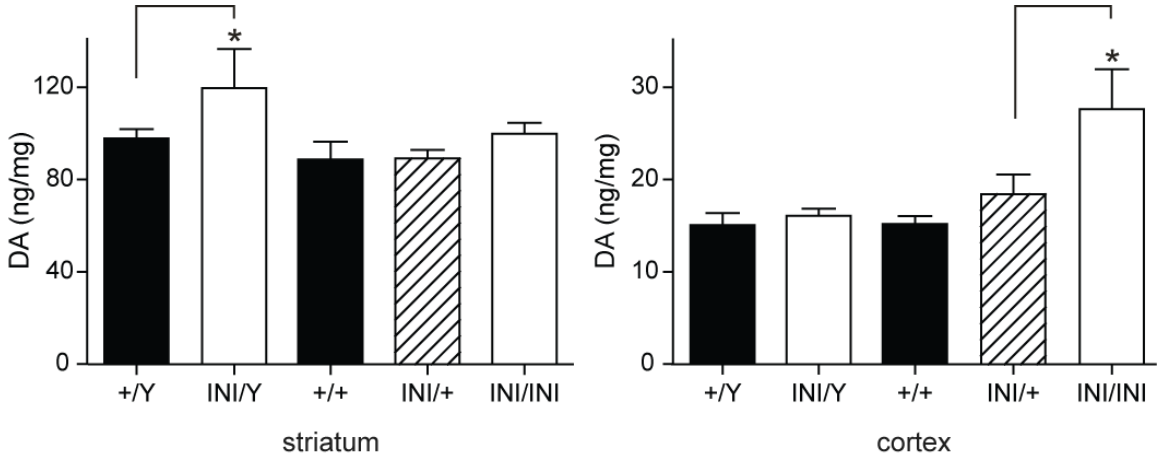

Figure 29: Biogenic amine analysis revealed differences in DA levels. INI/Y male mice have increased DA levels in striatum compared to wild-type male littermates (n=6, mean ± SEM) while INI/INI female mice have increased DA levels in cortex compared to their wild-type female littermates (n=6, mean ± SEM). *: $p<0.05$.

Discussion

The editing of the $5HT_{2C}$ receptor transcripts has been shown to modulate multiple aspects of receptor signaling, trafficking and expression in heterologous systems, yet the physiologic relevance of $5HT_{2C}$ receptor editing has been unexplored. Genetically-modified mice solely expressing the non-edited isoform of the $5HT_{2C}$ receptor show a 30% reducution in steady-state $5HT_{2C}$ receptor mRNA (Figures 24 and 25) and a 50-75% decrease in receptor protein expression (Figure 28). Beacause the INI isoform of the $5HT_{2C}$ receptor has been shown to display increased constitutive activity and G-protein coupling relative to more highly edited isoforms (Burns et al., 1997; Niswender et al., 1999; Niswender et al., 1998), the decreased levels of $5HT_{2C}$ mRNA and receptor levels may represent an adaptive compensatory alteration to maintain normal levels of receptor signaling. Previous pharmacologic studies have suggested that

$5HT_{2C}$ receptor editing represents a homestatic mechanism to retain the basic response properties of $5HT_{2C}$ receptors in the face of changing synaptic input to maintain receptor activation (Gurevich et al., 2002a; Schmauss, 2005). Disparities between the observed decrease in receptor mRNA and protein levels may be further explained by alterations in $5HT_{2C}$ alternative splicing patterns, as mutant mice show a 25% reduction in synthesis of RNA2 (Figure 27), the mRNA isoform responsible for generating the full-length, functional receptor protein. Recent studies have indicated that the alternative splicing of $5HT_{2C}$ receptor pre-mRNAs can be regulated by editing at the E, C and D sites to promote the expression of RNA2 (Flomen et al., 2004), consistent with the observed increase in RNA1 at the expense of RNA2 for non-edited transcripts in mutant mice (Figure 27).

It is important to note that saturating binding analyses with total membrane proteins may underestimate the degree of compensation that occurs due to the fact that such techniques detect total $5HT_{2C}$ receptor binding, rather than receptors localized to the cell surface. Recent studies have indicated that cell surface expression of the $5HT_{2C}$ receptor decreased in parallel increased constitutive activity (decreased editing) of the receptor (Marion et al., 2004); the subcellular distribution of the non-edited (INI) and highly edited (VSV and VGV) isoforms was dependent on their ability to interact with β-arrestin2, which correlated with the constitutive activity level of each isoform.

Despite the observed decrease in $5HT_{2C}$ receptor expression in mutant mice, we did not observe any changes in other components of the 5HT system including the $5HT_{1B}$ and $5HT_{2A}$ receptors and the serotonin transporter (SLC6A4) at the mRNA level (Figure 26). Such changes are not necessarily surprising given that $5HT_{2C}$ receptor KO animals do not display any significant alterations in these components of 5HT signaling (Lopez-Gimenez et al., 2002). We also do not observe any alterations in levels of 5HT or 5HIAA in $5HT_{2C}R^{INI}$ mutant animals; we do observe changes in DA levels in striatum and

cortex of INI/Y and INI/INI animals (Figure 29). These changes in DA levels appear to be regional as well as gender-specific so it is unclear what role, if any, these alterations may have. It is possible that INI-mutant mice have increased stores of DA, so future studies should address this. Nevertheless, these changes do implicate that the DA system is closely regulated with the 5HT system. Taken together, the observed alterations in $5HT_{2C}$ receptor mRNA, protein, alternative splicing and DA levels in mutant mice that solely express the non-edited isoform of the $5HT_{2C}$ receptor emphasize the importance of RNA editing in the CNS.

CHAPTER IV

PHENOTYPIC ALTERATIONS IN $5HT_{2C}R^{INI}$ DAMS

Introduction

Previous studies in heterologous cell systems have revealed differences in the efficacy of receptor:G-protein coupling between edited $5HT_{2C}$ receptor isoforms, yet the physiological relevance of this differential signaling has yet to be examined in an animal model system. Since the INI isoform of the $5HT_{2C}$ receptor exhibits the greatest basal activity and agonist potency via enhanced G-protein coupling (Berg et al., 2001; Herrick-Davis et al., 1999), we hypothesized that mutant mice bearing the INI allele would show enhanced signaling capacities compared to wild-type (WT) animals. $5HT_{2C}$ receptor-null mice have abnormalities in feeding behavior, seizures, learning and memory, anxiety and cocaine abuse (Nonogaki et al., 1998; Rocha et al., 2002; Tecott et al., 1998; Tecott et al., 1995) highlighting potential systems in which physiologic alterations may be observed. In mice solely expressing the INI isoform of the $5HT_{2C}$ receptor, an unanticipated deficit in maternal behavior was observed and phenotypic characterizations has focused upon altered behaviors in both dams and offspring (Chapter 5).

Maternal behaviors are defined as a series of events (e.g. nest building, pup retrieval, nursing) that are required for offspring survival and subsequent behavioral states in the offspring (Kuroda et al., 2007). The initiation and maintenance of maternal behaviors is regulated by a complex series of events that includes modulating hormonal balance in the dam prior to and immediately following delivery, as well as interaction with pups from birth until weaning. Early maternal behavior is controlled by hormones associated with parturition, particularly the protein hormones oxytocin and prolactin

(Theodosis et al., 2004; Wettschureck et al., 2004). Both of these hormones are required for effective nursing of young pups. Female oxytocin-null mice are unable to lactate and therefore their young must be cross-fostered to survive (Nishimori et al., 1996). Disruption of the prolactin gene causes infertility and an inability to lactate, but does not prevent mice from nurturing their young (Horseman et al., 1997). Interestingly, many prolactin-releasing factors such as thyroid-stimulating hormone (TRH) and oxytocin act through $G_{\alpha q/11}$-coupled receptors (Aragay et al., 1992; Freeman et al., 2000; Gimpl and Fahrenholz, 2001) and a forebrain-specific double knockout mouse for $G_{\alpha q/11}$ displays deficits in maternal behaviors such as nest building and pup retrieval (Wettschureck et al., 2004). $5HT_{2C}$ receptors preferentially couple to $G_{\alpha q}$, thus providing a potential link between endogenous signaling pathways and behaviors observed in $5HT_{2C}R^{INI}$ mice.

Maternal behavior is also influenced by the limbic system. Activation of c-fos, a marker of neuronal activation, is often seen in brain regions important for the development and maintenance of maternal behaviors including medial preoptic area of the hypothalamus (MPOA), nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), basolateral amygdala (BLA) and prefrontal cortex (PFC) (Fleming and Korsmit, 1996; Fleming et al., 1994; Numan et al., 2005; Sheehan et al., 2001; Stack et al., 2002; Stack and Numan, 2000). Reinforcement of maternal behaviors also depends on other limbic pathways, including the amygdala-nucleus accumbens pathways, a pathway important in rewarding behaviors (Fleming et al., 1994). Systemic administration of DA antagonists disrupts both ongoing maternal behavior (Giordano et al., 1990) and development of conditioned place preference to pup stimuli (Fleming et al., 1994). Recent studies using functional magnetic resonance imaging (fMRI) have further demonstrated that suckling stimulation in lactating dams activated the DA reward system. When lactating mothers were given cocaine, the resultant activation of these reward pathways was suppressed, indicating that pup stimulation was more reinforcing

than cocaine (Ferris et al., 2005). It has been suggested that activation of $5HT_{2C}$ receptors may substantially contribute to a serotonergic inhibition of mesolimbic DA activity (Di Giovanni et al., 2000; Gobert et al., 2000); activation of the mesolimbic DA system is considered critical for the reinforcing and psychostimulant effects of cocaine (Wise, 1984) and thus provides a potential link between alterations in the $5HT_{2C}$ receptor system and maternal behavior.

Experimental Procedures

Behavior

All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee and complied with Public Health Service Policy on the humane care and use of laboratory animals.

Rearing paradigms: cross-fostering and aunting

For cross-fostering studies, wild-type and heterozygous mutant (INI/+) female mice were mated with wild-type and hemizygous mutant (INI/Y) males, respectively. On P0, entire litters were exchanged between dams of different genotypes and litters were checked daily for pup survival. Beginning at P7, pups were marked and weighed daily until weaning on P21.

For behavioral studies requiring pups raised by communal nesting, separate groups of wild-type female mice were mated with wild-type males or hemizygous mutant (INI/Y) males. When females were visibly pregnant (~E18), wild-type and INI/Y-mated dams were placed in the same cage and allowed to cooperatively and communally raise their offspring, thereby allowing both wild-type and INI/+ female littermates to be reared by wild-type dams.

Nest building

For quantification of nest building behavior, individually housed multiparous, pregnant dams (wild-type and INI/+; E19) were provided a 5 x 5 cm piece of commercially available cotton nesting material (Ancare, Bellmore, NY) 90 minutes prior to the start of the dark cycle. At the end of the dark cycle, 12 hours later, a final estimation of the percentage of nesting material chewed, as well as nest depth at the thickest part of the nest, were recorded. The quality of nest building by wild-type and INI/+ dams were rated according to a 5-point scale developed by Lisk (Lisk et al., 1969) where: 0=no nest; 1=nest material in the corner of the cage; 2=some organization of nest material; 3=nest with semi-circular walls; 4=full nest with tall walls.

Maternal care and pup retrieval

Pup retrieval assays were conducted 8 hours after the onset of the light cycle in postpartum, multiparous dams (wild-type and INI/+; P0). Pups were removed and kept warm for a 1 hour period. Shortly before reunion of dams with offspring, the dam was removed from the cage and 3 of her own pups were placed into the corners, away from the nest. The dams were placed back in the cage with their pups and assayed for latency to approach the first pup as well as the total time to retrieve all pups into the nest (Brown et al., 1996). Retrieval was defined as picking up the pup and moving it into the nest; a pup that was picked up and then dropped was not classified as a successful retrieval event. Total time for completion of the retrieval task was limited to 15 minutes.

For pharmacologic rescue studies, pups were removed from newly postpartum dams as described above and dams were administered either vehicle (0.9% saline) or the $5HT_{2C}$ receptor inverse agonist, 3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2-b:4,5-b'] dipyrrole-1(2H)-carboxamide hydrochloride (SB206553; 5mg/kg body weight, Tocris

Bioscience, Ellisville, MO), by intraperitoneal injection one hour prior to retrieval testing. Retrieval studies were then performed as described above.

Locomotor activity

Locomotor activity was assayed using a commercially-available open field arena (ENV-520, MED Associates) measuring 27 x 27 x 20 cm with clear Plexiglas walls and white floors. Infrared detectors determined the mouse's position every 50 milliseconds (msec) and automatically recorded and analyzed distance traveled, rearings, circling, etc., in the open field.

Elevated Plus Maze

Anxiety-related behaviors were assayed using an elevated plus maze (EPM). This apparatus is plus-shaped consisting of two closed arms (platforms with tall walls) and two open arms (platforms with no walls) connected by a small center square (5 x 5 cm) built on 40 cm high legs and placed on the floor for testing. All arms measured 30 cm long x 5 cm wide with white Plexiglas floors; closed arms had 15 cm high walls made of black Plexiglas, while open arms were lined with a 0.25 cm white Plexiglas edge to decrease the likelihood of the mouse falling off the maze. Four white screens were placed around the maze to reduce spatial cues from the room and a white noise generator was present in the room for all experiments. A camera was placed directly above the maze to record the behavior of each animal.

For all experiments, individual animals were placed in center of the maze and permitted to freely explore for five minutes. The maze was illuminated in dim lighting conditions (~60 lux) to increase the likelihood of exploring the maze. Measurements were performed by investigators blind to genotype who hand coded the videotaped behavior using ProcoderDV (Vanderbilt University, Nashville, TN). The number of entries into the open and closed arms, duration spent in arms and the center of the maze was

also measured for each animal. Entries and exits from maze arms were defined as all four paws crossing into or out of an arm.

Novelty

To assess the response to a novel object, multiparous female dams were placed into a test cage for five minutes. At the end of a five minute habituation period, a 15mL conical tube (novel object) was introduced. Mice were permitted to explore the novel object for 10 minutes and behavior was recorded. Latency to approach novel object as well as time spent assessing the object was scored.

Olfaction

To assess whether mutant mice were anosmic, assays of olfaction were performed by placing multiparous female dams into a test cage for five minutes for habituation. After 5 minutes, the mouse was briefly removed and a single chocolate chip was hidden in the bedding. The mouse was then returned to the test cage and the latency to approach the newly introduced scent and dig it up was recorded.

Mammary gland morphology

Inguinal mammary glands were harvested bilaterally at various stages of development including virgin, lactation day 1 (L1), lactation day 7 (L7) and involution. For nipple disconnection studies, virgin female mice underwent surgery to disconnect the left fourth inguinal gland at 10 weeks of age prior to breeding. After surgically disconnecting the nipple, glands were separated from the proximal connective mesenchyme associated with the adjacent dermis. The blood supply between the fourth and fifth inguinal gland was cauterized and the two glands were surgically separated. The fourth inguinal gland was then allowed to retract toward the distal region prior to closing. The distal region of the fourth inguinal mammary gland and associated vasculature remained

intact and attached to the adjacent mesenchyme during the procedure. 10 days after the procedure, mice were mated and both inguinal glands were collected from L1 dams. Harvested glands were fixed in 10% neutral buffered formalin overnight at 4°C. Following fixation, glands were transferred to 70% ethanol then sectioned (5uM) for subsequent Hematoxylin and Eosin (H&E) staining and histopathological analyses. H&E stained sections were visualized on an AxioplanII microscope and Axiocam HR (Carl Zeiss, Thornwood, NY). Images were digitally captured using Axiovision (4.1) software and exported as TIFF files for subsequent analysis.

Quantitative analysis of mRNA transcripts associated with parturition

To examine potential changes in prolactin and oxytocin mRNA, pituitary gland and hypothalamus were harvested from dams on lactation day 1 (L1). Total RNA was isolated as described in Chapter 2 and a quantitative real-time RT-PCR strategy was utilized using specific Taqman probes (prolactin, assay ID Mm00599957_m1; oxytocin, assay ID Mm00726655_s1).

Statistical Analyses

All statistical analyses were performed using GraphPadPRISM (GraphPad Software, Inc., La Jolla, CA). For comparisons between 2 groups, Student's t-test was performed; comparisons between 3 or more groups utilized one-way analysis of variance with appropriate post-hoc tests as indicated. Values are reported as means ± SEM, and *P* values of <0.05 were considered significant.

Results

Growth in INI/+ reared offspring

Initial observations of home cage behavior for INI/+ dams identified lethality for offspring born to mutant dams, yet a normal Mendelian ratio for pups was observed at weaning. To distinguish between neonatal lethality associated with altered maternal behavior and phenotypic changes in mutant offspring, we took advantage of a selective breeding strategy in which wild-type females were mated to INI/Y males and INI/+ females were mated to wild-type males, allowing both wild-type and INI/+ dams to raise mixtures of wild-type and mutant offspring. Following birth, litters were culled to five pups and the growth rates of offspring were monitored daily from P7 through weaning (P21). Both wild-type and mutant offspring raised by INI/+ dams weighed significantly less than offspring raised by wild-type femeales (Figure 30A), indicating that the observed deficits in growth rate resulted from an altered maternal environment rather than genotype-dependent growth deficits in the offspring themselves.

To eliminate the possibility that differences in intrauterine environment might explain the delayed growth rate for INI/+-raised offspring, we also employed a cross-fostering paradigm in which mice were bred using the same selective breeding paradigm, but within 2 hours of birth, entire litters were cross-fostered between wild-type and INI/+ dams. Results from this litter manipulation revealed a significant decrease in the number of pups that survived beyond P3 when raised by INI/+ dams (60.5%) as compared to wild-type dams (94.3% survival) (Figure 30B). Furthermore, a decrease in the weight of the surviving offspring fostered to INI/+ dams (8.3±1.3g for wild-type-reared offspring versus 7.6±0.4g for INI/+-reared offspring; $n \geq 23$, $p < 0.001$) was observed. To assess long-term effects on offspring growth, male and female offspring were generated using the selective breeding strategy; pups were separated at weaning, housed with littermates of the same gender and weights were monitored weekly from P21 through

P84. Although no difference in pup body weight was observed at birth, both wild-type and mutant male offspring born to INI/+ dams displayed a 14.5% decrease in body mass by weaning and an 18% decrease in body mass by P84 (Figure 30C, left). Growth differences were also observed for female offspring born to INI/+ dams, though the effects were less pronounced (Figure 30C, right). These long-term alterations in growth observed in offspring of INI/+ dams indicate that early maternal environment can lead to subsequent growth disparities in adult animals.

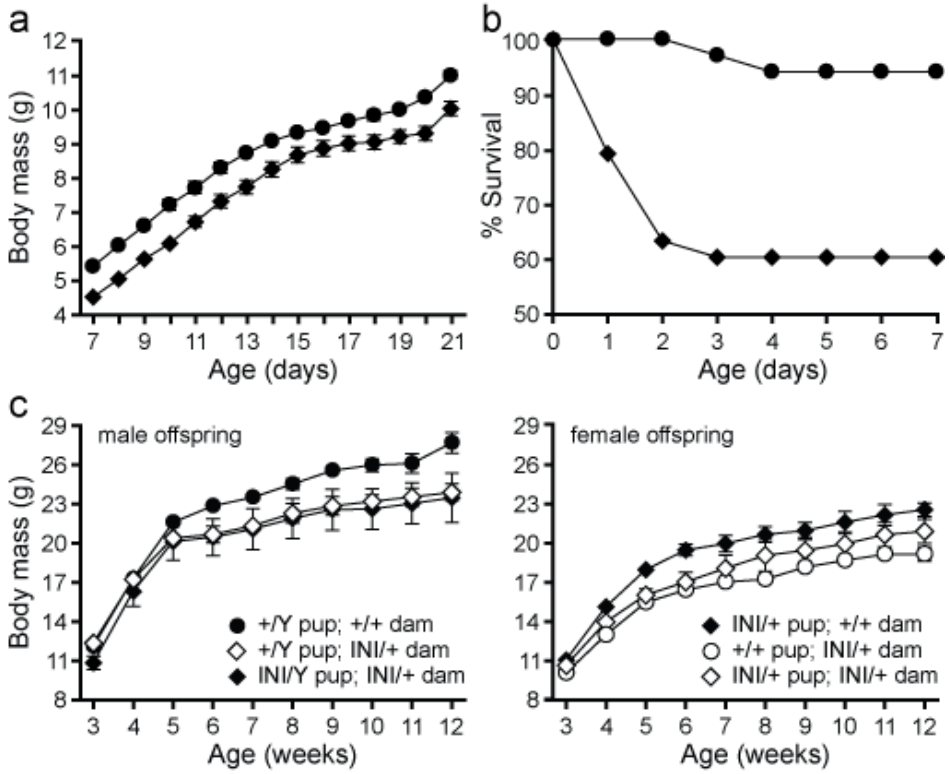

Figure 30: Growth deficits and neonatal lethality for offspring reared by INI/+ mutant dams. a) Pre-weaning growth curves are shown for litters culled to 5 pups at P0 for mixed gender offspring resulting from either the breeding of hemizygous mutant males x wild-type females (●) or wild-type males x heterozygous mutant females (♦). (n = 20 pups for each breeding strategy; mean ± SEM, p<0.0001). **b)** Survival curves for mixed gender offspring cross-fostered at P0 to wild-type dams (●, n=28 pups) and wild-type offspring cross-fostered at P0 to heterozygous mutant dams (♦.n=35 pups). **c)** Post-weaning growth curves of selectively bred male (left) and female (right) offspring reared by +/+ and INI/+ dams (n≥5 pups for each pup/dam combination, mean ± SEM, p<0.0001 for all curves by two-way ANOVA).

Mammary gland morphology in INI/+ dams

To determine if the failure to thrive phenotype in offspring raised by INI/+ dams was related to an inability to nourish the pups, we sought to determine if +/+ and INI/+ dams differed in their mammary gland structure and hence their ability to lactate. No anatomical differences between the inguinal mammary glands of virgin +/+ and INI/+ mice were observed. Females were then mated and the inguinal glands were harvested on the first and seventh day of lactation (L1 or L7). On L1, +/+ mammary glands contained ~50% ductal and alveolar structures (Figure 31A), however mammary glands harvested from L1 INI/+ dams contain ~80% ductal and alveolar structure (Figure 31E). Further examination at higher magnification revealed that INI/+ dams had few lipid droplets within the alveolar lumen and epithelial cells (Figure 31F) when compared to wild-type tissue (Figure 31B). However, examination of +/+ and INI/+ dam mammary glands reveal no differences by L7.

Since alterations in mammary gland structure can be the result of dam behavior (decreased time spent with pups, decreased pup suckling) or biochemical alterations in the dam, we surgically disconnected one inguinal gland in virgin females and allowed them to recover from surgery before mating. At L1, both inguinal glands were harvested and processed for H&E staining. The non-disconnected nipple (suckled nipple) revealed the previously observed differences between +/+ (Figure 31D) and INI/+ dams (Figure 31H) with few lipid droplets present in mutant animals. However, the disconnected nipple (unsuckled nipple) was identical between the two genotypes (Figure 31C and G), suggesting that the hormonal and biochemical circuitry necessary to establish normal gland morphology was intact.

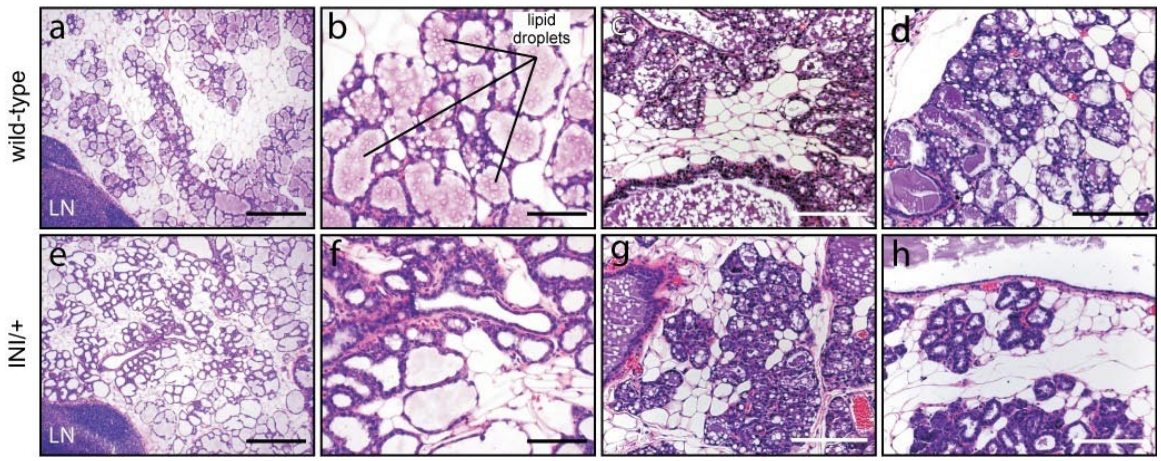

Figure 31: INI/+ mutant mice demonstrate altered mammary gland morphology. At lactation day 1, H & E staining of inguinal glands reveals vast differences between INI/+ mutant females and their wild-type littermates (n=6). Under low powered magnification, INI/+ mutant mammary glands **(e)** are comprised of approximately 80% aveoli and ductal structures as compared to wild-type glands **(a)** which contain 50% aveoli and ductal structures suggesting increased lactogenesis in INI/+ mutant mammary glands. At high powered magnification, INI/+ mutant females **(f)** have a noticeable lack of lipid within the aveoli and lumen as compared to wild-type littermates **(b)**. H & E staining of inguinal glands from the unsuckled nipple reveal no differences between wild-type **(c)** and INI/+ **(g)** dams while suckled nipple **(d)** displays characteristics of increased lactogenesis and lack of lipid in INI/+ dams **(h)**.

Changing levels of prolactin and oxytocin at parturition have been implicated in their role in nurturing behaviors (Bridges, 1994; Rosenblatt et al., 1988). Suckling activity by pups leads to the release of prolactin, to stimulate production of milk (lactogenesis), and oxytocin, which triggers milk let-down (Figure 32) (Hansen and Gummesson, 1982; Juss and Wakerley, 1981). To determine whether alterations in prolactin or oxytocin expression between +/+ and INI/+ dams could explain the observed differences in mammary gland morphology or the failure to thrive observed in the offspring, we used qPCR to quantify prolactin (PRL) and oxytocin (OXY) mRNA levels in pituitary and hypothalamus, respectively, harvested from L1 dams. Results from this analysis revealed no difference in the levels of PRL and OXY transcripts in +/+ and INI/+ dams (Figure 33).

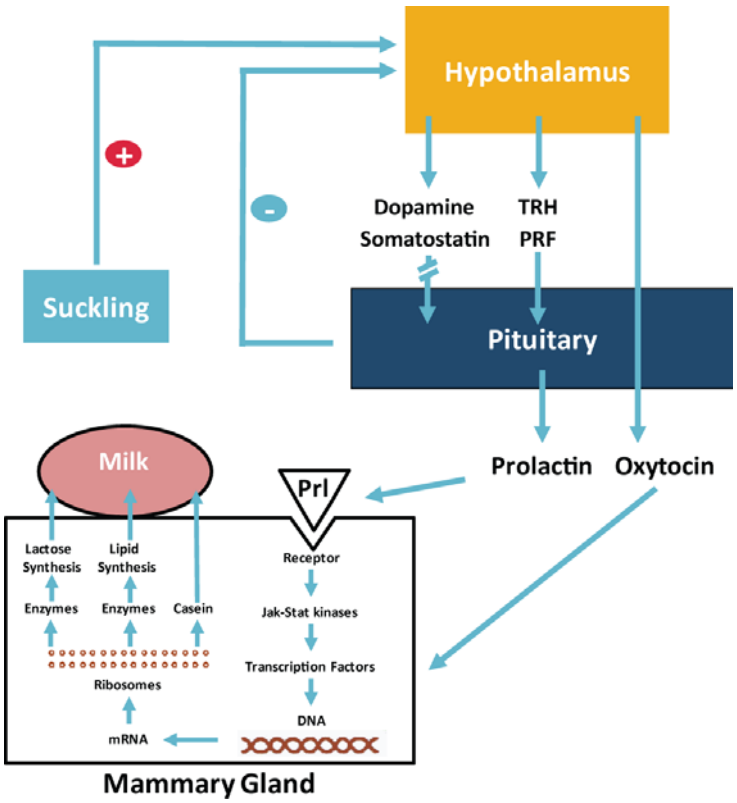

Figure 32: Schematic representation of the role of prolactin and oxytocin in milk production and letdown. Suckling behavior of the pup triggers the release of prolactin and oxytocin from the pituitary and hypothalamus, respectively. Prolactin binds to its receptors in the mammary gland to stimulate the production of milk while oxytocin acts on the mammary gland to produce milk ejection.

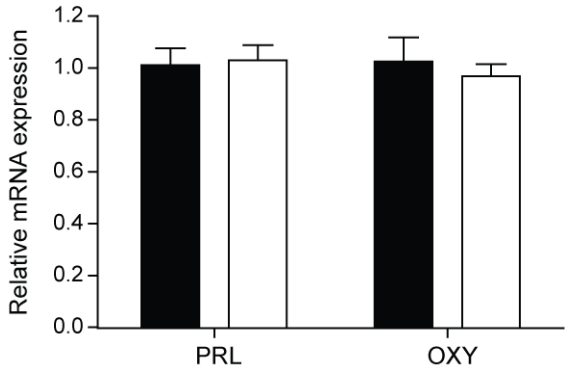

Figure 33: Prolactin and oxytocin mRNA levels in +/+ and INI/+ mutant dams. Quantitative PCR analysis of prolactin (PRL) and oxytocin (OXY) mRNAs isolated from pituitary gland and hypothalamus, respectively, of L1 dams is presented for +/+ (black) and INI/+ mutant (white) dams (n=8, $p>0.05$).

General behavior of INI/+ dams

To assess potential behavioral alterations in anxiety-related behavior or locomotor activity in INI/+ dams that accompany the abnormal maternal behavior, we assessed the behavior of wild-type and heterozygous mutant dams. Analyses of a 60 minute open-field exposure revealed that INI/+ dams did not differ from their wild-type littermates in total distance traveled (Figure 34A). To measure anxiety-related behaviors, we used the elevated-plus maze (EPM) task. +/+ and INI/+ dams did not differ in the amount of total entries or entries into either arm of the maze, but INI/+ dams spend less time in the closed arms (Figure 34B) with corresponding increased time in the center of the maze.

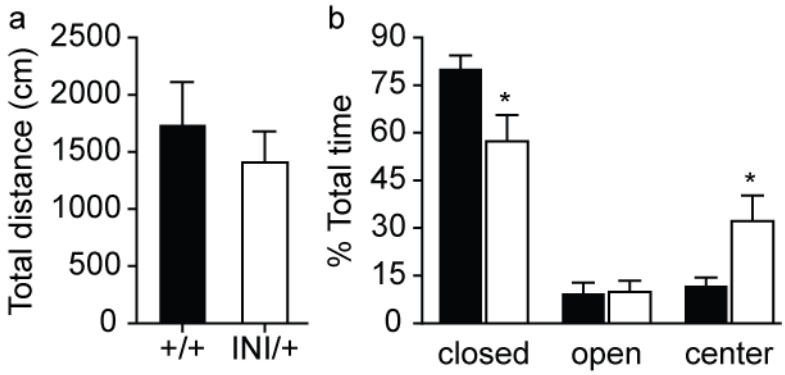

Figure 34: Behavioral analyses of +/+ and INI/+ dams. a) Activity behavior assessed by open field analysis is presented for +/+ (black) and INI/+ (white) dams; the total distance traveled in 60 minutes is presented (mean ± SEM, n=10 for each genotype). **b)** Elevated plus maze analysis of anxiety-like behavior is presented for +/+ (black) and INI/+ (white) dams; the percentage of time spent in the closed and open arms as well as the center portion of the maze is presented (mean ± SEM, n=10 for each genotype *:p<0.05 by Student's two-tailed t-test).

To control for the influence of an olfactory or exploratory deficit on the disruption of maternal care, we also assessed INI/+ mice for deficits in olfaction and their response to novel objects. +/+ and INI/+ females also did not differ in the latency to approach a novel object (Figure 35A) or in the percentage of total time spent investigating the object

(+/+: 45.4 ± 1% versus INI/+: 45.5 ± 0.4%, p>0.05). +/+ and INI/+ mice also did not differ in the latency to discover a chocolate chip buried within the bedding (Figure 35B), suggesting that olfactory and exploratory deficits do not play a role in the observed disruption of maternal care in $5HT_{2C}R^{INI}$ dams.

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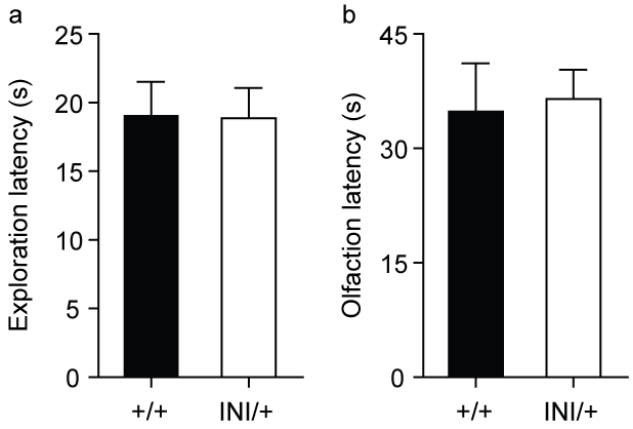

Figure 35: INI/+ mutant dams show normal responses to novel objects and olfactory cues. a) Latency to approach a novel object is presented for +/+ (black) and INI/+ (white) dams (mean ± SEM, n=6 for each genotype, p>0.05). **b)** Latency to find a hidden olfactory cue is presented for +/+ (black) and INI/+ mutant (white) dams (mean ± SEM, n=6 for each genotype, p>0.05).

Maternal behavior in INI/+ dams

After ruling out obvious anatomic or physiologic alterations as potential explanations for the observed deficits in maternal behavior, we next examined potential alterations in nurturing of the dams. In rodents, nurturing behavior is quite stereotyped and includes nest building, cleaning the pups, retrieving them to the nest and crouching over them to nurse (Barnett and Burns, 1970). Observations of home cage behavior revealed that offspring born to INI/+ dams were found scattered around the cage (Figure 36A) rather than huddled beneath the dams and INI/+ dams failed to remove placental membranes and groom pups following birth. To assess futher dimensions of maternal care, we compared nest-building behavior between wild-type and $5HT_{2C}R^{INI}$ mice. Wild-

type and INI/+ multiparous pregnant (E18) females were provided with cotton nesting material 90 minutes prior to onset of the dark cycle and photographs were taken 24 hours later to quantify nest quality. Nests prepared by INI/+ dams were not only of reduced quality compared to wild-type dams, but also 80% of INI/+ dams failed to chew the nesting material (Figure 36B).

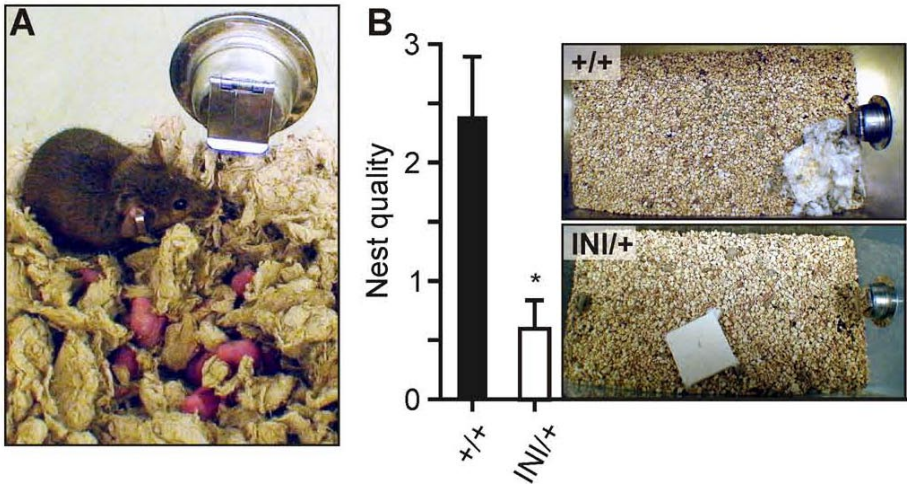

Figure 36: INI/+ mutant dams demonstrate altered maternal behaviors. a) Representative photograph of an INI/+ mutant dam with scattered pups shortly after giving birth (P0). **b)** The quality of nest building was assessed (n=6, mean ± SEM) and representative photographs of nests taken 24 hours after addition of a cotton nestlet to the cages of mice are presented for +/+ and INI/+ mutant dams.

To assess another aspect of maternal behavior, pup retrieval behavior was examined in wild-type and INI/+ dams. The latency to approach the first pup was significantly less ($p<0.001$) for wild-type dams (29±18 seconds; n=6) compared to INI/+ mothers (418±289 seconds; n=6). All wild-type dams successfully retrieved three pups each, wheras only one mutant dam retrieved all of her pups and the remaining INI/+ dams failed to retrieve a single pup (Figure 37), indicating a severe disruption in maternal retrieval behavior.

Altered behavior could result from signaling changes in $5HT_{2C}R^{INI}$ mice since this isoform displays the greatest level of constitutive activity and G-protein coupling efficacy (Niswender et al., 1999). To determine if decreased retrieval was related to an alteration in $5HT_{2C}$ receptor signaling, we attempted to rescue pharmacologically the pup retrieval deficit in INI/+ dams by treatment of newly postpartum females with a $5HT_{2C}$ receptor inverse agonist. Dams were treated with SB206553 (5mg/kg, IP) or vehicle following removal of pups from the cage; after 1 hour of separation, pup retrieval behavior was assessed. As observed previously, wild-type dams retrieved all of their pups even in the presence of the inverse agonist, but unlike vehicle-treated INI/+ dams that did not retrieve a single pup, all treated INI/+ dams retrieved at least two of three offspring (Figure 37).

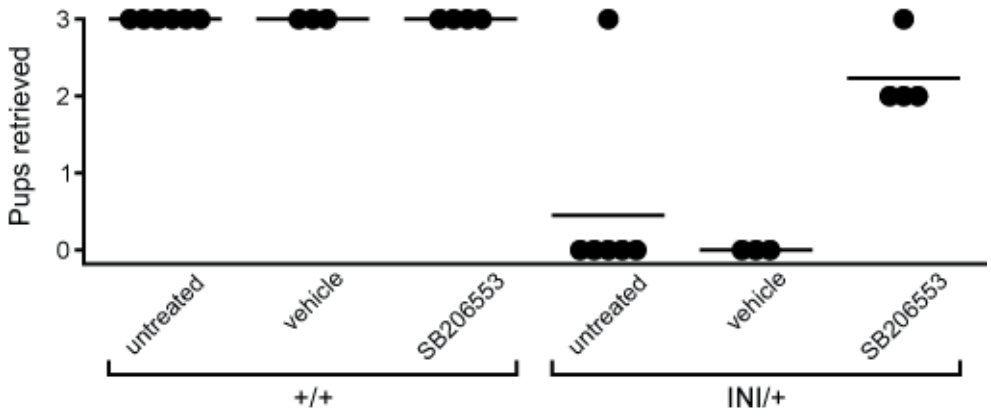

Figure 36: Retrieval deficits in INI/+ dams can be rescued with treatment of a $5HT_{2C}$ receptor inverse agonist. The number of pups retrieved by wild-type and INI/+ dams within 15 minutes is presented for untreated (n=6), vehicle-treated (n=3) or inverse agonist-treated (SB206553, 5mg/kg; n=4). Data was analyzed by Friedman's non-parametric two-way ANOVA ($p<0.05$).

Discussion

As compared to wild-type dams, heterozygous mutant dams display deficits in maternal care including failure to build nests, to clean pups at birth or to retrieve

scattered pups (Figures 36 and 37). Offspring born to dams demonstrating poor maternal care often have decreased survival and growth rates (Brown et al., 1996; Thomas and Palmiter, 1997) as well as long-term behavioral deficits (Champagne and Meaney, 2001; Meaney, 2001).

Accordingly, the offspring of $5HT_{2C}R^{INI}$ dams have both decreased survival and those that survive demonstrate long term effects of maternal neglect including early growth rate deficits (Figure 30A) that continue throughout adulthood (Figure 30C and D). Cross-fostering studies revealed that wild-type offspring fostered to INI/+ dams had a decreased survival rate (Figure 30B) as well as decreased body weight compared to those raised by wild-type dams, confirming that the alterations in growth are due to poor maternal care rather than the genotype of the pups. Failure to thrive can lead to long-term growth deficits in offspring as well as higher incidences of behavioral abnormalities (Black et al., 2007; Oates et al., 1985). The early quality of care that offspring receive can also have long-term effects in later life; individuals raised in environments with poorer maternal care develop vulnerability to illness throughout later life (Taylor and Seeman, 1999), are at greater risk for mental illness (Bifulco et al., 1991), diabetes and heart disease (Felitti et al., 1998).

If the INI mutation caused abnormal mammary gland function, it could be responsible for the failure to thrive phenotype observed in offspring of INI/+ dams. No differences in the morphology of the inguinal mammary glands were observed in virgin females, but inguinal glands harvested from dams at L1 revealed that the glands from INI/+ dams had increased aveoli and ductal structures (Figure 31E) as well as decreased lipid droplets (Figure 31F). These differences could not be attributed to altered biochemical circuits within INI/+ dams (Figure 31C, D, G and H) or prolactin or oxytocin transcript levels within the pituitary and hypothalamus, respectively (Figure 33). Since surgically disconnected nipples of INI/+ dams appeared normal at L1, it is most

likely that these morphological differences are due to decreased suckling by the pups as a result of poor maternal care, rather than direct alterations in structure/function of the mammary glands caused by the gene mutation. We did not directly measure suckling time by the pups; however it has been demonstrated in other models of poor maternal care that the extreme maternal neglect is often accompanied by less time spent on the nest as well as decreases in nursing time (Brown et al., 1996; Thomas and Palmiter, 1997).

Maternal neglect and nurturing deficits are observed in other mutant mouse models [for a review see: (Gammie, 2005)] suggesting that a common neural circuit underlies these behaviors. Sensory abnormalities can lead to possible nurturing defects as the processing of olfactory cues has been shown to be critical for pup retrieval (Smotherman et al., 1974). The main olfactory bulb appears to be the key mediator for olfactory signals as lesions of the accessory olfactory system has no effect on maternal care (Jirik-Babb et al., 1984; Lepri et al., 1985). Our mice performed normally on an olfaction assay demonstrating that, at the least, they are able to detect their pups (Figure 35B). Highly anxious animals or animals which have either heightened or reduced responsiveness to novelty also sometimes display abnormal maternal behavior. Inbred strains of mice often vary in their quality of maternal care, for example, as compared to C57BL/6J dams, BALB/cJ dams spend less time in the nest, exhibit less licking and grooming of pups and display less time nursing pups (Anisman et al., 1998; Carlier et al., 1982). These mouse strains also differ in their anxiety-like states with BALB/cJ mice displaying increased levels of anxiety (Falls et al., 1997; Hackler et al., 2006; Yilmazer-Hanke et al., 2003) and elevated glucocorticoid responses to stress (Anisman et al., 1998; Shanks et al., 1990; Tannenbaum et al., 2003) suggesting that anxiety-like states alter the quality of maternal care. However, INI/+ mice do not display alterations in

anxiety as measured by the EPM or differences in response to a novel object test (Figures 34 and 35A).

Another possible explanation for poor maternal care is disruption of the circuitry that mediates reward. Postpartum dams will bar press for pups in an operant response paradigm at a higher-rate than virgin animals (Lee et al., 2000), will cross electric grids to receive pups and will choose pups over food (Fahrbach and Pfaff, 1982). Dams will also develop conditioned place preference to pup stimuli (Fleming et al., 1994). However, these behaviors are dependent upon the dam's maternal state as non-maternal virgins often will not exhibit these behaviors (Fleming and Luebke, 1981; Fleming et al., 1994). Administration of DA antagonists disrupt both ongoing maternal behavior (Giordano et al., 1990) and infusions of 6-hydroxydopamine (6-OHDA), a neurotoxin that destroys catecholaminergic neurons, into lactating rat dams results in deficits in pup retrieval (Hansen et al., 1991). Observations that the retrieval behavior of $5HT_{2C}R^{INI}$ mutant dams can be rescued with a selective $5HT_{2C}$ receptor inverse agonist (SB206553) indicate that the observed deficits in retrieval behavior result from increased 5HT signaling (Figure 37) rather than reduced $5HT_{2C}$ expression. Interestingly, SB206553 has been shown to increase DA release in nucleus accumbens and striatum *in vivo* (Berg et al., 2005; De Deurwaerdere et al., 2004), presumably modulating a negative serotonergic tone on mesolimbic DA activity (Gobert et al., 2000) via GABAergic interneurons (Eberle-Wang et al., 1997). Therefore, we postulate that the disruption of normal maternal behavior observed in INI-mutant dams may be due to excess constitutive activity of non-edited $5HT_{2C}$ receptors, which leads to abnormal DA signaling. This represents a novel role for RNA editing of the $5HT_{2C}$ receptor in the regulation of serotonergic control over reward behavior and highlights the necessity for expressing multiple, edited isoforms for normal nervous system function.

CHAPTER V

PHENOTYPIC ALTERATIONS IN OFFSPRING OF $5HT_{2C}R^{INI}$ DAMS

Introduction

Early social experiences can have long-term behavioral and emotional consequences in mammals, including non-human primates and rodents. In the 1960s, Harlow *et al.* demonstrated that total social deprivation throughout infancy usually results in behavioral abnormalities and severe social and emotional deficits, especially evident in aggressive, reproductive and parenting activities (Harlow et al., 1965). In both primates and rodents, infants deprived of maternal care, either as the result of complete maternal deprivation or neglect, exhibit increased fearfulness, inappropriate and often excessive aggressive patterns of social behavior and impaired cognitive development (Ladd et al., 2000; Suomi, 1997). The decreased mother-infant contact, resulting from extended periods of maternal separation, seems likely to be critical for the effects on development, but even relatively 'mild' forms of early maternal deprivation can have significant short- and long-term behavioral and physiological consequences (Suomi, 1997).

Similarly, maternally deprived females are more likely to exhibit neglectful and/or abusive treatment of their own offspring (Suomi, 1997). Natural variations in maternal licking/grooming (LG) in rat pups during the first week of life have been shown to alter gene expression, physiology and behavior of both male and female offspring (Caldji et al., 1998; Liu et al., 2000; Liu et al., 1997). Cross-fostering of offspring from high LG mothers to low LG mothers (and vice versa) has shown that the adult phenotype of these offspring can be influenced by quality of postnatal care (Francis et al., 1999a). These variations in maternal care are passed from one generation to the next, providing

a behavioral mechanism of inheritance of stress response and social behavior (Champagne and Curley, 2005).

Given the deficits in maternal care observed in INI/+ dams, we sought to determine what effects this rearing environment exerts, if any, on the behavior of offspring. We utilized a series of behavioral paradigms to investigate activity and anxiety-related behaviors that may be present, both as a factor of the maternal environment and as a result of the introduced mutation.

Experimental procedures

Unless otherwise indicated, animals used for these series of experiments were naïve male age-matched animals (12-14 weeks of age, n=10/genotype) generated by heterozygous mutant females mated to hemizygous mutant males (INI/+ x INI/Y) to generate +/Y and INI/Y male littermates raised by INI/+ dams. Where indicated, each group of experiments included wild-type males reared by wild-type dams as a control.

Elevated-plus maze analysis of anxiety-related behavior

Elevated-plus maze experiments were conducted as described previously in chapter 4.

Open Field

Open field experiments were performed as described previously with animals exposed to the open field for 100 minutes. See chapter 4.

Light-Dark (emergence neophobia)

For assays of light-dark preference, open-field chambers were fitted with an acrylic insert (black walls and ceiling) to partition the chamber into an enclosed dark chamber and an open light chamber of equal size. Mice were placed in the center of a

brightly light chamber and permitted to freely pass between light and dark chambers during the duration of the five-minute assay. The total time spent in each chamber, in addition to the number of entries into each chamber, was recorded.

Y-maze

The Y-maze is a y-shaped apparatus in which the three arms were equal in length. Each arm consisted of clear, circular Plexiglas that was 30.5 cm long x 4.8 cm wide x 4.3 cm tall with the bottom of the Plexiglas tube removed so that the maze sat flat on a grey rubber surface. The end of one arm was removable for placement of mice into the maze. The arms of the maze joined in the center with each arm at a 120° angle from the next. Spatial cues were available to the mice during the testing. A videocamera was mounted overhead to record animal behavior.

Each animal was placed into the apparatus for eight minutes and permitted to freely explore the maze. Two investigators, blind to genotype, recorded the order of entry into each of the arms, with an entry defined as all four paws entering into an arm of the maze. If an entry order discrepancy was present between the two investigators, the animal was then analyzed using the videotaped behavior. Spontaneous alteration behavior was defined as non-repetitive entry into each of the maze arms and number of total entries was also measured.

Cocaine-Induced Locomotor Activity

To assess changes in locomotor activity in response to cocaine, mice were placed in an open-field arena and movement was detected by a series of infrared beams as described in Chapter 4. Baseline activity was determined by administration of IP injections of saline, followed by assessment of locomotor activity for 2 hours per day for successive 3 days. On the 4^{th} day, mice were treated (IP) with 10 mg/kg cocaine (Sigma)

or saline vehicle and returned to the open-field arena for 2 hours of testing. Following testing, mice were rapidly sacrificed and perfused as described earlier. Brains were removed and postfixed overnight at 4°C, then immersed in a series of sucrose (10%, 20%, 30%) solutions and saved at 4°C for future studies.

Results

It is widely accepted that poor maternal environment can lead to long-term defects in offspring (Caldji et al., 1998; Francis et al., 1999b; Meaney, 2001). To characterize potential deficits in offspring, we performed behavioral analyses on male animals reared by wild-type and INI/+ dams and also included a cohort of wild-type reared wild-type male offspring.

Open field

To assess potential alterations in locomotor activity in offspring, we utilized an open field assay. When offspring were analyzed in an open-field, only INI/Y males reared by INI/+ dams displayed significant changes in activity. INI/Y males were initially hyperactive on the first two days of the test paradigm, but by the third day of testing, the total distance traveled by all animals was not significantly different regardless of their genotype or the genotype of the dams by which they were raised (Figure 38).

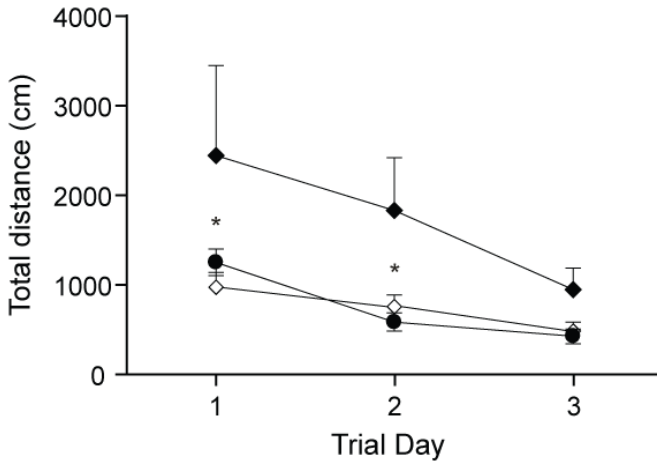

Figure 38: Open-field analysis of offspring locomotor activity. Activity behavior assessed by open-field analysis is presented for INI/Y mice (⧫) and +/Y mice (◊) reared by INI/+ dams as well as +/Y mice reared by +/+ dams (●); the total distance traveled in 100 minutes over three consecutive trial days is presented. (mean±SEM, n=10 for each genotype; *p<0.05 by one-way ANOVA).

Elevated-plus maze

The relationship between early life events and health in adulthood appears to be mediated by parental influences on the development of neural systems which underlie the expression of behavioral and endocrine responses to stress (Caldji et al., 1998; Francis et al., 1999a). To assess if poor maternal care resulted in anxiety-related behaviors for adult mice raised by INI/+ dams, we utilized the elevated-plus maze. Results from this analysis revealed that both wild-type and INI/Y mice raised by mutant dams spent significantly less time in the open arms of the maze and had less open arm entries that mice raised by wild-type dams (Figure 39), consistent with an increase in anxiety-related behavior as a consequence of deficits in maternal care.

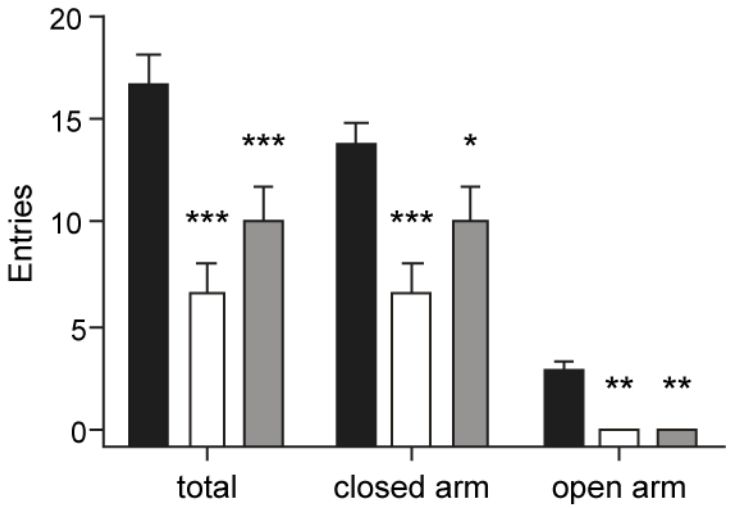

Figure 39: Anxiety-related behavior is observed in mice raised by INI/+ mutant dams. Elevated plus maze analysis of anxiety-like behavior is presented for INI/Y (white) and +/Y (grey) mice reared by INI/+ dams as well as +/Y mice reared by +/+ dams (black); the total number of entries as well as entries into closed and open arms is presented (mean ± SEM, n=10 for each genotype; *:p<0.05, **: p<0.01, ***:p<0.001 by one-way ANOVA).

Y-maze

The Y-maze takes advantage of the exploratory drive of rodents to investigate the newest areas of their environment. When animals from the selective breeding paradigm were subjected to the y-maze, animals reared by INI/+ dams make less total entries in the maze than animals reared by wild-type dams (Figure 40A). When we examine spontaneous alternation behavior, we find no differences between any of the genotypes (Figure 40B).

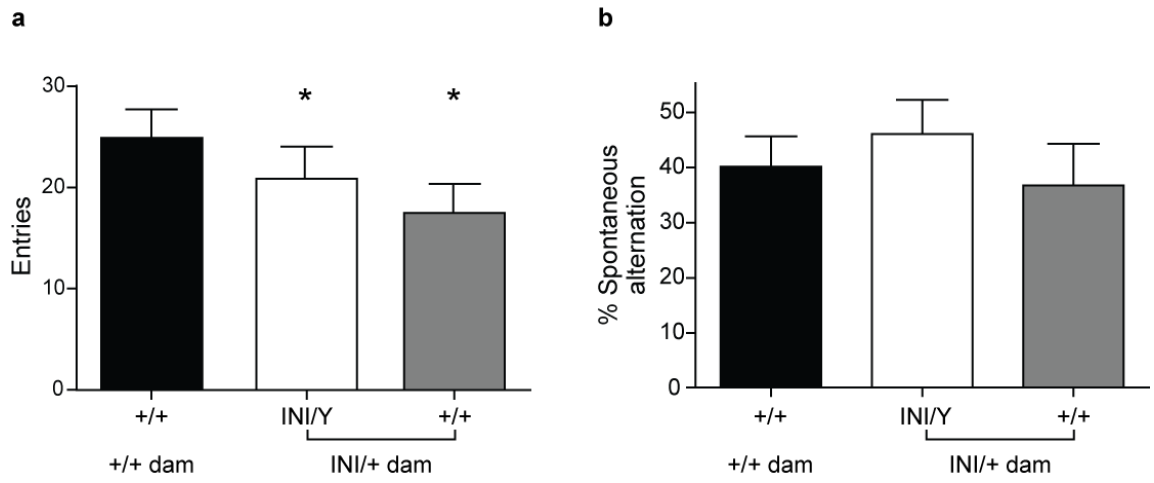

Figure 40: Activity and spontaneous alternation by offspring in the Y-maze. Y-maze analysis of activity **(a)** and spontaneous alternatition **(b)** behavior is presented for INI/Y (white) and +/Y (grey) mice reared by INI/+ dams as well as +/Y mice reared by +/+ dams (black); the total number of entries (a) as well percent spontaneous alternation is presented (mean ± SEM, n=10 for each genotype; *:p<0.05 by one-way ANOVA).

Light-dark exploration

An additional behavioral measure of anxiety is light-dark exploration (emergence neophobia) in which an open field arena can be bisected into light and dark chambers. Traditionally, mice with increased anxiety-related behaviors will spend more time in the dark side of the chamber. In the light-dark exploration task, INI/Y mice reared by INI/+ dams traveled more total distance in the entire arena as compared to their wild-type littermates (Figure 41A). This increase in total distance is due to increased distance traveled in both the light and dark chambers. Despite this hyperactivity observed in both sides of the chamber, INI/Y animals spent less time in the light area of the arena (Figure 41B) indicating increased anxiety-like states as well as hyperactivity.

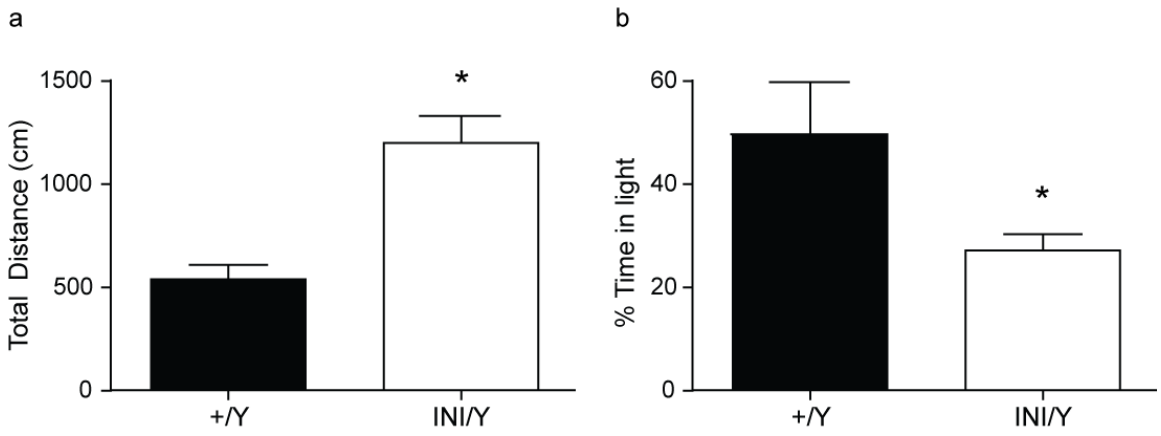

Figure 41: Emergence neophobia behavior in +/Y and INI/Y animals. Emergence neophobia analysis of anxiety-like behavior is presented for INI/Y (white) and +/Y (black) mice reared by INI/+ dams; the total distance traveled **(a)** as well as percentage of total time spent in the light portion of the apparatus **(b)** is presented (mean ± SEM, n=10 for each genotype; *:p<0.05 by one-way ANOVA).

Cocaine-induced locomotor activity

Cocaine administration in rodents typically results in increased spontaneous locomotor behavior and the expression of behavioral stereotypy. Discrete circuits within the CNS are proposed to play a role in each of these behaviors with striatal dopamine mediating stereotypy behaviors while locomotor activity is thought to be mediated by the nucleus accumbens (Kalivas and Stewart, 1991; Koob and Bloom, 1988; Pulvirenti et al., 1991). Given the potential role of DA reward systems in the initiation and maintenance of maternal behaviors (Ferris et al., 2005; Stern and Keer, 1999), we sought to determine if offspring of wild-type and INI/+ displayed alterations in cocaine locomotor responses. Following a three day acclimation period, mice were assessed for their locomotor responses to cocaine and compared to a saline treated group of the same genotype. As expected saline-treated animals did not show any increase in activity over baseline. All cocaine injected animals did display increases in activity, but responses were dependent upon genotype. As a group, INI/+ reared offspring had greater increases in cocaine-

induced locomotion than wild-type reared offspring (Figure 42). In addition to these observed changes in activity, INI/+-reared offspring also had increased stereotypic behaviors.

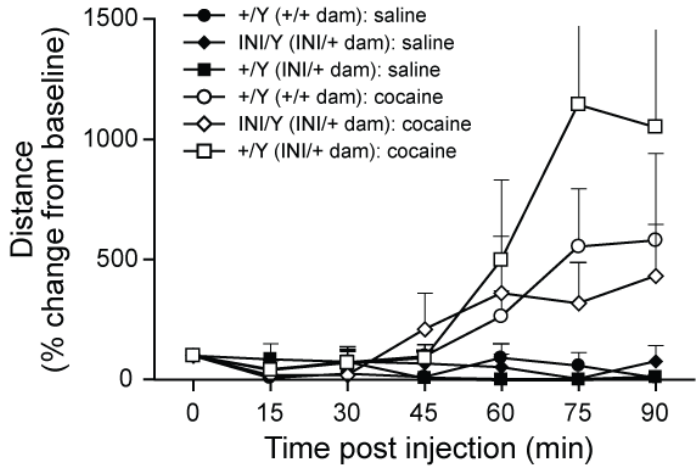

Figure 36: Locomotor responses following a single dose of cocaine in offspring of +/+ and INI/+ dams. Open-field analysis is presented for locomotor responses following saline or cocaine treatment. The total distance traveled (as a percent change from baseline) is presented in fifteen minute bins (mean±SEM, n=5 for each genotype; $p>0.05$ by one-way ANOVA).

Discussion

The experiments described in this chapter add further support to existing data underscoring the effects of maternal environment on the development and behavior of offspring. As a result of their deprived maternal environment, offspring of INI/+ dams display abnormalities in most behavioral paradigms examined irrespective of genotype, implicating rearing effects of INI/+ dams rather than the genotype of the offspring themselves.

We observe decreased levels of activity in both wild-type and mutant offspring of INI/+ dams in several behavioral paradigms including y-maze and elevated plus maze. Decreased activity in all of these paradigms is known to correlate with anxiety, suggesting a pervasive anxious phenotype in offspring of INI/+ dams. For traditional

measures of anxiety, we utilized the elevated plus maze and the emergence neophobia paradigm. EPM analyses revealed that offspring of INI/+ dams display remarkable anxiety-like traits including making less total entries and complete failure to enter the open arms of the maze (Figure 39). In the emergence neophobia paradigm, INI/Y mice spent less time in the open area of the field, also suggesting increased anxiety-like states in these animals (Figure 41B). Differences observed in spontaneous alternations within the Y-maze may indicate deficits in hippocampal memory as the animal may not recall which arms have been previously explored. Offspring of INI/+ dams make less total entries within the y-maze, indicating a decreased propensity to explore (and likely increased anxiety-like states); no differences were observed in spontaneous alternations suggesting that hippocampal memory is intact and these differences are not explained by a memory deficit (Figure 40).

Behavior of INI/+ and wild-type offspring diverge only with respect to baseline activity, environmental reactivity and magnitude of cocaine-induced hyperactivity. Measures of baseline activity reveal that offspring of INI/+ dams are typically hyperactive, though differences are observed depending on behavioral paradigm. In an open-field arena, INI/Y males raised by INI/+ dams are hyperactive when measured on a single day as observed in the emergence neophobia paradigm. This hyperactivity persists across longer trials (100 minutes) over several days (three), but by the third day INI/Y animals have similar levels of activity to wild-type littermates (Figure 38), suggesting that these animals are hyper-reactive to novel situations.

A cocaine-induced locomotor paradigm was utilized to determine if INI-mutant animals showed abnormal responses to the locomotor effects of a stimulant (cocaine) (Figure 42). While this experiment was statistically underpowered to detect any effects, we only observed hyperactive behavior in INI/Y offspring of INI/+ dams following repeated exposure to an open-field. As an entire group, INI/+ reared offspring displayed

greater increases in activity in response to cocaine administration. Since INI/Y mice have a higher baseline activity rate, their increased activity in response to cocaine parallels the increases seen in +/Y offspring of +/+ dams. Follow up studies using a larger sample size are needed to determine if cocaine differentially exerts its effects on offspring of +/+ and INI/+ dams.

Generalization of these findings is limited by the fact that $5HT_{2C}R^{INI}$ animals are currently maintained on a 129S6/SvEvTac background strain, a mouse strain that is suboptimal for behavioral assays due to previous observations indicating that this background strain displays increased levels of anxiety as compared to other strains (Bothe et al., 2004; Voikar et al., 2004). All behavioral studies should be repeated on animals that have been fully backcrossed to the C57BL/6J strain, a mouse strain widely used in mouse behavioral studies displays less anxiety-related traits (Bothe et al., 2004; Voikar et al., 2004) as well as more reliable responses to the locomotor effects of cocaine (Miner, 1997; Schlussman et al., 1998), before interpretations of $5HT_{2C}R^{INI}$ mice, relative to other mutant mouse lines, can be made.

Taken together, these data suggest that offspring of INI/+ dams are highly anxious. Since these behavioral alterations are observed in both +/+ and INI/Y littermates reared by INI/+ dams, these anxiety-related behaviors must be due to early maternal differences rather than effects resulting from the introduced INI mutation. These observations are consistent with existing data that neglectful maternal behavior promotes anxiety phenotypes in offspring (Caldji et al., 1998; Francis et al., 1999b).

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Summary

RNA editing refers to a group of post-transcriptional modifications that involve base modification, substitution or deletion of RNA sequences. The editing process can create vast genomic diversity in the human transcriptome by generating multiple protein isoforms from a single genomic locus (Gott and Emeson, 2000). In mammals, A-to-I RNA editing has previously been shown to alter the amino acid coding potential of the mRNA, leading to functional changes within the encoded protein. In addition to modifying protein sequences, RNA editing can also alter the structure, stability, translation efficiency and alternative splicing patterns of edited transcripts (Bass, 2002; Gott and Emeson, 2000).

Numerous lines of mutant mice have been generated to study the functional impact that RNA editing may have *in vivo*. The best characterized editing site, the Q/R site within transcripts encoding the GLUR2 subunit of the AMPA receptor, is edited to near completion in the postnatal brain (Sommer et al, 1991). Mice that express only the fully edited version of the protein ($GluR2^R$) were generated and homozygous mutant mice appeared phenotypically and electrophysiologically normal (Kask et al., 1998). Conversely, mutant mice that express a modified GluR2 allele that cannot be edited ($GluR2^Q$) die by P14-P21 due to seizures triggered by the increased permeability of the AMPA receptor to Ca^{2+} (Brusa et al., 1995). Taken together, these results suggest that RNA editing of the GLUR2 subunit of the AMPA receptor is required for normal function in the CNS.

The Q/R site within transcripts encoding the GLUR5 subunit of kainate receptors also modulates Ca^{2+} permeability and channel conductance (Bernard and

Khrestchatisky, 1994; Kohler et al., 1993). Genetically modified mice that solely expressed the fully-edited version of this subunit ($GluR5^R$) were generated to determine whether regulated editing at this site is necessary for normal CNS development and function. Heterozygous and homozygous mutant mice were viable and phenotypically normal; electrophysiologically there were no obvious consequences of expressing a fully edited GLUR5 subunit, suggesting that editing at this site is not essential for viability (Sailer et al., 1999).

The Q/R site of the GLUR6 subunit of the kainate receptor also modulates Ca^{2+} permeability (Egebjerg and Heinemann, 1993) and may play a role in the modulation of synaptic transmission and seizure susceptibility (Bernard and Khrestchatisky, 1994). To address these functional questions, a mutant strain of mouse that solely expressed the non-edited version of GLUR6 was generated (Vissel et al., 2001). Homozygous mutant mice were viable and did not display any overt phenotypic differences, yet electrophysiogical analyses revealed that mutant mice displayed an increased susceptibility to kainate-induced seizures as well as enhancements in NMDA-independent LTP in the perforant pathway (Vissel et al., 2001). These results suggest that RNA editing of the GLUR6 subunit leads to lowered seizure threshold and altered modulation of synaptic plasticity, and is therefore required for normal CNS function (Vissel et al., 2001).

Mutant mice have also been generated to study both ADAR substrates and the ADAR enzymes themselves. Several lines of ADAR1 knockout mice have been produced. The first line revealed the most extreme phenotype, with embryonic lethality observed in chimeric animals (Wang et al., 2000a). Conditional knockouts of ADAR1 were later developed using Cre-mediated recombination (Hartner et al., 2004; Wang et al., 2004), which lead to the survival of chimeric and heterozygous ADAR1-null mice. However, global ablation of ADAR1 expression resulted in embryonic lethality in

homozygous null animals (Hartner et al., 2004; Wang et al., 2004). Embryonic lethality occurred between E11 and E12.5 due to deficits in liver development and was characterized by hematopoietic failure, as well as widespread apoptosis in the heart, liver and vertebra, suggesting the involvement of ADAR1 in the development and function of these systems (Hartner et al., 2004; Wang et al., 2004).

ADAR2 null animals have also been successfully generated with a normal Mendelian ratio at birth indicating that ADAR2 does not lead to embryonic lethality, however homozygous null mice died of seizures by P20 (Higuchi et al., 2000). Given the similar phenotype of ADAR2 null animals with that observed in mice unable to edit the Q/R site of the GLUR2 subunit of the AMPA receptor, it was suggested that editing of this site must be mediated by ADAR2 (Higuchi et al., 2000). In an attempt to rescue the phenotype of ADAR2 null animals, an edited GLUR2 allele(s) was introduced into these animals (Higuchi et al., 2000). ADAR2 null animals that expressed a single edited copy of GLUR2 had delayed lethality until P35 and null animals with 2 copies of edited GLUR2 appeared phenotypically normal (Higuchi et al., 2000), suggesting that the postnatal lethality observed in ADAR2 null animals is due to the loss of editing at the Q/R site of GLUR2 transcripts, highlighting the requirement for normal ADAR2 expression and function (Higuchi et al., 2000).

ADAR2 has been shown to edit its own pre-mRNA, suggesting an autoregulatory mechanism by which ADAR2 can modulate its expression as a means of controlling editing levels within the organism (Rueter et al., 1999). To test this hypothesis, a mutant strain of mouse was generated in which the conserved duplex structure (ECS) was disrupted with the potential for conditional ablation of ADAR2 autoediting (ΔECS) (Feng et al., 2006). Homozygous ΔECS were viable with no obvious phenotypic abnormalities; detailed analysis revealed that autoediting of ADAR2 was successfully ablated leading to an increase in ADAR2 protein in multiple tissues and increased levels of editing for

ADAR2 substrates (Feng et al., 2006). These results support the hypothesis that autoediting serves as a negative-feedback mechanism to modulate ADAR2 protein expression and function (Feng et al., 2006), yet the physiological consequences resulting from autoediting dysregulation have not yet been identified.

The functional relevance of RNA editing for the $5HT_{2C}$ receptor has been well-characterized in vitro, but parallel studies in vivo were lacking. Analysis of 5HT dose-response curves in transfected NIH-3T3 cells demonstrated that serotonergic agonists were 15- and 40-fold less potent when acting through the edited VSV and VGV receptor isoforms, respectively, suggesting that edited isoforms couple less efficiently to the intracellular signaling machinery (Burns et al., 1997; Niswender et al., 1998). Competition binding studies using human isoforms of the $5HT_{2C}$ receptor revealed a guanine nucleotide-sensitive high-affinity state, only observed in the non-edited (INI) receptor, providing direct evidence that RNA editing generates $5HT_{2C}$ receptor isoforms that differ in G-protein coupling efficiency (Niswender et al., 1999), affecting both basal activity and ligand potency (Herrick-Davis et al., 1999).

To further characterize the role of RNA editing *in vivo*, we evaluated the developmental regulation of RNA editing and the editing enzymes, ADAR1 and ADAR2. By using complementary approaches, we demonstrate spatiotemporal regulation of expression patterns for ADAR1 and ADAR2 in the mouse forebrain. ADARs are expressed at low levels early in development and increase gradually during a developmental time course at both the mRNA and protein levels. Regionally, ADAR1 mRNA displayed the lowest expression levels in the choroid plexus, cortex and hippocampus, and the highest levels in the thalamus (Figure 11A). Similarly, ADAR2 mRNA expression levels are lowest in cortex and hippocampus, with increased expression in the thalamus (Figure 11B). We have also reported complete ADAR

colocalization with NeuN indicating that ADAR expression is restricted to neuronal populations (Figures 14 and 15).

Additionally, we have determined the editing patterns of several known edited substrates throughout the developmental time course. In agreement with previous studies, we confirmed the nearly complete editing of the Q/R site of GLUR2 by E15 (Figure 22B). Other substrates had low levels of editing at E15 and gradually increased throughout development. Exceptions to this pattern were the I/V site of Kv1.1 in which significant editing did not occur until after P0 and the 5 individual sites of the $5HT_{2C}$ receptor. The D-site of the $5HT_{2C}$ receptor was 50% edited at E15 and increased to maximal editing levels by P21; the remaining sites had relatively low editing at E15 and reached adult levels by P0 (Figure 22A). In adult animals, we investigated site-specific editing in several brain regions (choroid plexus, cortex, hippocampus, hypothalamus, olfactory bulb, striatum) using Pyrosequencing on individual cDNA clones. Editing patterns revealed a very similar pattern of $5HT_{2C}$ receptor edited isoforms to that observed in rat with VNV representing the most predominate isoform in whole brain and the non-edited, INI, isoform representing only about 10% of total isoform expression (Table 1).

Given the distinct expression patterns of the $5HT_{2C}$ receptor editing isoforms in mouse brain, each with differing functional properties, we sought to determine the physiological role of RNA editing in vivo by generating a mutant strain of mouse that solely expressed the non-edited isoform of the $5HT_{2C}$ receptor ($5HT_{2C}R^{INI}$). Mutant mice are viable and fertile with a normal Mendelian distribution observed. However, in INI-expressing animals we observed decreases in receptor expression at the mRNA and protein levels. INI-mutant mice have a 30% decrease in mRNA (Figures 24 and 25), with a larger decrease observed for protein levels (Figure 28). Additionally, we observe a change in splicing pattern with $5HT_{2C}R^{INI}$ animals preferentially generating more non-

functional RNA1 transcripts at the expense of full-length RNA2 transcripts (Figure 27). These results suggest a compensatory mechanism in $5HT_{2C}R^{INI}$ mice in response to the increased signaling capacity of the non-edited receptor.

Despite alterations in receptor expression that may serve to balance enhanced signaling by decreasing $5HT_{2C}$ receptor mRNA and protein levels, $5HT_{2C}R^{INI}$ animals are not phenotypically normal. The most overt phenotypes exhibited by INI-mutant animals were deficits related to maternal care. As compared to wild-type dams, heterozygous mutant dams fail to build nests for their young or retrieve their scattered pups into a common nest (Figures 36 and 37). This alteration in maternal environment leads to growth deficits (Figure 30) and altered behavior in offspring reared by heterozygous mutant dams (Figures 38-42).

Offspring, regardless of genotype, reared by INI/+ dams display growth retardation as early as P7 (Figure 30A). Offspring maintain this 15-20% growth impairment throughout their lifespan and never successfully “catch up” to wild-type offspring reared by wild-type dams (Figure 30C and D). In addition to deficits in growth, offspring reared by INI/+ dams have increased anxiety-related behaviors, indicating that the early maternal environment provided by INI/+ dams transmits long term effects to offspring (Figures 39-42).

Aberrant maternal care could not be explained by deficits in olfaction or novelty-related tasks (Figure 35) or by alterations in mammary gland structures (Figure 31), but apparently by the altered signaling properties resulting from the sole expression of the non-edited (INI) $5HT_{2C}$ receptor. This is underscored by the fact that treatment of newly postpartum dams with a single dose of a $5HT_{2C}$ receptor inverse agonist, SB206553, restores near-normal pup retrieval behavior (Figure 37).

Future Directions

We have generated one of the first mutant strains of mice with altered $5HT_{2C}$ receptor editing. Previous *in vitro* studies indicated that the INI isoform confers the greatest basal activity and agonist potency via enhanced G-protein coupling (Berg et al., 2001; Herrick-Davis et al., 1999) and $5HT_{2C}R^{INI}$ animals exhibit phenotypes that would confirm these in vitro findings.

Given that $5HT_{2C}R^{INI}$ animals have decreased mRNA expression, it would be compelling to assess mRNA stability. Primary neuronal cells could be cultured from cortex and hippocampus, treated with an inhibitor of transcription such as Actinomycin D (Beach and Ross, 1978; Chen et al., 1993; Sehgal et al., 1975; Singer and Penman, 1972) and harvested at various timepoint to quantify mRNA and thus determine half-life. These methods are relatively toxic so alterative approaches (e.g. pulse-chase labeling) could also be considered. It may also be possible to assay mRNA stability within the animal, but typically the tissue of interest for these studies is usually the liver. Given that $5HT_{2C}$ receptor expression is limited to the CNS, these methods would have to be validated for analyses of brain transcripts and potential issues of toxicity would also need to be addressed.

Similarly, $5HT_{2C}R^{INI}$ animals also have decreased receptor protein expression, most likely due to a decreased mRNA population from which to translate protein, but it is also possible that INI mutant animals have alterations in translation efficiency or decreased protein stability. Primary neuronal cultures could be treated with an inhibitor of translation such as cycloheximide, harvested at various time points and western blots could be performed for detection of protein levels.

$5HT_{2C}R^{INI}$ mice have altered $5HT_{2C}$ receptor mRNA splicing; specifically, INI-mutant animals have increased expression of the non-functional RNA1 isoform at the expense of decreased expression of full-length, fully-functional RNA2 isoform. While

mRNA splicing for this receptor is not fully understood, previous studies have shown that the snoRNA, MBII-52 (and its human ortholog, HBII-52) regulates alternative splicing of the $5HT_{2C}$ receptor by binding to a silencing element within exon 5b and also promotes the formation of non-edited isoforms by masking a splicing silencer (Kishore and Stamm, 2006). In mutant mice that only express the non-edited isoform, it is possible that MBII-52 is directing the formation of RNA1 isoforms. Assays can be performed to determine MBII-52 expression in INI mice and elucidate whether MBII-52 is binding directly, thereby promoting RNA1 isoform formation.

While this dissertation has focused on preliminary characterizations of maternal deficits using assays of nest building and pup retrieval, future studies should include a more detailed analysis of maternal behavior including time and position during nursing as well as assays of maternal aggression. While INI-mutant dams do not have alterations in prolactin or oxytocin mRNA within the pituitary and hypothalamus (Figure 33), respectively, differences may still exist in circulating hormones that could potentially address some of these deficits in maternal behavior.

Our attempts at cross-fostering were unsuccessful as wild-type offspring fostered to wild-type dams had decreased growth rates and altered behavior as compared to non-fostered wild-type offspring. Most likely these effects are due to the background strain of the animal, 129S6/SvEvTac, which is known to have higher levels of anxiety-related behaviors as compared to other strains (Bothe et al., 2004; Voikar et al., 2004). Backcrossing studies are nearly complete to introduce the $5HT_{2C}R^{INI}$ mutation onto the C57BL/6J background and all behavioral assays should be repeated to confirm our initial findings.

The most intriguing findings reported in this thesis are related to the identification of the potential mechanism underlying the deficits in maternal care observed in $5HT_{2C}R^{INI}$ dams. Treatment of INI/+ dams with a single dose of a $5HT_{2C}$ receptor inverse

agonist, SB206553, can rescue the observed retrieval deficits. Inverse agonists are highly effective in silencing the constitutive activity that some receptor subtypes exhibit; in vivo administration of SB206553 has been shown to increase basal DA release within nucleus accumbens and striatum (De Deurwaerdere et al 2004, Berg et al 2005). $5HT_{2C}$ receptors are expressed on GABAergic interneurons within these brain regions (Figure 43) (Eberle-Wang, 1997) and are responsible for serotonergic inhibition of mesolimbic DA activity (Di Giovanni et al., 2000; Gobert et al., 2000).

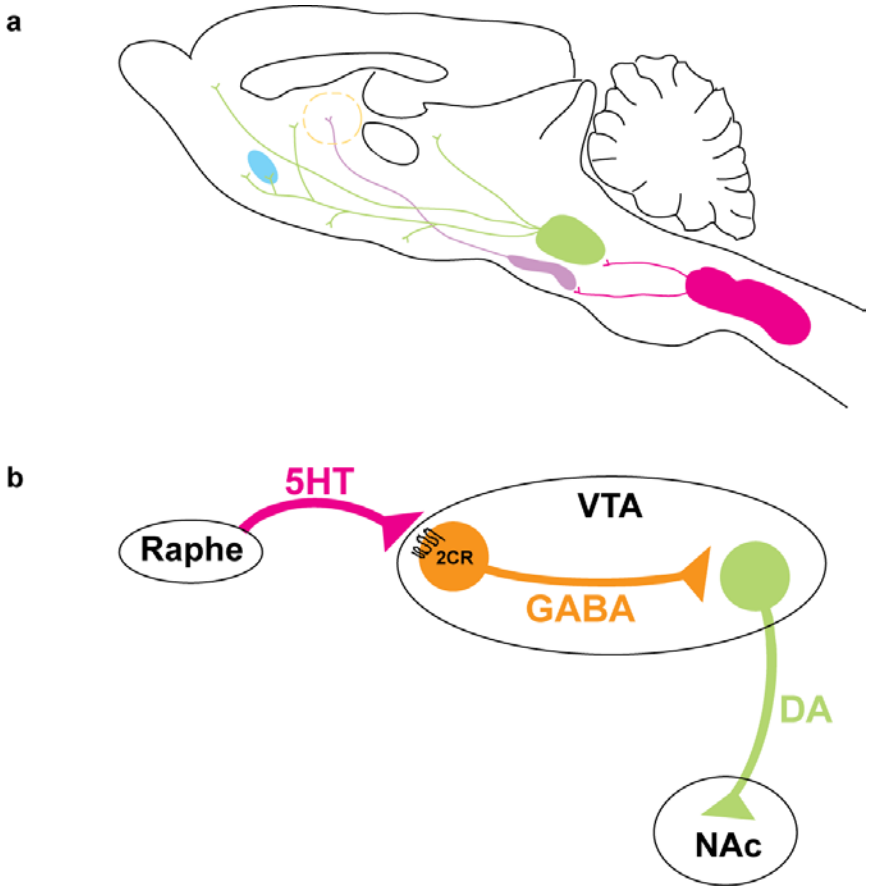

Figure 43: Schematic representation of interactions between 5HT and DA pathways. a) 5HT neurons project from the raphe nuclei (pink) in the brainstem to two major DA pathways, the nigrostrital tract (purple) and the mesocortical tract (green). The nigrostriatal tract orginates in the substantia nigra and projects to the striatum (outlined in yellow). The mesocortical tract originates in the ventral tegmental area (VTA) of the midbrain and projects to cortical and limbic structures, including the nucleus accumbens (NAc, blue). These projections from the VTA to NAc are thought to mediate rewarding behaviors. **b)** Within the VTA, GABAergic interneurons (orange) express $5HT_{2C}$ receptors and are able to modulate DA release within the NAc.

Given that mesolimbic DA release is critical for reinforcing and rewarding behaviors, including maternal care (Ferris et al., 2005), and mice lacking the $5HT_{2C}$ receptor exhibit increased NAc DA levels and therefore enhanced sensitivity to the psychostimulant and reinforcing effects of cocaine (Rocha et al., 2002), we hypothesize that the DA reward system is blunted or hypoactive in $5HT_{2C}R^{INI}$ animals. The inverse agonist, SB206553, is able to increase basal DA release and therefore reinstate normal maternal behaviors. Future studies should focus on testing this hypothesis, including assays to determine if the DA reward system is intact (conditioned place preference and self-administration assays) as well as microdialysis measurements of DA release in the NAc and VTA under basal and drug-treated (agonist, antagonist, inverse agonist) conditions.

This body of work helps to characterize the developmental regulation of RNA editing of the $5HT_{2C}$ receptor and the phenotypic effects of editing loss, chiefly manifested by impaired maternal nurturing behavior. These data are in agreement with literature arguing that inadequacy of maternal care predicts phenotypic changes in the offspring (Caldji et al., 1998; Francis et al., 1999b) and our data suggest that an appropriately edited serotonin 2C receptor could effect expression of maternal behaviors through modulation of the mesolimbic DA reward pathway. Dissecting the intricacies of mother-child attachment has far-reaching implications, suggesting that potential pharmacological targets for the treatment of depressed or anxious mothers and underscoring the importance of psychosocial interventions to facilitate a nurturing infant environment.

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