DEVELOPMENTAL PERSPECTIVES ON SEROTONIN AND SUSCEPTIBILITY TO MOOD AND ANXIETY DISORDERS

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by
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ABSTRACT

Evidence for the involvement of the serotonin neurotransmitter system in the etiology and treatment of mood and anxiety disorders has accumulated over the past three decades. Developmental influences of serotonin have been forthcoming in putting into perspective how alterations early in life can modify the trajectory of serotonergic circuitry involved in establishing adulthood emotionality. Studies presented in this thesis focused on investigating early life alterations in the function of the serotonin transporter (SERT), the substrate for current antidepressant treatments, using pharmacological and genetic mouse models. These studies provide information that is clinically relevant to antidepressant exposure during pregnancy and human SERT gene polymorphisms, respectively. Mice exposed to the serotonin-selective reuptake inhibitor (SSRI) antidepressants S-citalopram (S-CIT) and fluoxetine (FLX) during a critical postnatal development window and mice with constitutive SERT deletions were compared in terms of emotion-related behavior, physiology, and neurochemistry during late adolescence or adulthood. Findings indicate that postnatal administration of S-CIT but not FLX resulted in reduced anxiety, potentiated 5-HT1A autoreceptor responses, and reductions in extracellular serotonin levels in late adolescent and adult mice. By contrast, lifelong SERT deficiency was associated with increased anxiety, blunted 5-HT1A responses, and increased levels of extracellular serotonin at all ages examined. From these findings, it can be concluded that these two models of early SERT disruption produce dissimilar outcomes and warrant attention to determine the benefits and risks
associated with the safety of antidepressants during pregnancy versus untreated maternal depression. Additionally, real-time quantitative PCR assays to determine mRNA levels of genes relevant to mood and anxiety disorders were developed and carried out. In the first study, brain-derived neurotrophic factor (BDNF), a complex gene associated with mechanisms of stress and antidepressant treatment, was studied in SERT-deficient mice. In the mouse, the Bdnf gene contains up to nine splice variants, all encoding the same mature protein, that can be differentially regulated by stress and/or antidepressant therapies. Findings show that SERT-deficient mice having increased anxiety and hypersensitivity to stress show reductions in total BDNF levels, as well as levels of splice variants containing Exons I and V. A second study to investigate SERT expression in rhesus macaques with respect to a SERT gene polymorphism (5-HTTLPR) associated with enhanced anxiety traits in carriers of the short allele of this polymorphism was undertaken. Although functional changes in SERT (reductions in uptake) were observed with respect to short allele carriers, these changes were correlated with surface SERT expression but not SERT mRNA levels. Together, these studies suggest that while the serotonin neurotransmitter system is undoubtedly involved in the pathophysiology and treatment of emotional disorders, many complexities and questions remain to be addressed. Nonetheless, understanding developmental roles of serotonin provide a means to gain a better understanding as changes occurring during this period are sufficient to generate persistent alterations in serotonergic circuitry.
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Preface

The chapters contained in this thesis are modifications of previously published work or are manuscripts being prepared for submission to peer-reviewed journals as detailed below:


**Chapter 2:** Select figures from a manuscript in preparation with Anne M. Andrews.

**Chapter 3:** Manuscript in preparation for submission with coauthors Hongyan Yang, Hannah J. O’Brien, Marisela F. Valdez, Julie G. Hensler, Damla Senturk, and Anne M. Andrews.

**Chapter 4:** Review article reproduced with minor modifications and permission from *ACS Chemical Neuroscience.* Altieri SC, Garcia A, Leonardo ED, and Andrews AM. (2012) Rethinking serotonin 1A receptors: Emerging modes of complex inhibitory feedback of relevance to emotion-related behavior. *ACS Chemical Neuroscience,* DOI: 10.1021/cn3002174.

**Chapter 5:** Select data/graphics from rhesus macaque experiments (as indicated within the chapter) reprinted with permission from *Translational Psychiatry.* Singh YS*, Altieri SC*, Gilman TL, Michael HM, Tomlinson ID, Rosenthal SJ, Swain GM, Murphey-Corb MA, Ferrell RE, and Andrews AM, (2012) Differential serotonin
transport is linked to the rh5-HTTLPR in peripheral blood cells. *Co-first authors*
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“We can only be said to be alive in those moments when our hearts are conscious of our treasures.” —Thornton Wilder
Chapter 1

Serotonergic Pathways in Depression

1.1 Introduction

The magnitude of evidence supporting a role for the serotonin neurotransmitter system in major depressive disorder has been forthcoming over the last five decades and is indispensable to our understanding of this complex and heterogeneous illness. In this chapter, historical perspectives that led to the monoamine hypothesis of depression, and specifically, ideas regarding the role of serotonin in the etiology and pathophysiology of depression will be discussed. Recent views on factors influencing susceptibility to depression, including the discovery of gene polymorphisms in humans and studies focused on the developmental sensitivity of the serotonin system, which provide approaches to unraveling the complexity of depression will be included. Additionally, investigations on the mechanisms of action of different classes of antidepressant drugs have also contributed insight into the role of serotonin in depression. Finally, the topic of peripheral biomarkers of the central serotonin system that might lead to
personalized medicine approaches to improve therapeutic outcomes will be introduced.

1.2 Serotonin: The Basics

1.2.1 Discovery and Biosynthetic Pathway

Serotonin (5-hydroxytryptamine, 5-HT) was first discovered in 1937 by Vialli and Erspamer and given the name enteramine. These investigators observed enteramine in the vesicles of enterochromaffin cells of the gut and determined that enteramine was associated with smooth muscle contraction (Vialli and Erspamer, 1937, 1940). In an unrelated study, Rapport and coworkers (1948) isolated and crystallized a compound from beef serum with vasoconstricting properties, which they named serotonin (serum tonic). It was realized afterwards that enteramine and serotonin were the same compound (Erspamer and Asero, 1952, 1953). The discovery of serotonin in mammalian brain extracts led to its recognition as a chemical neurotransmitter (Twarog and Page, 1953; Amin et al., 1954).

Serotonin is synthesized from tryptophan (L-tryptophan; TRP), an essential dietary amino acid (Fig. 1-1). Tryptophan crosses the blood brain barrier via the large neutral amino acid transporter (LNAA) and is converted into 5-hydroxytryptophan (5-HTP) in serotonergic neurons, which selectively express the rate-limiting synthetic enzyme tryptophan hydroxylase (TPH) (Fig. 1-1). Tryptophan depletion or pharmacologic inhibition of TPH has been used to alter brain serotonin levels and to study the involvement of serotonin in depression. There are two isoforms of TPH termed TPH1 and TPH2, which are now recognized as products of two different
genes. The TPH2 isoform is predominantly expressed in the central nervous system in humans and rodents (Gutknecht et al., 2009), while 5-HT is synthesized via TPH1 mainly in the gastrointestinal system. The enzyme aromatic L-amino acid decarboxylase converts 5-HTP into serotonin to complete the two-step synthesis process (Fig. 1-1). Only a small proportion of available plasma tryptophan is actually converted to serotonin, thus, the overall concentration of serotonin in the brain is low (Tagliamonte et al., 1973).

Serotonin is broken down by monoamine oxidase (MAO) into 5-hydroxyindoleacetic acid (5-HIAA) (Fig. 1-1). There are two isoforms of MAO referred to as MAO-A and MAO-B, which are co-expressed in serotonergic neurons during development (Vitalis et al., 2002). The MAO-B isoform predominates in serotonergic neurons later in life. Investigation of postmortem brain tissue, cerebrospinal fluid (CSF), platelets, and brain imaging studies have been used to detect variations in the key components of the serotonin system as they relate to depression. Evidence from these studies and their contributions to depression research are described below.

1.2.2 Serotonin Receptors and Transporters

Once synthesized, serotonin is sequestered into presynaptic vesicles via vesicular monoamine transporters (VMAT1 and VMAT2), whereby its stimulated and/or constitutive release into the extracellular space produces extensive effects through interactions with a remarkably large class of receptors. The extracellular actions of serotonin are terminated primarily via uptake by the serotonin
transporter (SERT; 5-HTT) (Fig. 1-1). To date, there are fifteen molecularly identified serotonin receptor subtypes categorized into seven major families termed 5-HT1 (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F), 5-HT2 (5-HT2A, 5-HT2B, 5-HT2C), 5-HT3 (5-HT3A, 5-HT3B), 5-HT4, 5-HT5 (5-HT5A, 5-HT5B), 5-HT6, and 5-HT7 (Hoyer et al., 2002). Serotonin receptors are known to exhibit complexity at a number of different levels. For instance, 5-HT1B and 5-HT1D receptor subtypes, which exhibit high sequence homology, were originally thought to occur exclusively in rats and mice or guinea pigs, pigs, dogs, cows, and primates, respectively. However, molecular cloning revealed that 5-HT1D receptors in humans comprised two separate subtypes first named 5-HT1Dα and 5-HT1Dβ receptors. The discovery of the 5-HT1Dβ gene in humans prompted reclassification of 5-HT1B receptors to include 5-HT1B receptors and 5-HT1Dβ receptors, as these differ by only a single amino acid (Hannon and Hoyer, 2008). The 5-HT1Dα receptor subtype is now referred to as 5-HT1D receptors across species. Another example of serotonin receptor heterogeneity involves the 5-HT2C subtype, which was discovered to undergo RNA editing (adenosine→inosine) at multiple sites to produce several receptor proteins with different affinities for serotonin (Gurevich et al., 2002; Sanders-Bush et al., 2003). The presynaptic serotonin system is regulated by multiple autoreceptors including 5-HT1A, 5-HT1B, and 5-HT1D receptors, which also function as heteroreceptors. The 5-HT3 receptor family includes various combinations of 5-HT3A and 5-HT3B subtypes that form pentameric ligand-gated ion channels. All other subtypes of serotonin receptors function as G-protein coupled receptors (GPCRs).
Serotonin interactions at 5-HT1 and 5-HT5 receptor families activate Gαi proteins, while 5-HT4, 5-HT6, and 5-HT7 classes activate Gαs proteins to produce opposing effects to reduce or to increase, respectively, the activation of cyclic adenosine monophosphate (cAMP), which further influences downstream protein targets. The 5-HT2 family of receptors is coupled to Gαq and regulates the activity of phospholipase C (PLC) with the downstream consequences of protein kinase C (PKC) phosphorylation and/or alteration of intracellular calcium levels. The large variety of receptor subtypes present in the serotonin system, in combination with their multiple G-protein coupled pathways and overlapping regional and cellular expression patterns, highlights the complexity and the number of targets within the serotonin system that might be related to the etiology and/or pathophysiology of depression. Many of these serotonin receptors have not been fully characterized regarding localization and functional features. For those receptors whose actions have been investigated in greater detail and for which functions have been identified, their involvement in depression and its treatment has been explored. The predominant focus here will be on members of the 5-HT1 and 5-HT2 serotonin receptor families (predominantly 5-HT1A and 5-HT2A subtypes) as these receptors have been extensively studied in the context of depression. Information on the mechanisms of additional serotonin receptors can be found in (Hensler, 2006; Hannon and Hoyer, 2008).

The serotonin transporter is a central modulator of serotonergic neurotransmission. The SERT is a 12-transmembrane domain protein localized on soma and presynaptic terminals of serotonergic neurons, and also glia. The SERT
uses co-transport of Na\(^+\) and Cl\(^-\) and counter-transport of K\(^+\) to drive the clearance of serotonin from the extracellular space (Kanner and Schuldiner, 1987). In addition to its role in modulating the pre- and post-synaptic effects of extracellular serotonin, the SERT is also the principal target of widely prescribed classes of antidepressants, including the tricyclic antidepressants (TCAs), discovered in the 1960’s, the serotonin-selective reuptake inhibitors (SSRIs), and also the mixed serotonin and norepinephrine reuptake inhibitors (SNRIs) (White et al., 2005). Other important antidepressant targets within the serotonin system include the 5-HT1 and 5-HT2 classes of receptors, MAO-A and MAO-B, and TPH2, and these are also being investigated for their roles in the pathophysiology and etiology of MDD.

Serotonin is present in most brain regions in the central nervous system and in multiple peripheral organs and cell types. Serotonin and other constituents of the serotonin pathway appear early in embryonic development (during the first trimester in humans and higher-order primates and at day 10 of rodent gestation) and they continue to mature throughout early postnatal development (Levitt and Rakic, 1982; Hendricks et al., 1999). In the brain, serotonergic neurons originate deep within the brainstem. Serotonin-containing neurons are anatomically grouped into caudal (B1-B5) and rostral (B6-B9) nuclei, with the former projecting to areas of the deep cerebellar nuclei, cortex, and spinal cord, while the latter extends an axonal network throughout the forebrain and cortices (Fig. 1-2). Major targets of the rostral raphe nuclei include limbic regions (e.g. hippocampus and amygdala), hypothalamus, thalamus, and neocortex (Hornung, 2003). A similar organization is observed in rodents and other mammals (Dahlstrom and Fuxe, 1964). Of particular
relevance to mechanisms of depression are projections to structural correlates of emotionality including the amygdala, prefrontal and cingulated cortices, hypothalamus, and thalamus (Fig. 1-2). Recently, serotonin neurons have been classified based on genetic lineages. Specifically, serotonin neuronal progenitors can be subdivided into subsets defined by rhombomeres (r1, r2, r3, r5), which are discriminated by differing genetic cues (transcription factors) during development (Jensen et al., 2008).

The broad functions of serotonin in the central nervous system include its involvement in the modulation of aggression, sleep, appetite, mood, thermoregulation, and sexual function; however, serotonin levels in the brain account for only 10% of the total serotonin in the body (Berger et al., 2009). In the periphery, serotonin is predominantly found in enterochromaffin cells of the gut, where it functions in muscle contraction and gut motility (Spiller, 2008). Additionally, serotonin is taken up into platelets, where it is released to cause vasoconstriction and is involved in cardiovascular regulation (Ramage and Villalon, 2008). Serotonin is also found in lymphocytes and monocytes and is associated with immune system function (Gordon and Barnes, 2003). Peripheral tissues express the SERT and multiple serotonin receptors and thus, posses many of the components of the central serotonin system (Berger et al., 2009). As discussed later, correlations between central and peripheral serotonin system components are important for advances in depression research whereby peripheral cells might be used as diagnostic tools to study treatment responses in cases where direct brain measurements are not feasible.
1.3 The Monoamine Hypothesis of Depression

1.3.1 Discovery of the Role of Serotonin

Early evidence for the involvement of monoamine neurotransmitters, including serotonin, in depression and other affective disorders arose serendipitously. Reserpine (*Rauwolfia serpentina*), was first used as an ayurvedic treatment for insanity, producing calming effects and lowering blood pressure (Sen and Bose, 1931; Gupta et al., 1947; Vakil, 1949). Wilkins and coworkers (1953) confirmed the effects of reserpine to control hypertension. Later reports identified additional sedative properties of this drug, along with evidence that reserpine might be useful for alleviating anxiety and symptoms of obsessive compulsive disorder (Kline, 1954). By contrast, another study identified depressogenic properties of reserpine. Here, five patients displayed depressive symptoms after receiving a chronic regimen of reserpine but acquired remission upon discontinuation of treatment (Freis, 1954). Shortly thereafter, it was found that reserpine administration leads to a severe depletion of monoamine neurotransmitters in the mammalian brain (Holzbauer and Vogt, 1956; Paasonen and Vogt, 1956). Based on these findings, the monoamine hypothesis was formulated, which stated that decreases in monoamine neurotransmitters in the brain might be causative in depression.

Further support for the role of monoamines in depression occurred inadvertently via the discovery of two other drugs, iproniazid, a monoamine oxidase inhibitor (MAOI) and imipramine, a monoamine uptake inhibitor. Clinical trials
using iproniazid, a compound originally designed as an antibacterial agent, also reported antidepressant-like effects (Saunders et al., 1959). The mechanism of action of iproniazid was later shown to involve inhibition of monoamine oxidase, which is responsible for the degradation of monoamine neurotransmitters (Davison, 1957). These findings suggested that antidepressant effects could be achieved by increasing the concentrations of monoamines. Subsequently, the investigation of imipramine, a drug designed to mimic the effects of the antipsychotic agent chloropromazine, likewise revealed its antidepressant properties (Kuhn, 1958). Axelrod and coworkers (1961) went on to show that imipramine increases monoamine concentrations by blocking the reuptake of serotonin and norepinephrine into presynaptic terminals. These early studies on iproniazid and imipramine together reinforced the monoamine hypothesis of depression.

Research carried out by Pollin and colleagues (1961) proposed that out of the monoamine neurotransmitters (serotonin, dopamine, and norepinephrine), serotonin contributes foremost to the etiology of depression. While studying the effects of amino acids in schizophrenic patients, a paradigm was employed whereby amino acid mixtures in the presence or absence of iproniazid were administered to potentiate levels of endogenous monoamines. These investigators observed that only tryptophan resulted in an elevation in mood in schizophrenic patients, and that these changes were altered after removal of tryptophan causing sudden increases in hostility and depression in some patients. Similar experiments in depressed patients receiving the MAO inhibitor tranylcypromine showed accelerated improvements when given in combination with tryptophan compared to tranylcypromine alone.
(Coppen et al., 1963). Together, these studies suggested that the key neurochemical disruption in depression and the therapeutic action of antidepressants are related to variations in serotonin levels.

1.3.2 Serotonin Metabolite Levels in Depression

Serotonin is broken down into 5-hydroxyindoleacetic acid (5-HIAA) by the actions of monoamine oxidase and aldehyde dehydrogenase (Fig. 1-1). Thus, 5-HIAA has been used as an indirect marker for investigating alterations in serotonin neurotransmission. The concentration of 5-HIAA in cerebrospinal fluid (CSF) has been used primarily to make inferences about brain function because of its high concentration compared to serotonin, the accessibility of CSF, and the high degree of correlation between CSF and brain 5-HIAA levels in non-human primates (Eccleston et al., 1968) and humans (Stanley et al., 1985).

In initial studies examining 5-HIAA as an index of brain serotonin concentrations, decreases in CSF 5-HIAA levels were reported in psychiatric patients suffering from depressive psychosis compared to patients with neurological disorders without depression (Ashcroft and Sharman, 1960). This finding prompted several subsequent studies aimed at measuring CSF 5-HIAA in patients diagnosed with psychiatric disorders to understand further the involvement of the serotonin system. However, the results of these studies were inconclusive, with findings ranging from significant decreases (Agren, 1980a, b) to a trend towards decreases (Papeschi and McClure, 1971) and to no changes (Oreland et al., 1981) in 5-HIAA levels in depressed patients compared to healthy controls.
Investigations in the relationship between CSF 5-HIAA levels and the severity of depression symptoms has also yielded conflicting results, with some studies observing significant associations (Peabody et al., 1987), while others did not detect significant correlations between CSF 5-HIAA levels and self-report scores on the Hamilton Depression Rating Scale (HDRS) (Papeschi and McClure, 1971; Banki and Arató, 1983).

Further studies have suggested a bimodal distribution of CSF 5-HIAA levels in depressed patients (Asberg et al., 1976b). In a longitudinal study, Träskman-Bendz and coworkers (1984) replicated this finding by observing a significant increase in 5-HIAA levels after recovery in a subset of depressed patients who exhibited lower CSF 5-HIAA concentrations before improvements. A subset of depressed patients with low 5-HIAA levels revealed a strong association between severity of depression, the amount of 5-HIAA in CSF (Asberg et al., 1976b), and increased vulnerability to suicide attempts (Asberg et al., 1976a). This was further supported by findings reporting a negative relationship between suicidal tendencies or seriousness of intent to harm oneself with CSF 5-HIAA levels in unipolar and bipolar depressed patients (Agren, 1980a). Many studies in depressed individuals have reported low CSF 5-HIAA levels in patients that attempt suicide (Oreland et al., 1981; Edman et al., 1986; Jones et al., 1990; Nordström et al., 1994; Samuelsson et al., 2006) or have suicidal ideation (Lopez-Ibor et al., 1985; Mann et al., 1996) compared to depressed non-suicidal patients or healthy controls. Furthermore, suicide attempters with no history of diagnosed depression also have been reported to have low CSF 5-HIAA levels compared to healthy controls (Träskman et al., 1981).
However, some studies in affective disorder patients have found no association between suicide attempt and low CSF 5-HIAA levels (Roy-Byrne et al., 1983; Secunda et al., 1986; Hou et al., 2006; Sullivan et al., 2006).

Together, the information obtained from these studies suggest that low CSF 5-HIAA levels might be a marker for decreased serotonin neurotransmission, and particularly as associated with suicide. However, how CSF 5-HIAA levels definitively pertain to major depressive disorder (MDD) is as yet unclear. It appears as though low CSF 5-HIAA is linked to a higher probability for suicidal ideation or attempt, which is sometimes but not always co-morbid with depression. The high variability in CSF levels in healthy subjects (Kennedy et al., 2002) might contribute to the variability of the results of studies in this area. Furthermore, CSF levels can be affected by environmental factors such as daily diet or the time of day of sampling (Grimes et al., 2000; Kennedy et al., 2002).

1.3.3 Postmortem Studies of Brain Tissue

In comparison to CSF measurements, postmortem brain tissue provides direct access to central serotonin levels, in addition to other important serotonin system markers, including the expression and function of the SERT and serotonin receptors. Early research on postmortem tissue serotonin and 5-HIAA levels in multiple brain regions of depressed and control subjects supported the monoamine hypothesis of depression. In depressed patients, significant reductions in serotonin were reported in brain stem (Shaw et al., 1967; Pare et al., 1969; Lloyd et al., 1974; Birkmayer and Riederer, 1975), nucleus accumbens, caudate, putamen, substantia
nigra, and amygdala (Birkmayer and Riederer, 1975). Moreover, this deficit in tissue serotonin content was not observed in brain tissue from remitted depressed cases, suggesting that treatment restored serotonin levels (Birkmayer and Riederer, 1975). Similarly, decreases in tissue 5-HIAA levels were also observed in depressed patients (Pare et al., 1969; Birkmayer and Riederer, 1975; Beskow et al., 1976). These results from postmortem human brain tissue were in agreement with the hypothesis of impairment of the serotonin system in depression. However, other reports of postmortem findings measuring neurochemicals in the brain fail to detect changes in serotonin (Bourne et al., 1968; Beskow et al., 1976; Gottfries et al., 1976) and 5-HIAA levels (Crow et al., 1984; Ferrier et al., 1986; McKeith et al., 1987) in depressed patients. The collection and processing of human postmortem tissues is lengthy (~6-12 hours) and serotonin and other monoamines, which are highly susceptible to biological and chemical oxidation, might undergo substantial and variable degradation thereby contributing to these discrepancies (Roubein and Embree, 1979; Siddiqui et al., 1990).

In addition to serotonin and 5-HIAA levels, SERT, which is the molecular target for a variety of antidepressants, has been investigated in postmortem human brain. The majority of studies have observed a significant decrease in SERT in depressed patients in many serotonergic-rich brain regions including the hippocampus (Perry et al., 1983), putamen (Lawrence et al., 1989) occipital cortex (Perry et al., 1983), frontal cortex (Leake et al., 1991), prefrontal cortex (Mann et al., 2000), and dorsal raphe nuclei (Arango et al., 2001). However, other studies have reported no significant alterations in SERT in the hippocampus, frontal cortex, mid-
brain (Little et al., 1997; Bligh-Glover et al., 2000), and prefrontal cortex (Thomas et al., 2006).

Decreases in 5-HT1 receptor family expression were first observed in the frontal cortex of depressed patients (McKeith et al., 1987). In subsequent research focusing on 5-HT1A autoreceptor expression in the dorsal raphe nuclei, some studies report reductions in depressed patients (Drevets et al., 1999; Arango et al., 2001; Boldrini et al., 2008) while another report indicates an increase in 5-HT1A autoreceptor expression in this same brain region (Stockmeier et al., 1998). Discrepancies have been attributed to a reduced number of SERT-positive neurons in the dorsal raphe of depressed patients (Arango et al., 2001) and differences in brain tissue volume. In terms of postsynaptic 5-HT1A receptors in depressed patients, 5-HT1A receptor mRNA and protein expression are decreased in the hippocampus (Lowther et al., 1997; Lopez et al., 1998; Drevets et al., 1999). Variable results have been observed in the frontal cortex with one group reporting a trend towards an increase in postsynaptic 5-HT1A binding (Lowther et al., 1997), while others failed to observe differences in this brain region (Owen et al., 1983).

Alterations in the 5-HT2 family of receptors in depressed patients were first reported by Stanley and coworkers (1983). They observed a significant increase in 5-HT2 binding in the frontal cortex of depressed suicide victims. However, these results are in contrast to a similar study where no differences were found in 5-HT2 binding in the frontal cortex (Owen et al., 1983). Moreover, in the case of suicide victims, reports were again divided in that some found a significant increase in 5-
HT2 receptor expression in the frontal cortex (Yates et al., 1990; Arango et al., 1992; Hrdina et al., 1993), while others failed to find similar changes (Cheetham et al., 1988). Investigations into other brain regions including the hippocampus and amygdala have also produced inconsistent findings. For example, in the amygdala, one study has reported an increase in 5-HT2 receptor binding (Hrdina et al., 1993), while another showed no change (Cheetham et al., 1988) in depressed suicide victims. Cheetham and coworkers (1988) observed a trend toward increases in 5-HT2 receptor binding in the frontal cortex and amygdala, which were accompanied by a decrease in 5-HT2 binding in the hippocampus of violent suicide cases.

The bulk of postmortem studies have been carried out in suicide victims or elderly depressed patients. However, these groups of patients might not represent the general population of depressed subjects and instead, be specific subsets with different serotonergic alterations compared to the majority of individuals with depression. Additionally, early studies did not take into account patient histories of antidepressant treatment. Only a few studies within the last decade have separated patients into antidepressant-exposed versus antidepressant-naïve groups when considering changes in the expression of key components of the serotonin system, despite the observation that prior antidepressant exposure alters expression of these components (Messa et al., 2003; Parsey et al., 2006b; Hirvonen et al., 2008). Lastly, recent findings regarding associations between gene × environment interactions having an effect on the serotonin system warrant interpreting postmortem data with caution. Although it appears as though SERT expression in the dorsal raphe and serotonin and
5-HIAA levels in midbrain regions are reduced in depressed patients in a majority of studies, inconsistencies in these findings warrant further investigation while controlling for prior antidepressant history, substance abuse, suicidal ideation, and other factors.

1.3.4 Brain Imaging Studies

Recent advances in functional brain imaging methods including positron emission tomography (PET) and single photon emission computed tomography (SPECT), in addition to the design of radioactive ligands for use with these techniques, allow for noninvasive measurements of different aspects of the serotonin system to be carried out in vivo in humans. Currently, the targets that can be investigated using PET and SPECT include SERT, 5-HT1 and 5-HT2 receptor families, and serotonin synthesis (for review see (D’Haenen et al., 1992; Zipursky et al., 2007)).

In vivo visualization and measurement of SERT using PET and SPECT have revealed a significant decrease in SERT binding potential in the midbrain region in depressed patients (Malison et al., 1998; Cannon et al., 2006; Parsey et al., 2006b; Oquendo et al., 2007). However, information obtained from other brain regions has not resulted in straightforward findings. Some studies describe a decrease in SERT binding in the amygdala (Parsey et al., 2006b; Oquendo et al., 2007), hippocampus, thalamus, putamen, and anterior cingulate cortex (Oquendo et al., 2007) in depressed patients compared to healthy subjects. By contrast, in other studies, increases in SERT binding in the thalamus, dorsal cingulate cortex (Miller et al.,
2009b), and medial frontal cortex have been reported in depressed patients (Cannon et al., 2006). Furthermore, subjects with a history of suicide attempt showed a pronounced reduction in SERT binding potential in the midbrain and an increase in SERT binding in the anterior cingulate cortex compared to both depressed patients without suicidal histories and healthy controls (Cannon et al., 2006). These results are in agreement with the hypothesis that individuals with suicidal tendencies might represent a specific subset of depressed patients characterized by serotonergic dysfunction. However, Parsey and coworkers (2006b) failed to find differences in SERT binding in any of the brain regions examined when comparing suicide attempters and non-attempters.

In an example of how environmental factors might influence serotonergic function, Miller and colleagues (2009a) found using PET imaging that depressed patients with a history of childhood abuse displayed significant decreases in SERT binding in all brain regions, which were not present in depressed patients with no history of childhood abuse. Similar results were observed in rhesus monkeys, where decreased SERT binding in the brain stem, thalamus, and striatum was evident in peer-reared monkeys compared to mother-reared animals (Ichise et al., 2006). These findings suggest that environmental factors such as stress induced by abuse or maternal separation can modulate the serotonergic system and potentially manifest themselves as risk factors for developing depression.

Compared to investigations on the expression of SERT, results from studies of 5-HT1A binding are more variable. Some groups have reported a global decrease
in 5-HT1A binding in antidepressant-näive depressed patients (Hirvonen et al., 2008), while others have observed a global increase in 5-HT1A binding (Parsey et al., 2006a; Miller et al., 2009b), although in both cases, significant changes in 5-HT1A binding potential were not pinpointed to specific brain regions. By contrast, 5-HT1A binding has been reported to be reduced only in the dorsal raphe in elderly depressed patients compared to age-matched healthy control subjects (Meltzer et al., 2004). The relationship between 5-HT1A binding potential and the severity of depression has also been taken into consideration whereby findings indicate a high correlation (Meltzer et al., 2004), although this has not been a consistent finding either (Hirvonen et al., 2008). Prior antidepressant treatment can also affect the results of imaging studies. Antidepressant-naïve depressed patients show increased 5-HT1A binding potential compared to healthy controls, but no changes were reported in depressed patients with a history of antidepressant exposure compared to healthy individuals (Parsey et al., 2006a; Miller et al., 2009b).

Initial PET imaging studies aimed at investigating 5-HT2 receptors in depressed patients showed higher binding in the parietal cortex (D’Haenen et al., 1992) and reduced binding in the right hemispherical region of the infero-frontal cortex (Biver et al., 1997). However, in another study of unmedicated depressed patients without a history of suicidal ideation, there were no changes in 5-HT2 receptor binding (Meyer et al., 1999). When looking specifically at 5-HT2 receptor subtypes, there are reports of decreased 5-HT2A receptor binding in the hippocampus (Sheline et al., 2004), and frontal, occipital, and cingulate cortices (Messa et al., 2003) in antidepressant-naïve depressed patients compared to
controls, but similar findings were not observed in elderly depressed patients compared to age-matched healthy controls (Meltzer et al., 1999).

*In vivo* brain imaging techniques provide valuable information on changes in neuronal systems in relation to human disease and its treatment with the possibility for longitudinal studies. Nonetheless, the complexity of the serotonin system, most notably the heterogeneity of its receptor subtypes, necessitates the continued development of highly selective ligands. Additionally, single nucleotide polymorphisms (SNPs) and other common polymorphisms are rapidly being identified in serotonergic genes. This increasing genetic complexity will need to be considered in terms of its influence on the expression and function, including ligand binding, of *in vivo* imaging targets. A related issue is the fact that sample size is an increasingly important factor in the design and interpretation of depression-related studies, including brain imaging studies, and particularly in light of genetically heterogeneous human populations.

1.3.5 Blood Platelet Serotonergic Markers

Another common avenue to study the serotonin system in humans is through the use of blood cells, plasma, and serum. Platelets and lymphocytes express the SERT, as well as a number of different types of serotonin receptors including 5-HT1A receptors (Yang et al., 2006; Yang et al., 2007). SERT-mediated uptake of serotonin in platelets has been correlated with maximal uptake rates in brain in postmortem and SPECT imaging studies (Rausch et al., 2005; Uebelhack et al., 2006). In depressed patients, a number of studies have reported decreases in SERT
expression in platelets using [³H]-imipramine binding (Briley et al., 1980; Paul et al., 1981; Ellis et al., 1990; Nemeroff et al., 1994); however, not all studies are in agreement and some have reported no changes in SERT expression in depressed individuals compared to control subjects (Gentsch et al., 1985; Lawrence et al., 1993). In terms of correlating the severity of depression with platelet SERT function, consensus also has not been reached and results refuting (Kanof et al., 1987) or in support of this association are reported (Ellis et al., 1990; Fisar et al., 2008). Use of the SERT-selective ligand, [³H]-paroxetine has also produced contrasting results (Kanof et al., 1987; D'haenen et al., 1988; Lawrence et al., 1993; Nemeroff et al., 1994). Additional factors hypothesized to affect the outcome of platelet studies include prior antidepressant exposure of patients, seasonal effects on SERT expression, genetic polymorphisms, and gender (Nemeroff et al., 1994; D'Hondt et al., 1996; Parsey et al., 2006a). A few studies have controlled for some of these factors, including seasonal effects (Kanof et al., 1987) and antidepressant exposure (Lawrence et al., 1993; Nemeroff et al., 1994) but none have controlled for all of these variables.

1.3.6 Serotonin Depletion Studies

Pharmacologic depletion of serotonin has been used as an approach to evaluate the hypothesis of the association between low serotonin levels and depressed mood. There are a number of steps in the serotonin synthesis pathway that can be manipulated, e.g., decreasing the bioavailability of the serotonin precursor, L-tryptophan or inhibiting the rate-limiting enzyme in serotonin
synthesis. Factors to consider when using this type of paradigm include the relative specificity of drugs used to block these targets, the ethics of carrying out studies in humans (particularly patients), and interpreting behavioral changes. Despite these complexities, information has been obtained through pharmacologic studies that have contributed to our knowledge about the role of serotonin in mood disorders.

L-Tryptophan, the precursor for serotonin synthesis, is an essential dietary amino acid (Rose et al., 1954). Experimental protocols have been developed to try to reduce central levels of serotonin by decreasing plasma tryptophan levels, thereby depleting the amount of tryptophan available to cross the blood brain barrier (Biggio et al., 1974). Brain tryptophan levels are dependent on plasma levels of other large neutral amino acids (LNAAAs) as all compete to cross the blood brain barrier using the same LNAA transporter (Hood et al., 2005). Reduced plasma tryptophan:LNAA ratios decrease the likelihood of tryptophan being transported into the brain. By orally administering an amino acid mixture to fasted subjects that contains large amounts of all LNAAAs except tryptophan, it is possible to reduce plasma tryptophan levels, plasma tryptophan:LNAA ratios, brain serotonin synthesis as measured by PET, and cerebrospinal fluid levels of 5-HIAA (Nishizawa et al., 1997; Carpenter et al., 1998; Klaassen et al., 1999; Moreno et al., 2000; Booij et al., 2003).

Recent reviews and meta-analyses highlight the reproducibility of results obtained using acute tryptophan depletion (ATD) methods to investigate the role of serotonin and other monoamines in depressive states (Booij et al., 2003; Fusar-Poli et al.,
The use of ATD does not appear to stimulate depressed mood in healthy individuals lacking an existing or previous diagnosis of depression nor does it increase the severity of depressed mood in patients clinically diagnosed with depression who are not currently receiving antidepressant treatment (Delgado et al., 1994; Neumeister et al., 1997; Murphy et al., 2002; Evers et al., 2005; Robinson and Sahakian, 2009). However, ATD has been found to reinstate a transient depressed mood state in remitted patients with a prior diagnosis of MDD and who are actively receiving antidepressant therapy (Delgado et al., 1990; Moreno et al., 2006; Merens et al., 2008). Additional evidence suggests that female participants show greater responses to ATD compared to males as indicated by self-reports of affective measures. Furthermore, a family history, i.e., first-degree relatives with MDD, serves as a risk factor for vulnerability to the depressogenic effects of ATD, irrespective of a clinical diagnosis of depression (Ellenbogen et al., 1996; Moreno et al., 2006). Moreover, in response to ATD, “s” allele carriers of the 5-HTTLPR SERT-gene polymorphism (vide infra), particularly females, tend to be more vulnerable to relapse into a depressive state, and also to perform worse in motivational tasks and to have slower responses to antidepressants (Roiser et al., 2006; Walderhaug et al., 2007).

Animal models have also been used to investigate the effects of brain serotonin depletion on depression-related behavior. Degeneration of serotonergic neurons in the dorsal and medial raphe nuclei has been achieved using intracerebral administration of the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). The tryptophan hydroxylase inhibitor p-chlorophenylalanine (pCPA) also has been used
to deplete serotonin levels, similar to the ATD paradigm in humans. These agents produce reductions in brain serotonin levels in rodents without affecting other monoamines (Lorens et al., 1976). However, decreased brain serotonin levels caused by neurotoxins or electrolytic lesion have not been associated with increases in behavioral despair, a depression-related endophenotype modeled in rats and mice by increases in immobility times in the forced swim test (Borsini, 1995; Lieben et al., 2006). By contrast, neonatal rats administered 5,7-DHT displayed increases in behavioral despair in adulthood (Kostowski and Krzaścik, 2003). The latter result supports the hypothesis that developmental disturbances in serotonin system function produce persistent changes in behavior in adulthood, and these ideas are developed below.

1.3.7 Genetically Engineered Animal Models

In light of the difficulties associated with investigating the monoamine hypothesis of depression in humans, a number of animal models having targeted loss of expression of specific genes have been utilized and found to be particularly advantageous in understanding the role of serotonergic pathway components and their involvement in depression. Gardier et al. (2009) reviewed strategies to create knockout animal models for the study of neurochemical imbalances in the etiology of depression. Mice engineered to lack one or two functional copies of the SERT gene (SERT+/− and SERT−/−, respectively) (Bengel et al., 1998) possess a complex phenotype most often characterized by increased anxiety-like behavior that is dependent on background strain and environmental influences such as stress (Li et
al., 1999; Tjurmina et al., 2002; Holmes et al., 2003; Joeyen-Waldorf et al., 2009). SERT deficient mice also show increases in depression-like behaviors that are strain and test dependent and modest hypolocomotion (Holmes et al., 2002a; Kalueff et al., 2006). Constitutive reductions in SERT are associated with gene-dose dependent decreases in brain serotonin uptake rates (Montanez et al., 2003; Perez and Andrews, 2005; Perez et al., 2006) and increases in extracellular serotonin levels (Fabre et al., 2000; Mathews et al., 2004) with the highest levels observed in SERT-/- mice. In addition, 5-HT1A and 5-HT1B receptor function is desensitized (Li et al., 1999; Fabre et al., 2000; Murphy and Lesch, 2008). For recent review of the complex central and peripheral phenotypic changes associated with loss of SERT expression in mice see (Murphy et al., 2008). Together, findings in SERT deficient mice have led to the hypothesis that increases in extracellular serotonin occurring throughout life, and particularly during early development, result in behavioral changes that are different from and sometimes opposite to those associated with antidepressant administration in adults (Murphy et al., 2004; Sibille and Lewis, 2006; Murphy and Lesch, 2008). Along the same lines, inactivating the genes encoding MAO, the enzyme that breaks down monoamine neurotransmitters including serotonin, might initially be predicted to result in an antidepressant effect. However, mice genetically lacking MAO-A are characterized by enhanced aggression (Cases et al., 1995), whereas MAO-B deficient mice show exaggerated responses to stress (Grimsby et al., 1997).

Altering other serotonergic genes in mice to investigate their roles in depression has produced valuable insights. As discussed above, there are two
isoforms of tryptophan hydroxylase produced from two separate genes, termed TPH1 and TPH2, with the latter being the predominant brain isoform (Gutknecht et al., 2009). Mice lacking functional copies of TPH1 (TPH1/- mice) show reduced serotonin levels restricted to the periphery (Cote et al., 2003; Savelieva et al., 2008) and deficits in the normal functioning of peripheral organs (Trowbridge et al., 2010). The discovery of TPH2 occurred more recently (Walther et al., 2003), and the initial report of behavioral changes in TPH2/- mice, although somewhat inconclusive, suggests contrasting changes in depression-like behaviors across tests of behavioral despair that are more prominent in mice lacking both isoforms of TPH (Savelieva et al., 2008). In mice where the gene encoding the 5-HT1A receptor (HTR1A) has been deleted, the primary phenotype is one of enhanced anxiety and stress responsiveness, similar to that found in SERT/- mice (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Interestingly, a strategy to restore 5-HT1A function in postsynaptic neurons in brain regions involved in emotional regulation (hippocampus, frontal cortex) reversed the increased anxiety-like phenotype associated with constitutive 5-HT1A receptor loss suggesting that postsynaptic 5-HT1A receptors modulate anxiety and that these effects occur during specific developmental timeframes (Gross et al., 2002). Additional serotonin receptor subtypes have been investigated using knockout strategies producing a range of behavioral phenotypes, although their potential roles in depression remain uncertain (Gaspar et al., 2003).
1.3.8 Genetic and Environmental Interactions

Genetic factors can increase predisposition to depression by ~30% (Fava and Kendler, 2000). However, understanding the contribution of different genes is complicated by many factors including the high degree of heterogeneity of the pathophysiology of depression, the involvement of multiple neurotransmitter systems and brain regions, the growing multitude of genetic variants being identified and the small effects that most of these variants contribute, and a variety of possible environmental influences (Burmeister, 1999; Caspi et al., 2010). Despite these limitations, genes that code for important regulators of the serotonin system have been investigated to attempt to identify hereditary influences that might increase vulnerability to depression.

In depression, the most highly investigated gene variants occur in the SERT (SLC6A4) gene, and in particular, include the serotonin transporter-linked polymorphic region (5-HTTLPR) first reported by Heils and coworkers (1996). This polymorphism occurs only in humans and higher order primates (Lesch et al., 1997). It is hypothesized that the short ‘s’ allele is associated with reduced transcriptional efficiency and thus, decreases in SERT expression that manifest as decreased serotonin transport (Lesch et al., 1996; Singh et al., 2010). Furthermore, there is a correlation between stress-associated depression and increased neuroticism in carriers of the ‘s’ allele, (Caspi et al., 2003; Uher and McGuffin, 2008) although this has not always been consistently reported (Flory et al., 1999). Discrepancies might arise as a result of the genetic complexity of this polymorphic
region and additional functional allelic variants have been described (Hu et al., 2005; Wendland et al., 2008). For example, a SNP that results in a base-pair difference in the repeat elements, termed LA or LG, has been discovered within the 5-HTTLPR. The LG variant is functionally similar to the low expressing ‘s’ allele and is likewise linked to stress-induced depression (Zalsman et al., 2006).

Brain imaging studies using PET or SPECT are associated with contrasting results regarding the association between SERT binding and 5-HTTLPR genotype (Murthy et al.; Heinz et al., 2000; Willeit et al., 2001; van Dyck et al., 2004; Parsey et al., 2006c). Brain imaging might be poor at detecting modest changes in SERT binding for a number of reasons including low sensitivity, small sample sizes, and the presence of other polymorphisms and SNPs within the SERT gene.

Ongoing studies provide further evidence for the complexities of the SERT gene and the genetics of depression. Recent genome wide association studies from the STAR*D trial (described below) have sought to identify key genetic components involved in the etiology and treatment of depression, although definitive results have not been obtained due to the involvement of multiple genetic factors (alone or in combination) that may manifest in heterogeneous diseases such as depression (Garriock and Hamilton, 2009; Laje et al., 2009).

1.3.9 Serotonin Function During Development

In addition to its role as a neurotransmitter, serotonin is important in driving the development of neuronal circuitry and associated phenotypes. As discussed above, serotonin and markers for serotonergic neurons are expressed beginning in
early embryonic development (Gaspar et al., 2003). Additionally, some neurons express SERT only during brief periods of early postnatal life, although these particular neurons do not continue to express SERT or become serotonergic neurons in the adult brain (Lebrand et al., 1996; Verney et al., 2002). Transient expression of SERT is limited to sensory thalamic neurons (Lebrand et al., 1996), and neurons in the hippocampus (Lebrand et al., 1998b), anterior cingulate cortex (Lebrand et al., 1998b), and the superior olive (Cases et al., 1998). In humans, transient SERT is reported to occur in thalamocortical neurons, although it has been more difficult to ascertain additional subsets of neuronal populations expressing SERT during development in human tissues (Verney et al., 2002). The machinery in neurons with a transient partial serotonergic phenotype is limited to expression of SERT and VMAT (Lebrand et al., 1998a). Thus, while these neurons lack the capability to synthesize serotonin, serotonin uptake and release at these sites might have a morphogenic effect (Lebrand et al., 1996). Studies to identify a role for SERT during embryonic stages through the first few weeks of postnatal life have revealed that transient SERT expression is necessary for the development of the barrel field cortex in rodents (Vitalis et al., 1998). Other roles for developmentally sensitive SERT expression have yet to be fully identified but there is evidence to suggest that disruptions in SERT during key windows of development might be important in mediating persistent adulthood phenotypes.

A critical window (the first few weeks of postnatal development in rodents) during which the serotonin system can be disrupted via the administration of antidepressants has been associated with changes depression-related behaviors in
adulthood. Clomipramine administered to rats during early life is correlated with reduced sexual activity (Neill et al., 1990; Vogel et al., 1990), increased rapid eye movement sleep (Mirmiran et al., 1981), and increased immobility in tests of behavioral despair (Velazquez-Moctezuma and Diaz Ruiz, 1992). In mice, administration of the SSRIs fluoxetine or escitalopram during early postnatal development produces adult phenotypes characterized by reduced exploratory locomotor behavior (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008), reduced sucrose consumption (a measure of anhedonia) (Popa et al., 2008), and increased immobility in behavioral despair tests (Popa et al., 2008). This early influence of altered serotonin reuptake on adult mood and anxiety-related behaviors in rodents might be used to further our understanding of how the serotonin system shapes affect-associated brain circuitry in development. Research in this area also has implications for pregnant women taking SSRIs and for establishing recommendations for treating childhood and adolescent mood and anxiety disorders with serotonergic drugs. For example, clinically relevant drug levels have been detected in amniotic fluid and the umbilical cords of pregnant women treated with SSRIs, (Hostetter et al., 2000; Hendrick et al., 2003b), and newborns display SSRI withdrawal effects and low birth weights (Hendrick et al., 2003a; Sanz et al., 2005). Some motor coordination effects have also been reported in young children (Casper et al., 2003), although it is not clear yet how human behavior might be influenced by exposure to prenatal antidepressants but also by untreated maternal depression. Maternal serotonin has recently been suggested to be a necessary component for morphogenesis as embryos arising from TPH1
deficient mice show developmental deficiencies (Côté et al., 2007). The effects of low serotonin levels on pups from mothers lacking the TPH2 gene are as yet unknown. The developmental influence of serotonin is a growing avenue of investigation in the field of depression research that has the possibility to elucidate susceptibility factors and developmental pathways that predispose individuals to MDD.

1.4 Treatment Strategies for Depression

1.4.1 Overview of Antidepressants

Agents developed decades ago, including MAOIs (e.g., selegiline, moclobimide) and TCAs (e.g., imipramine, amitriptyline) remain effective for the treatment of MDD. However, newer classes of drugs, including those that have been specifically designed to target the serotonin system, are now the most widely prescribed antidepressants. Recently developed drug classes include SSRIs (e.g., fluoxetine, fluvoxamine, paroxetine, citalopram), mixed serotonin and norepinephrine reuptake inhibitors (SNRIs; e.g., venlafaxine, duloxetine), norepinephrine reuptake inhibitors (NRIs; e.g., reboxetine, mazindole), and drugs that have sites of action at both serotonin receptors and uptake transporters (e.g., trazodone, nefazodone) (Ghanbari et al.). The ongoing search for novel classes of antidepressants is driven by efforts to decrease side effects and to increase therapeutic effectiveness, partly by reducing the delay in the onset of antidepressant effects but also with hope of treating higher percentages of patients, including those who are resistant to current therapeutic approaches. Compared to SSRIs and SNRIs,
the use of MAOIs and TCAs is associated with more serious adverse side effects and potential toxicity caused by interactions with other transmitter systems, such as cholinergic and histaminergic receptors in the case of the TCAs (Jefferson, 1975; Remick et al., 1989) and hypertensive crisis associated with the effects of dietary biogenic amines in the case of the MAOIs. Reduced side-effect profiles of SSRIs and SNRIs result in better compliance, but not necessarily increased percentages of response and remission compared to older antidepressants.

Most antidepressant drugs have a primary mechanism of action to inhibit the uptake of monoamine neurotransmitters, primarily serotonin, but also norepinephrine and dopamine, or to prevent monoamine catabolism. Thus, all current antidepressants are hypothesized to produce elevated synaptic neurotransmitter levels, which are then available to elicit further homeostatic modifications in downstream targets (Langer and Schoemaker, 1988). In contrast to this prevailing hypothesis of antidepressant mechanism of action, half of the ~50 studies that have been carried out using in vivo microdialysis fail to report increases in extracellular serotonin levels in response to chronic antidepressant administration in mice and rats (Andrews, 2009; Luellen et al., 2010). Electrophysiological studies have shown that acute SSRI treatment leads to activation of autoreceptors to reduce serotonergic firing rates, while chronic SSRI administration results in desensitization of autoreceptors, which allows normalization of the rates of serotonergic firing (Blier et al., 1986; Chaput et al., 1986a; Pineyro and Blier, 1999). These data, along with many other studies, highlight the need to continue investigations into the long-term molecular
mechanisms of antidepressant medications. Moreover, despite the reductions in side effects associated with newer classes of antidepressants, treatment with all existing antidepressants continues to be characterized by two major problems: (1) Prolonged treatment from weeks to months is required to observe clinical effects, and (2) a large fraction of patients suffering from major depression, recently estimated to be as high as 50%, fail to respond or respond poorly to current antidepressant therapies (Rush, 2007; Sinyor et al., 2010).

1.4.2 Mechanisms for Delayed Therapeutic Responses to Antidepressant Treatment

While it is generally accepted that SSRIs and many TCAs have a primary mechanism of action associated with their ability to inhibit SERT, adaptive changes in brain function that develop with continued administration accounting for delayed efficacy are still not well understood, particularly those occurring beyond the serotonin system. However, many studies point to a variety of presynaptic and postsynaptic mechanisms that are evoked following prolonged antidepressant administration. The combination of SSRIs and 5-HT1A antagonists, such as WAY100635, reportedly hastens or potentiates changes in serotonin neurotransmission by inhibiting the negative feedback inherent in 5-HT1A autoreceptor function (Hjorth et al., 2000), and the enhanced serotonergic tone that develops more quickly might be correlated with faster clinical benefits (Richardson-Jones et al., 2010). Alternatively, it might also be that 5-HT1A receptor desensitization is one of the mechanisms responsible for producing therapeutic effects independent of extracellular serotonin levels (Popa et al., 2010). Research in
rodents has established that chronic treatment with SSRIs desensitizes 5-HT1A autoreceptor function (Chaput et al., 1986b; Kreiss and Lucki, 1995; Pineyro and Blier, 1999; Newman et al., 2004). Further investigation into compensatory changes has revealed that while fluoxetine-induced 5-HT1A desensitization affects G-protein function (Hensler, 2002; Pejchal et al., 2002), the same is not true for all SSRIs (Rossi et al., 2008).

Presynaptic 5-HT1A receptors have been studied with regard to antidepressant mechanisms owing to their purported role in regulating serotonin neurotransmission. However, postsynaptic 5-HT1A receptors have also been investigated and changes in these receptors are better correlated with the mechanism of action of TCAs compared to SSRIs (Chaput et al., 1991; Rossi et al., 2006). Given that the majority of serotonin receptors operate through G-protein coupled mechanisms, there are many downstream molecular targets associated with postsynaptic receptors that might be modulated in response to chronic antidepressant administration. For example, the enhanced production of cAMP and the recruitment of the transcription factor cAMP response element-binding protein (CREB) have been suggested to alter levels of brain-derived neurotrophic factor (BDNF) (Tanis et al., 2007). In human suicide victims diagnosed with depression, Dwivedi et al. (2003) reported a decrease in BDNF mRNA and protein levels in postmortem hippocampus and frontal cortex. Regarding peripheral BDNF levels, two recent meta-analyses conclude that serum BDNF protein levels in unmedicated patients with depression are subnormal and recovery of serum BDNF occurs after 8-12 weeks of antidepressant therapy (Brunoni et al., 2008; Sen et al., 2008). These
data suggest that increases in central and/or peripheral BDNF levels might be an important long-term therapeutic modification resulting from antidepressant treatment. Other postsynaptic alterations (e.g., changes in the expression, function, and/or activation of other serotonin receptor subtypes and respective signaling cascades) are also likely to play a role in delayed treatment efficacy.

1.4.3 Non-responders to Current Antidepressants

In an effort to delineate the heterogeneity of antidepressant responsiveness among individuals suffering from depression, pharmacogenetic studies have been carried out seeking relationships between gene polymorphisms and variable responses to antidepressant treatment. Early studies showed promise in associating 5-HTTLPR short ‘s’ allele carriers with poor/slower antidepressant response. However, some recent studies have failed to replicate these associations (for review see Table 1 in (Kraft et al., 2007)). Contradictory findings have been attributed to a number of factors, not the least of which is underpowered sample sizes. Nonetheless, mixed reports continue to emerge from the large Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial involving 2876 patients. For example, Kraft et al. genotyped STAR*D participants for transcriptionally active 5-HTTLPR variants, as well as 8 marker SNPs, but found no significant single locus or haplotype associations with response to citalopram (Kraft et al., 2007). Hu et al. examined associations between 5-HTTLPR genotypes using a triallelic 5-HTTLPR classification and similarly reported a lack of significant association with citalopram response (Hu et al., 2007). However, a weak association
between 5-HTTLPR genotype and remission was reported in white non-Hispanic subjects (Mrazek et al., 2009). Discrepancies arising from the STAR*D studies are hypothesized to involve different definitions of remission, considerations (or lack thereof) of ethnicity, and other subtle differences in study design and interpretation (Garriock and Hamilton, 2009; Mrazek et al., 2009). Overall, findings from pharmacogenetic studies imply that common genetic polymorphisms in SERT appear only modestly associated, at best, with SSRI responses in patients with depression.

Multiple factors, many of which remain unidentified, influence SERT expression (Lipsky et al., 2009). In human lymphoblasts, SERT mRNA levels vary by 5-10-fold even when controlling for triallelic genotype with only 8% of the variance arising from measurement considerations (Hu et al., 2006). In the postmortem brain, individuals segregated by the 5-HTTLPR genotype showed 10-fold differences in SERT mRNA and SERT binding levels in mid brain regions (Little et al., 1998) but not in hippocampus (Naylor et al., 1998). Thus, genome-wide polymorphisms, in addition to those identified in the SERT gene, likely contribute to individual variations in SERT expression. Furthermore, nongenetic influences, e.g., epigenetic factors (Philibert et al., 2008), posttranslational modifications, and plasma membrane localization are expected to contribute to additional variability in SERT expression and function (Blakely et al., 2005; Jayanthi and Ramamoorthy, 2005; Rudnick, 2006; Iceta et al., 2008). Thus, identifying and accounting for all factors in terms of their combined potential to influence SERT and relationships to antidepressant response will require a substantially greater effort.
Nonetheless, variability in the expression and ultimately, the function of SERT, which is the proximal molecular target for SSRIs, is expected to impact treatment effectiveness. Reduced SERT function during treatment might prevent adequate increases in extracellular serotonin levels from developing in response to SSRIs. Moreover, decreased SERT function beginning early in life has the potential to alter the development of pre- and postsynaptic targets (Hariri et al., 2002; Pezawas et al., 2005; Sibille and Lewis, 2006; Murphy and Lesch, 2008) such that the associated circuitry is less responsive to increases in extracellular serotonin caused by SSRIs in adulthood. Preclinical studies in mice and rats with intermediate and complete constitutive reductions in SERT expression support these hypotheses (Holmes et al., 2002b; Kim et al., 2005; Ansorge et al., 2008; Popa et al., 2008).

Additional genetic variations that might contribute to differential responses to antidepressants include those associated with the gene encoding TPH2. A SNP has been identified in rodents whereby the amino acid proline is substituted with arginine (Pro447Arg; C1437G). This TPH2 SNP is associated with a reduction in enzyme activity and central serotonin content (Zhang et al., 2004; Sakowski et al., 2006). Differential responses to the SSRI citalopram in mice were accounted for by allelic variations across mouse strains at this locus in the TPH2 gene (Cervo et al., 2005). However, evidence linking a similar TPH2 SNP to major depression and antidepressant responses in humans (Zhang et al., 2005) has been hindered by the rarity of this SNP and inconsistent identification across sample populations (Zhang et al.; Bicalho et al., 2006; Delorme et al., 2006; Garriock and Hamilton, 2009).
1.5 Future Prospects

From the discovery of serotonin in 1937 until the present, numerous lines of investigation have revolved around identifying the peripheral and central nervous system functions of this evolutionarily ancient signaling molecule but notably, as they pertain to the origins and treatment of human major depressive disorder. Taken on the whole, the studies discussed here, which necessarily represent only a subset of the work carried out in this area of research, strongly suggest that serotonin plays an important role in the etiology and pathophysiology of depression, despite the inability to pinpoint precise serotonergic pathways. Many studies in depressed patients describe alterations in one or more aspects of the serotonin system, with SERT playing a central role. However, the high degree of variability in the results of studies attempting to correlate various serotonergic markers with depression likely reflects the heterogeneous nature of the illness and of human populations. Indeed, as depression is currently diagnosed based on sets of unrelated symptoms, which do not necessarily correspond with underlying biological mechanisms, it remains likely that human studies will continue to be confounded by biological heterogeneity.

The SSRIs and other newer antidepressants continue to represent drugs of choice for the treatment of depression because they improve symptoms for many people while having low side effect profiles and a large safety margin. However, current antidepressant therapies, regardless of drug class, are also associated with a
high rate of inadequate response and medication intolerance. For many patients, it takes months of treatment and multiple trials of different drugs and/or combinations of drugs before a satisfactory outcome is attained. Even the STAR*D trial, the largest of its kind to date, which sought to optimize treatment strategizes, was unable to produce high response rates. Thus, improving therapeutic approaches remains a high priority in depression research. Elucidating the downstream mechanisms associated with chronic administration of current antidepressants will lead to a better understanding of the multiple etiopathological origins of depression and to the identification of new targets for the development of future antidepressant therapies having improved efficacies and side effect profiles.

Although a great deal of knowledge has been gained in the last decade related to the neurobiology of depression, our understanding of underlying causes, pathophysiology, current antidepressant mechanisms are still lacking. The search for potential biomarkers to guide treatment selection is a promising new avenue of research that will help to improve the clinical efficacy of current and future drugs used to treat depression. Investigations focused on biomarkers of brain structure and function have yielded fruitful results in terms of making associations with features of depression (e.g., reduced gray matter in the hippocampus and anterior cingulate cortex) and for tracking early treatment responsiveness (Leuchter et al., 2009), but they are not predictive such that they might guide individualized therapeutic approaches (McKinnon et al., 2009; Leuchter et al., 2010). Furthermore, gene expression studies have identified candidate genes expressed in the amygdala that might be used as biomarkers for depression (Sibille et al., 2009). Alternately,
peripheral blood cells such as lymphocytes and platelets that express serotonergic components whose expression and function have been suggested to be correlated with serotonergic function in the brain have the potential to allow for genetic screening (Blakely, 2001) and to investigate the effects of drugs *ex vivo*. 
Figures

Tryptophan

tryptophan hydroxylase (TPH)

5-Hydroxytryptophan (5-HTP)

aromatic amino acid decarboxylase

Serotonin (5-HT)

monoamine oxidase (MAO) aldehyde dehydrogenase

5-Hydroxyindoleacetic acid (5-HIAA)
**Figure 1-1: Key Components of the Serotonergic System.** Serotonin is synthesized from the dietary amino acid L-tryptophan in a two-step process. Tryptophan is first transported into the brain via the large neutral amino acid transporter (LNAAT), where it is converted to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH). In the second step of the process, 5-HTP is converted to 5-hydroxytryptamine (5-HT, serotonin) by amino acid decarboxylase (AADC). Newly synthesized serotonin is transported into synaptic vesicles within presynaptic neurons via vesicle monoamine transporters (VMATs). Upon neuronal activation, serotonin is released from vesicles into the extracellular space where it interacts with a number of different serotonin receptors that are located both pre- and postsynaptically. The actions of serotonin are terminated by (1) transport back into presynaptic neurons by the serotonin transporter (SERT) where serotonin can be repackaged into vesicles or (2) breakdown into 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO).
Figure 1-2: Serotonin Projections in the Human Brain. Anatomically, serotonin cell bodies are clustered in small groups called nuclei in the brain stem. From the rostral raphe nuclei arise projections to many areas of the cerebral cortex and midbrain, whereas projections from caudal raphe nuclei extend to the spinal cord and portions of the cerebellum.
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Chapter 2

Adolescent Anxiety- and Depression-like Behaviors in Mouse Models of Early Serotonin Transporter Disruption

2.1 Introduction

Serotonin and its plasma membrane transporter exert essential morphogenic influences during central nervous system development (Gaspar et al., 2003; Trowbridge et al., 2011). In rodents, serotonin transporter (SERT) expression emerges in serotonergic neurons during early embryonic development (embryonic day 12) and achieves near adult levels by the third week of postnatal life (Galineau et al., 2004; Narboux-Nème et al., 2008). Serotonin transporter expression also occurs in nonserotonergic thalamo-cortical neurons between embryonic day 15 and the third week of postnatal life, and this transient expression of SERT is coupled primarily to glutamatergic neurons involved in higher order somatosensory mapping (Lebrand et al., 1996; Esaki et al., 2005; Vitalis et al., 2007). Similar patterns in the serotonin system are present in developing human embryos with serotonin appearing early (first trimester) and brief periods of SERT expression occurring in nonserotonergic regions (Verney et al., 2002). Thus, the developmental trajectory of the serotonin system is controlled temporally and spatially during
sensitive early time frames (Hendricks et al., 2003; Savelieva et al., 2008; Alenina et al., 2009). Furthermore, variations in developmental patterns of SERT expression and function have been shown to influence adult neuronal circuitry and behavior.

Administration of serotonin reuptake inhibiting antidepressants (SRIs) to rodents during early postnatal development, roughly corresponding to the third trimester through 2 years of age in humans, produces an adult phenotype associated with alterations in anhedonia (Popa et al., 2008), behavioral despair (Popa et al., 2008), exploratory behavior (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008), sexual activity (Vogel et al., 1990), rapid eye movement (REM) sleep (Vogel et al., 1990), and learning (Bhagya et al., 2008). In humans, antidepressant administration during pregnancy produces potential risks associated with the ability of these drugs to cross the placenta; high levels of antidepressants have been detected in amniotic fluid (Hostetter et al., 2000), and infants display withdrawal effects (Nordeng et al., 2001) and delayed motor coordination (Casper et al., 2003). Moreover, children exposed to SRIs in utero exhibit increased internalizing behaviors and altered HPA axis function indicating potential vulnerability to altered mood states (Oberlander et al., 2008; Oberlander et al., 2009; Oberlander et al., 2010).

In addition to the potential effects of exposure to SERT inhibiting antidepressants early in life, human gene variants that influence the expression and function of SERT including the serotonin-transporter linked polymorphic region (5-HTTLPR) and single nucleotide polymorphisms (SNPs) associated with this region
(Hu et al., 2006; Wendland et al., 2006; Wendland et al., 2008) have been shown to influence a myriad of functions including alterations in anxiety-related personality traits (Canli and Lesch, 2007; Murphy and Lesch, 2008). Rodent models of reduced SERT deficiency have been generated (Murphy et al., 2008; Kalueff et al., 2010), and mice and rats lacking SERT have consistently reported to display phenotypes predominately associated with increased anxiety-related behaviors and stress responsiveness (Holmes et al., 2003b; Olivier et al., 2008; Kalueff et al., 2010). Nonetheless, correlations between the 5-HTTLPR and the expression and function of SERT continue to be debated (Lim et al., 2006; Parsey et al., 2006). Contradictory findings on association of the 5-HTTLPR and SERT expression and function together with developmentally sensitive timeframes for SERT (Sibille and Lewis, 2006) challenge the idea of persistent changes in SERT in adulthood. However, evidence that SERT function increases linearly between 2 and 10 months of age (Daws et al., 2007) along with functional changes in SERT correlated to the 5-HTTLPR in adult rhesus macaques (Singh et al., 2010) indicate potential influential effects of serotonin persist later in life.

Given the complex nature of the developmental influence of serotonin, we questioned whether exposure to SRIs limited to the first few weeks of postnatal life results in a behavioral phenotype that fully recapitulates that resulting from constitutive SERT deficiency. We administered the SRI S-(+)-citalopram (S-CIT) from PD5-21. Mice were tested on common measures of anxiety-, depression-, and locomotor-related behaviors at two months of age, a critical period immediately following periadolescent development (Spear and Brake, 1983; Klein et al., 2003),
which has not been thoroughly explored previously. We investigated gender-related effects by assessing behavior in female vs male animals. We further investigated functional responses of presynaptic 5-HT1A receptors because this is a critical receptor subtype controlling serotonergic neurotransmission. Behavioral and pharmacologic responses were directly compared to those of two-month old female and male SERT-deficient mice in the same background as S-CIT-treated mice. Our results highlight important differences emanating from these two models of early SERT disruption as evidenced by opposing anxiety-like behaviors and sensitivity of 5-HT1A function in late adolescent mice.

2.2 Materials and Methods

2.2.1 Animals and treatment

Animals were generated by breeding mice lacking one functional SERT allele (SERT+/- mice) to produce SERT+/-, SERT+/-, and SERT-/ littermate mice. The mice were on a congenic CD-1 × 129SvEv background (Bengel et al., 1998; Mathews et al., 2004; Perez and Andrews, 2005). These mice were housed and tested at facilities located at the University of California, Los Angeles. Mice with constitutive reductions in SERT on a CD-1 × 129SvEv background show robust increases in anxiety-related behavior in the elevated plus maze that are stable into adulthood and independent of test site (Fig. A-2, Appendix A). Behavior in this background strain of SERT-deficient mice is also highly similar to SERT-deficient mice on a
C57BL/6 background, which have been evaluated by different groups at different locations (Holmes et al., 2003b; Joeyen-Waldorf et al., 2009).

For the postnatal antidepressant treatment portion of the study, SERT+/+ mice were bred for one generation to produce animals on the same genetic background as SERT deficient mice. These mice, including unhandled SERT+/+ controls, were generated, housed, and tested at the Pennsylvania State University. Mice at both testing locations were maintained on a 12h-light/dark cycle with *ad libitum* access to food and water. Procedures involving animals were approved by the University of California, Los Angeles Chancellor’s Animal Research Committee or the Pennsylvania State University Institutional Animal Care and Use Committee, and were in accordance with the National Institutes of Health Animal Care and Use Guidelines.

Wildtype neonates designated for postnatal antidepressant treatment were weighed daily (Fig. 2-2) beginning on postnatal day (P)5 and ending on P21. Following weighing, mice were injected subcutaneously (sc) with saline or S-(+)-citalopram oxalate (10 mg/kg/d calculated as the free base and dissolved in sterile endotoxin-free saline at 1 mg/mL; Anawa Trading, Wangen, Switzerland). Injections occurred 1 h prior to the beginning of the dark cycle. This regimen of S-CIT has been shown to alter emotion-related behaviors in adult mice (Popa et al., 2008). Group sizes were *N*=14 for female saline-treated, *N*=16 for female S-CIT-treated, *N*=10 for male saline-treated, and *N*=8 for male S-CIT-treated mice. A separate group of mice from SERT+/+ pairings (*N*=8 female and *N*=10 male) was not
injected or handled except for routine cage changes by the animal care staff.

Unhandled SERT+/+ mice were tested at the same time as S-CIT- and saline-treated SERT+/+ mice to determine the effects of postnatal injection procedures on behavior. Nests containing all pups were briefly separated from mothers (~5 min) for the injection procedure and were immediately returned to mothers in their homecage afterward. Treatments were randomized across pups within a litter. During the maternal separation, pup body temperatures were maintained by during being placed in containers of warmed dry rice.

Mice from SERT+/- pairings (N=11-17 per genotype for females and N=15-24 per genotype for males) underwent the same behavior-testing paradigm as postnatal antidepressant-treated mice. However, offspring of SERT+/- parents were neither handled nor injected as mice deficient in SERT display heightened sensitivity to stressful stimuli, including a single saline injection (Li et al., 1999a; Tjurmina et al., 2002). Moreover, previous studies on emotion-related behavior in SERT-deficient mice have not involved postnatal injections.

Postnatal antidepressant-treated and SERT-deficient mice were tested on measures of anxiety-, depression-, and locomotor-related behaviors starting at P50, a critical period encompassing late adolescence (Spear and Brake, 1983; Klein et al., 2003). Behavior testing was carried out during the dark phase with dim light. All behavior tests were performed by S. C. Altieri at both study locations. At least three days separated each behavior test, which were conducted in the order presented below.
2.2.2 Elevated plus maze

Anxiety-like and exploratory behaviors were measured using the elevated plus maze (Walf and Frye, 2007). A single maze raised 38.5 cm from the floor was used throughout the study at both testing locations. The maze was constructed of two opposing open arms (30 cm × 5 cm) with a 0.5 cm lip around the edges to prevent animals from falling (Lapiz-Bluhm et al., 2008), two opposing enclosed arms (30 cm × 5 cm × 15 cm), and a center platform (5 cm × 5 cm). The walls of the closed arms were made of clear Plexiglas to allow for even lighting across the apparatus. One continuous piece of black Plexiglas lined the floor of the maze. Mice were placed onto the apparatus facing a closed arm. Mice were permitted to freely explore the maze for 5 min and behaviors were videotaped. In between each mouse, 70% ethanol was used to thoroughly clean the apparatus. Scoring was completed by a trained observer blind to postnatal treatment or genotype. Anxiety parameters including latency to enter an open arm and the time spent and number of entries (four-paw) into an open arm were measured. Additionally, locomotor behavior was assessed by determining total arm entries (open + closed). Exploratory behaviors including rears and head dips were also scored. Times in the open/closed arms were calculated as percents of total arm time excluding time spent in the center platform.

2.2.3 Open field test

Locomotor activity was analyzed using Digiscan Activity Monitors (Omnitech Electronics, Columbus, OH) as previously described (Unger et al., 2006) or a 3-point
digital video tracking system and Viewer® software (Biobserver, Bonn, Germany). Digiscan Monitors (40 cm × 40 cm × 30 cm) containing photobeams and receptors were housed in clear Plexiglass boxes. The center of the open field was defined as a 10 cm × 10 cm area. Mice receiving postnatal treatment with S-CIT or saline and respective unhandled SERT+/+ controls were tested in these apparatuses at Penn State University. The open field arenas coupled to the video tracking system were 50 cm × 50 cm × 40 cm and used for testing SERT-deficient mice at UCLA. Here, the center region was defined by a 22 cm × 22 cm square. Center areas were defined differently due to differences in the ways activity is measured between the two apparatuses. In the Digiscan Activity Monitors, animals are considered to have entered the center area once their noses cause a beam break at the perimeter of the center area. In the video tracking system, center parameters were recorded when the midpoint of the body crossed into the central region. Thus, the size of the center area was adjusted for the latter apparatuses to provide measurements of center area activity that were roughly equal to those measured in the Digiscan Activity Monitors. In all cases, mice were placed in a corner of the open field and allowed to freely explore for 1 h. Behaviors parameters to assess locomotor activity and anxiety-like behavior including the total distance traveled (cm), the ratio of center distance (cm) to total distance and time spent exploring the center region was analyzed. Data were collected in 5-min noncumulative bins. Open field arenas were thoroughly cleaned with 70% ethanol between mice.
2.2.4 Forced swim test

The forced swim test was used to measure depression-like behaviors (behavioral despair) using previously developed methods with some modifications (Porsolt et al., 1977; Shen et al., 2010). Mice were gently placed in a clear glass cylinder (27 cm high × 13 cm diameter) filled with water (RT 22 ± 2°C; depth of 15 cm). Videotaped 6 min swim sessions were conducted. Following the swim session, mice were placed in a warmed environment until their fur was completely dried and subsequently returned to their homecages. A retest was performed 24 h following the first swim session (Porsolt et al., 1977; Borsini et al., 1989) as repeat exposure has been shown to alter depression-like behavior in certain genetic backgrounds of SERT-deficient mice (Carroll et al., 2007). The glass cylinders were thoroughly cleaned with 70% ethanol and filled with fresh water in between each mouse. A trained observer blind to postnatal treatment or genotype scored mice for immobility during the last 4 min of each testing period.

2.2.5 Presynaptic 5-HT1A receptor function

A 5-HT1A agonist induced hypothermia using 8-OH-DPAT was used to study potential alterations in 5-HT1A autoreceptors responsiveness based on its role in maintaining serotonergic tone (Richer et al., 2002) and its association with mechanisms of antidepressant efficacy (Sinton and Fallon, 1988; Hjorth and Auerbach, 1994; Holmes et al., 2003c; Rossi et al., 2008a; Rossi et al., 2008b; Popa et al., 2010; Richardson-Jones et al., 2010). Previous reports have shown that 8-OH-DPAT-induced hypothermia is dependent on presynaptic 5-HT1A autoreceptors
(Martin et al., 1992; Li et al., 1999b; Richardson-Jones et al., 2011). Mice were injected with saline or the 5-HT1A receptor agonist 8-hydroxy-N,N-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT; 0.1 mg/kg calculated as the free base and dissolved in saline at 0.1 mg/ml; Sigma-RBI, Natick, MA) (Li et al., 1999b). Adult body weights were recorded at this time (Fig. 2-3). Mice were habituated to a rectal temperature probe by making one measurement 20 min prior to injection (Physitemp Instruments, Inc., Clifton, NJ). Baseline temperatures were recorded 10 min prior to injection. Core body temperatures were recorded 20 min after injection to determine maximal hypothermic responses (Li et al., 1999b) and again 60 min after injection.

2.2.6 Statistics

Elevated plus maze and forced swim test data were analyzed by two-way analysis of variance (ANOVA) with sex and postnatal treatment or genotype as the independent variables. Unhandled and saline-treated SERT+/- mice were compared by Student’s t-tests and pre-planned contrasts. No differences between these groups occurred for any variable using either method of analysis; therefore, data were combined and compared to S-CIT-treated mice by t-tests. Saline-treated mice were also compared to S-CIT-treated mice via pre-planned contrasts. Individual group comparisons for SERT-deficient mice were by pre-planned contrasts. Open field data were analyzed by 3-way ANOVA with sex and postnatal treatment or genotype as the between-subjects variables and time as the within-subjects variable. Temperature data were analyzed by 3-way ANOVA with pharmacologic challenge.
and postnatal drug treatment or genotype as the between-subjects variables and repeated measures on time. Individual groups were compared using least squares means for *a priori* hypotheses. For all data, interaction terms were examined first (Hatcher and Stepanski, 1994). Significant interaction terms are reported in the text and followed by analyses of simple effects. Nonsignificant interaction terms are generally not reported and main effects analyses follow. Statistical analyses were carried out using GraphPad Prism (GraphPad Inc., La Jolla, CA) or SAS Statistical Software (SAS Institute Inc., Cary, NC). All data are expressed as means ± standard errors (SEMs) with *P*<0.05 considered statistically significant.

### 2.3 Results

#### 2.3.1 Postnatal vs constitutive SERT disruption alter anxiety-like behavior in different directions

Wildtype (SERT+/+) mice treated with S-CIT or saline from P5-P21 and unhandled SERT+/+ mice on the same genetic background were compared on anxiety-related measures in the elevated plus maze. For percent open arm time, the main effect of postnatal treatment [F(2,60)=8.3, *P*<0.001] but not sex was significant. Both female and male S-CIT-treated mice displayed significant increases in percent open arm time compared to control groups (Fig. 2-4A). Mice with constitutive reductions in SERT showed significant changes with respect to genotype [F(2,91)=4.79, *P*<0.05] but not sex for percent open arm time. Here, individual group comparisons indicated that male SERT-/- mice spent less time
exploring the open arms of the maze compared to male SERT+/+ mice (Fig. 2-4B). A similar nonsignificant direction of change in percent open arm time was observed for female SERT-deficient mice.

Opposing changes in anxiety-like behavior were also observed for percent open arm entries. For postnatal antidepressant-treated mice, a significant effect of treatment \[F(2,60)=5.71, P<0.01\] but not sex was observed. Both female and male mice administered postnatal S-CIT made more entries into the open arms compared to controls (Fig. 2-4C). A main effect of genotype \[F(2,91)=4.15, P<0.05\] but not sex occurred for SERT-deficient mice. In contrast to postnatal antidepressant treatment, fewer open arm entries were observed in male SERT-/- mice compared to male SERT+/+ mice (Fig. 2-4D).

Mice deficient in SERT but not postnatal S-CIT-treated mice differed on latency to first open arm entry. For SERT-deficient mice, there was a significant effect of genotype \[F(2,91)=6.28, P<0.01\] but not sex. Individual group comparisons indicated that male SERT-/- mice had longer latencies to first open arm entry compared to male SERT+/+ mice (Table 2-1). Thus, prolonged open arm latencies further support an association between an anxiogenic phenotype and constitutive SERT deficiency. No changes in total arm entries were observed in either experimental cohort indicating that differences in anxiety-like behavior were not associated with changes in overall activity in the maze (Table 2-1).

Exploratory measures in the elevated plus maze indicated by numbers of rears and dips were also evaluated. Rears were not different across groups for
postnatal SERT inhibition or genetic SERT-deficiency (Table 2-1). Dips, whereby mice extend their heads below the level of an open arm, were not different after postnatal S-CIT treatment. However, a main effect of genotype [F(2,91)=4.80, P<0.05] with regard to SERT-deficient mice was found. Female SERT-/- mice made fewer head dips compared to female SERT+/+ mice (Table 2-1). Additional information on elevated plus maze data appears in Table 2-1.

2.3.2 Constitutive SERT deficiency but not transient postnatal SERT inhibition results in reduced open field activity

The open field test was used to assess locomotor activity and anxiety-like behavior by monitoring activity in the total field vs the center of the arena, respectively. Three-way interactions considering sex, time, and postnatal treatment or genotype were not significant for total distances traveled, center/total distance ratios, and center times. Data sets were broken out by sex and analyzed further.

For total distance traveled, postnatal antidepressant treatment was associated with significant main effects of time for female [F(11,407)=76; P<0.001] and male [F(11,264)=92; P<0.001] mice indicative of normal habituation to the open field (Fig. 2-5A,B). No significant effects of postnatal treatment were observed for either sex. For SERT deficient mice, a significant main effect of time was found for female mice (Fig. 2-5C; [F(11,352)=54, P<0.01]), while a significant time × genotype interaction occurred for male mice [F(22,484)=3.5; P<0.001] signifying that the effects of time were not the same across male genotypes. For male SERT-deficient mice, locomotor activity differed with respect to genotype during the first
20 min of the test, whereby male SERT-/− mice covered less distance than male SERT+/+ mice (Fig. 2-5D).

The ratio of center/total distance traveled was analyzed as a measure of anxiety-related behavior so as to account for changes in total distance. For example, if center and total distances are proportionally decreased, no change in the ratio occurs. This is interpreted as a global decrease in activity without a specific change in center avoidance. For postnatal antidepressant-treated mice, the main effects of time were significant with respect to center/total distance ratios for female [F(11,275)=3.8; \( P<0.001 \)] and male [F(11,407)=9.7; \( P<0.001 \)] mice (Fig. 2-6A,B), however, there were no significant main effects of postnatal treatment. For female SERT-deficient mice, there was a significant effect of time [F(11,352) = 2.1, \( P<0.05 \)] but not genotype for center/total distance ratios (Fig. 2-6C). By contrast, both the main effects of time [F(11,495)=2.8; \( P<0.01 \) and genotype [F(2,45)=3.9; \( P<0.05 \)] were significant for male SERT-deficient mice. Individual group comparisons indicated that male SERT-/- mice showed lower ratios of center/total distance traveled compared to male SERT+/+ mice at the 5-, 10-, 15-, 20-, 40-, and 45-min intervals (Fig. 2-6D). Male SERT+/− mice had lower center/total distance ratios at the 40- and 45-min intervals.

We also analyzed time spent in the center of the open field as another measure of anxiety-related behavior. Postnatal antidepressant treatment was not associated with significant effects of time or treatment in female or male mice. In SERT-deficient mice, no changes were observed for female mice while significant
main effects of time \( F(11,484)=2.3, P<0.01 \) and genotype \( F(2,44)=4.4, P<0.05 \) were found for male mice. Individual group comparisons revealed that male SERT-/- mice spent less time in the center of the open field compared to male SERT+/+ mice at the 5-, 10-, 15-, 20-, 40-, and 45-min intervals (data not shown). Male SERT+/− mice spent less time in the center compared to SERT+/+ mice at the 20- and 40-min intervals. Overall, mice of both sexes treated postnatally with S-CIT displayed few changes in locomotor and anxiety-related behavior in the open field test. By contrast, decreased activity and increased center avoidance were observed in male but not female SERT-deficient mice.

2.3.3 SERT-deficient mice but not postnatal antidepressant-treated mice show increased depression-related behavior during late adolescence

Postnatal antidepressant treatment had no effect on time spent immobile during the first (Fig. 2-7A) or second (Table 2-1) trials of the forced swim test. By contrast, a significant genotype × sex interaction was found for SERT-deficient mice \( F(2,90)=3.2, P<0.05 \). Male SERT-deficient mice showed a significant effect of genotype \( F(2,39)=6.2, P<0.01 \), whereby mice lacking SERT spent more time immobile than wildtype counterparts (Fig. 2-7B). Main effects of genotype \( F(2,90)=5.1, P<0.01 \) and sex \( F(2,90)=6.0, P<0.05 \) were observed in mice with constitutive SERT deficiency during the second exposure to the forced swim test, however, significant differences were not detected with respect to individual group comparisons (Table 2-1). Together, these results suggest that some aspects of depression-like behavior appear early in life relative to constitutive SERT deficiency,
particularly in male mice. Similar changes are not observed in late adolescence in mice experiencing transient postnatal SERT inhibition.

2.3.4 Hypothermic responses to presynaptic 5-HT1A-receptor stimulation differ between postnatal SSRI and SERT deficiency models

Reductions in core body temperature after 8-OH-DPAT were used to investigate in vivo responsiveness of 5-HT1A autoreceptors. Interactions between time × pharmacologic challenge were significant in female [F(2,68)=14; P<0.001] and male [F(2,34)=5.3; P<0.01] postnatal antidepressant-treated mice reflecting the hypothermic effect of 8-OH-DPAT vs saline-injection. By contrast, interactions between postnatal drug treatment × pharmacologic challenge were not significant for female and male mice indicating that 8-OH-DPAT produced similar levels of hypothermia across postnatal treatment groups. Significant reductions in temperature occurred 20 min after 8-OH-DPAT in female (Fig. 2-8A) and male (Fig. 2-8B) saline-treated and S-CIT-treated mice during late adolescence.

Conversely, constitutive SERT deficiency resulted in differential hypothermic responses. Significant time × genotype × pharmacologic challenge interaction terms were present for female [F(4,70)=8.3; P<0.001] and male [F(4,98)=8.6; P<0.001] SERT-deficient mice. Analyses at individual time-points revealed significant genotype × pharmacologic challenge interactions 20 min post-8-OH-DPAT for female [F(2,35)=13, P<0.001] and male [F(2,49)=8.7, P<0.001] mice. Body temperature was significantly reduced in female and male SERT+/+ and SERT+/-
mice 20 min after 8-OH-DPAT (Fig. 2-8C,D). By contrast, SERT-/- mice of both sexes showed no changes in body temperature in response to 8-OH-DPAT.

2.4 Discussion

Mice exposed to the serotonin reuptake inhibiting antidepressant S-CIT during early postnatal life display reduced anxiety-related behavior during late adolescence that can be interpreted as increased risk-taking behavior (Macri et al., 2002). No changes in depression-related or exploratory behaviors were observed in late adolescent mice treated postnatally with S-CIT. This finding stands in contrast to the increased anxiety-like phenotype observed in SERT-deficient mice of both sexes. Male SERT-deficient mice also displayed increased depression-related behavior and decreased exploratory behavior. These models of SERT disruption further differed with respect to 5-HT1A autoreceptor responsiveness. Hypothermic responses to presynaptic 5-HT1A-receptor stimulation were absent in mice lacking SERT yet were intact after transient postnatal SERT inhibition. Together, these findings support the hypothesis that disrupted SERT function limited to the early postnatal period does not replicate important aspects of the behavioral phenotype associated with genetic reductions in SERT expression.

Serotonin and the SERT exert essential morphogenic influences during development (Gaspar et al., 2003; Murphy and Lesch, 2008; Trowbridge et al., 2011). In rodents, Sert expression emerges in serotonergic neurons during mid-embryonic development (day 12) and nears adult levels by the third postnatal week.
Nonserotonergic thalamo-cortical glutamatergic neurons involved in somatosensory mapping also express SERT between embryonic day 15 and the second postnatal week (Lebrand et al., 1996; Esaki et al., 2005; Vitalis et al., 2007; Homberg et al., 2010). Similar patterns are present in developing human embryos with serotonin appearing during the first trimester and transient periods of SERT expression occurring in nonserotonergic regions (Verney et al., 2002; Homberg et al., 2010).

Studies in rodents dating back as early 1980’s have identified a critical time frame in development when drugs that inhibit SERT generate lasting changes in emotion-related behavior (Mirmiran et al., 1981; Hilakivi and Hilakivi, 1987; Fernandez-Pardal and Hilakivi, 1989; Vogel et al., 1990; Velazquez-Moctezuma and Diaz Ruiz, 1992; Frank and Heller, 1997; Ansorge et al., 2004; Maciag et al., 2006b; Maciag et al., 2006a; Ansorge et al., 2008; Bhagya et al., 2008; Popa et al., 2008; Karpova et al., 2009; Harris et al., 2011). None of these studies focused specifically on behavior in adolescent mice. Similar to humans, adolescence is a period in mice when changes in anxiety and risk-seeking behaviors in novel environments occur (Imhof et al., 1993; Macri et al., 2002; Laviola et al., 2003). In one recent report, male mice on a 129S6/SvEv background were administered postnatal fluoxetine and tested for changes in locomotor and anxiety-related behavior at 2-months of age in the open field test (Ansorge et al., 2008). Behavior was not altered leading to the conclusion that changes in emotion-related behaviors arising from postnatal SERT disruption do not manifest until adulthood, i.e., 3-months of age. Conversely, we observed a reduction in anxiety-related behavior in late adolescent female and male
mice administered postnatal S-CIT indicated by increased open arm parameters in the elevated plus maze. Discrepancies between these findings might arise from differences in genetic backgrounds, SSRIs, and the use of specific behavior tests. For example, Ansorge et al. (2008) only tested late adolescent mice in the open field test, where we also did not observe changes in behavior. The current findings further show that SERT-deficient mice exhibit increased anxiety-related behavior as early as late adolescence (P50) with phenotypic changes most pronounced in male mice at this age. Previous reports indicate that anxiety-like behavior becomes more pronounced in females at later ages (Joeyen-Waldorf et al., 2009) and this developmental trajectory might underlie slightly different behavioral phenotypes between late adolescent female and male SERT-/- mice.

Anxiety-related behavior in rodents is frequently assessed in the elevated plus maze with differences indicated by changes in open arm time and/or numbers of open arm entries (Walf and Frye, 2007). Adult mice on a C57BL/6 background with reduced SERT expression consistently display reductions in open arm time and open arm entries (Holmes et al., 2003b; Holmes et al., 2003a; Kalueff et al., 2007; Joeyen-Waldorf et al., 2009). Moreover, we observe increased anxiety-related behavior in male SERT-deficient mice in a congenic CD-1 x 129SvEv background during late adolescence (and adulthood; Fig. A-2, Appendix A) indicated by the same parameters in the elevated plus maze. By contrast, changes in anxiety-related parameters in the elevated plus maze most often observed in SERT-deficient mice are not reported in mice treated postnatally with SSRIs and tested as adults (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008; Karpova et al., 2009;
Harris et al., 2011) (Fig. 2-1). The interpretation of elevated plus maze parameters, i.e., open arm parameters vs total arm exploration, contribute to different conclusions regarding the similarity or lack thereof between postnatal and constitutive SERT disruption models (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008).

In contrast to increased depression-like behavior in adult animals (~P90) in the forced swim test (Popa et al., 2008), we did not observe changes in immobility in postnatal antidepressant-treated mice during late adolescence (P56), suggesting that this phenotype might not develop until adulthood (Ansorge et al., 2008). However, we did observe increases in immobility times in late adolescent male SERT-/- mice on a congenic CD-1 x 129SvEv background. Reports on changes in depression-related behaviors in adult SERT-/- mice are inconsistent (Kalueff et al., 2006). One group showed that SERT-/- mice on a 129S6/SvEv background exhibited increased immobility in the forced swim test (Lira et al., 2003) whereas another group examining SERT-/- mice on a C57BL/6J background showed similar effects of increased immobility in the forced swim test only during a repeated trial (Carroll et al., 2007). Additionally, others have observed that C57BL/6J SERT-deficient mice show no change in forced swim test behavior (Perona et al., 2008). Comparing strains is not straightforward as differences have been observed in baseline emotional behaviors between these two strains of SERT-deficient mice (Holmes et al., 2002; Zhao et al., 2006).
We hypothesized that differences in serotonergic circuitry involving 5-HT1A autoreceptors underlie contrasting phenotypes arising from postnatal SERT inhibition vs constitutive SERT deficiency. Previous work has consistently demonstrated that injection of 0.1 mg/kg 8-OH-DPAT results in reductions in body temperature in adult SERT+/+ and SERT+-/- mice but not SERT-/- mice supporting the idea that these receptors are desensitized in SERT-/- mice (Li et al., 1999b; Li et al., 2000; Holmes et al., 2003b). However, with respect to postnatal S-CIT exposure, hypothermic responses following a higher dose of 8-OH-DPAT (0.4 mg/kg) in adult mice were potentiated (Popa et al., 2008). In the present study, we did not observe increased hypothermic responses after postnatal S-CIT treatment in late adolescent mice, which might be due to the lower dose of 8-OH-DPAT used here. However, Popa et al. (2008) studied adult mice; 5-HT1A-autoreceptor-hypersensitivity might develop at later ages in mice exposed to SSRIss postnatally. Nonetheless, we hypothesize that the presynaptic molecular mechanisms driving differential phenotypes in adolescent mice are not the same.

While we kept protocols, equipment, and personnel similar across the two cohorts of mice studied, we cannot rule out potential influence of differences in animal room environments and open field equipment at the different institutions where mice were studied (Crabbe et al., 1999). Notably, complete standardization even within a test location is unattainable, e.g. differences occur in facility caretakers, social rank among group housed mice, and seasonal variability affecting barometric pressure and chow composition. Overly tight control within testing environments has been suggested to lead to variability in results reported by
different groups and to undue focus on volatile and irreproducible phenotypes (Richter et al., 2011). For behavior studies in mice with relevance to human behavior, it is advantageous to characterize generalizable and robust phenotypes and to interpret results from a battery of tests (Ramos, 2008; Guilloux et al., 2011).

Here, postnatal antidepressant-treated and SERT-deficient mice were compared statistically only to their own unhandled SERT+/+ control groups born to dams of the same genotype. It is in this light that we find differences in the relative directions of change across cohorts compelling. Ongoing studies by us comparing postnatal antidepressant treatment with constitutive decreases in SERT expression in mid- and late adulthood support the current interpretation. In summary, the present findings suggest that changes in anxiety- and depression-like behaviors and 5-HT1A autoreceptor function appearing as early as late adolescence in response to transient postnatal SERT inhibition are not representative of the most prominent phenotypic changes arising from constitutive reductions in SERT expression.
Expression of serotonin transcription factors, e.g. Pet1, Lmx1b, and GATA3

Serotonergic phenotype markers and SERT expression

Transient SERT Expression

Antidepressant Administration

Phenotype After SRI Administration

Developmental Patterns of SERT

Expression of serotonin transcription factors, e.g. Pet1, Lmx1b, and GATA3

Serotonergic phenotype markers and SERT expression

Transient SERT Expression

Antidepressant Administration

Phenotype After SRI Administration

Developmental Patterns of SERT
Figure 2-1: Serotonin transporter timeline. Key time frames in the developmental ontogeny of SERT (upper); phenotypes reported after transient SERT inhibition using SSRIs in mice (lower). Serotonin begins to be synthesized early in development (E10.5) (Hendricks et al., 1999) and is accompanied thereafter by SERT expression (E12) (Galineau et al., 2004; Narboux-Nême et al., 2008). Serotonin transporters are also expressed for a brief period in nonserotonergic cells (E15 through ~P14) (Lauder et al., 1988; Lebrand et al., 1996; Esaki et al., 2005; Vitalis et al., 2007). Although the expression of SERT reaches adult levels by weaning (P21), SERT function continues to increase throughout adulthood (2-10 months) (Daws et al., 2007). Exploration in the elevated plus maze (EPM) is assessed by total arm entries and number of rears, while exploration in the open field test (OFT) is defined by total distance traveled and ambulatory time. Abbreviations are as follows: Embryonic day (E); postnatal day (P); shock escape (SE); novelty-induced hypophagia (NIH); increased (↑), decreased (↓), no change (←→), and sexes analyzed together (♀/♂).
Figure 2-2: Postnatal body weight during treatment regimen. Body weights were recorded daily from postnatal day (P)5 through P21 during the treatment paradigm where female (a) and male (b) mice received either saline or S-citalopram (S-CIT). There was a significant main effect of S-CIT ($P<0.01$) with weights being lower in S-CIT-treated female pups beginning around PD11-16 (main effect of drug $P=0.1$).
Figure 2-3: Body weight in late adolescent postnatal SRI-treated and SERT-deficient mice. Body weights were recorded on P60 in (a) female and (b) male mice to assess changes in body weight during late adolescence resulting from early postnatal SRI administration or serotonin transporter deletion. No changes in body weight occurred with respect to postnatal treatment or genotype for either sex. Data are expressed as mean ± SEM.
Figure 2-4: Behavior in the elevated plus maze changes in opposing directions in postnatal antidepressant-treated mice compared to SERT-deficient mice. (a,b) Percent time spent exploring the open arms of the elevated plus maze is shown for female and male postnatal antidepressant-treated mice and SERT-deficient mice. Wildtype (SERT+/+) mice treated postnatally with S-CIT spent significantly more time in the open arms compared to same-sex saline-treated and unhandled mice, whereas male SERT-/- mice spent significantly less time exploring the open arms compared to SERT+/- mice. (c,d) Percent entries into the open arms are shown for postnatal antidepressant-treated and SERT-deficient mice. Mice treated with S-CIT made more entries into the open arms than their control counterparts. By contrast, male SERT-/- mice made fewer entries into the open arms compared to male SERT+/- mice. Values are means ± SEMs. †P<0.05 and ††P<0.01 with respect to same-sex combined control groups, i.e., unhandled and saline-treated; #P<0.05 vs same-sex saline-treated mice; **P<0.01 vs same-sex SERT+/+ mice.
Figure 2-5: Male SERT-deficient mice exhibit hypolocomotion in the open field during late adolescence. (a,b) Total distance traveled (cm) in the open field over the course of 1 h is shown for postnatal S-CIT-treated mice. No differences in activity levels were detected in female or male mice receiving postnatal S-CIT compared to saline-treated and unhandled SERT+/- groups. (c,d) Total distance traveled (cm) in the open field is shown for SERT-deficient mice. Male SERT-deficient mice exhibited significantly lower activity levels during the first 20 min of the test. Nonsignificant decreases in activity were also observed in female SERT-/- mice. Data are expressed as means ± SEMs at 5-min intervals. **P<0.01 and ***P<0.001 vs SERT+/+ mice.
Figure 2-6: Male mice with constitutive reductions in SERT display anxiogenic behavior in the open field. The ratio of center to total distance traveled was calculated to determine the proportion of activity spent investigating the center of the open field in late adolescent mice. (a,b) Center/total distance ratios for postnatal antidepressant-treated SERT+/+ mice show no significant differences in either sex. (c,d) Male SERT-/- mice traveled less in the center of the open field during the first 20 min of the test. Both SERT+/- and SERT-/- male mice displayed reduced center activity during the 40- and 45-min intervals. Data are means ± SEMs at 5-min intervals. *P<0.05 and **P<0.01 vs SERT+/+ mice.
Postnatal SERT Inhibition

(a)

Immunity (s)

unhandled
saline
S-CIT

female
male

Constitutive SERT Deficiency

(b)

Immunity (s)

SERT+/+
SERT+/-
SERT-/-

female
male

**
**Figure 2-7: Depression-related behavior is altered only in male SERT-deficient mice during late adolescence.** Mean immobility times during the last 4 min of a 6-min swim session are shown for postnatal S-CIT-treated mice (a) and SERT-deficient mice (b). Immobility was defined as the absence of swimming and/or climbing behaviors with the exception of minor movements needed to keep afloat. No changes in immobility were observed in SERT+/+ mice treated during P5-P21 with S-CIT. By contrast, male SERT-/- mice showed greater immobility than SERT+/+ mice. Values are means ± SEMs. **P<0.01 vs same-sex SERT+/+ mice.
**Figure 2-8: Presynaptic 5-HT1A receptor responses differ between postnatal antidepressant administration and constitutive SERT deficiency.** Mice were injected with saline or the 5-HT1A agonist 8-OH-DPAT at T=0. Core body temperatures were recorded at T=(−)10 min (baseline), and at T=20 min and T=60 min after injection. (a,b) Hypothermia occurred 20 min after 8-OH-DPAT in mice treated postnatally with saline- or S-CIT. No changes in temperature occurred in saline-injected unhandled, saline-treated, or S-CIT-treated mice (control groups not shown for clarity). (c,d) Gene-dose-dependent hypothermic responses occurred in female SERT-deficient mice. Hypothermic responses were similar in male SERT+/+ and SERT+/- mice. Both female and male SERT-/- mice failed to show reductions in body temperature in response to 8-OH-DPAT. No changes in temperature occurred in response to saline injection in female or male mice of all genotypes (data not shown for clarity). Horizontal gray bars represent means ± SEMs of body temperature in saline-treated groups. *P<0.05, **P<0.01, and ***P<0.001 vs saline-injected mice with the same postnatal treatment or of the same SERT genotype and ††P<0.01 and †††P<0.001 vs 8-OH-DPAT-injected SERT+/+ mice.
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<tr>
<td>Closed Arm Time (s)</td>
<td>140 ± 10</td>
<td>110 ± 7</td>
<td>93 ± 8 ††</td>
<td>68 ± 7</td>
<td>86 ± 7</td>
<td>99 ± 7*</td>
<td>120 ± 10</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>Closed Arm Entries (#)</td>
<td>17 ± 1</td>
<td>19 ± 0.9</td>
<td>13 ± 1 #†#††</td>
<td>11 ± 0.9</td>
<td>12 ± 1</td>
<td>12 ± 0.6</td>
<td>15 ± 2</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Total Arm Entries (#)</td>
<td>21 ± 1</td>
<td>24 ± 0.7</td>
<td>21 ± 1</td>
<td>18 ± 1</td>
<td>19 ± 1</td>
<td>17 ± 0.9</td>
<td>21 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Rarings (#)</td>
<td>18 ± 3</td>
<td>21 ± 2</td>
<td>19 ± 3</td>
<td>10 ± 2</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>14 ± 2</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>Head Dips (#)</td>
<td>10 ± 2</td>
<td>13 ± 1</td>
<td>15 ± 2</td>
<td>22 ± 3</td>
<td>18 ± 2</td>
<td>14 ± 2*</td>
<td>12 ± 3</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Immobility Trial 2 (s)</td>
<td>120 ± 20</td>
<td>120 ± 8</td>
<td>120 ± 10</td>
<td>170 ± 10</td>
<td>140 ± 20</td>
<td>190 ± 10</td>
<td>130 ± 20</td>
<td>100 ± 20</td>
</tr>
</tbody>
</table>
Table 2-1: Additional data for the elevated plus maze and forced swim tests.

Raw data for open and closed arm parameters in the elevated plus maze are shown for both cohorts of mice. Exploratory and locomotor behaviors including rears, head dips, and total arm entries are shown. Times spent immobile during the last 4-min period of the second trial of the forced swim session are indicated for all groups of mice. Data are means ± SEMs. #P<0.05 or ###P<0.001 vs postnatal saline-treated mice, †P<0.05, ††P<0.01, and †††P<0.001 vs same-sex combined control groups, and *P<0.05, **P<0.01, ***P<0.001 vs same-sex or SERT+/+ mice.
References


Frank MG, Heller HC (1997) Neonatal treatments with the serotonin uptake inhibitors clomipramine and zimelidine, but not the noradrenaline uptake


Chapter 3

Early Exposure to Antidepressants Does Not Recapitulate the Effects of Constitutive Serotonin Transporter Deficiency

3.1 Introduction

Administration of serotonin transporter (SERT) inhibiting antidepressants to rodents during early postnatal development has long been known to alter a range of adult behaviors including sexual activity, rapid eye movement sleep, exploratory behavior, behavioral despair, and learning (Vogel et al., 1990; Oberlander et al., 2009). In humans, this period of vulnerability corresponds to the third trimester of pregnancy through two years of age (Andersen, 2003; Watson et al., 2006; Clancy et al., 2007a; Clancy et al., 2007b; Thompson et al., 2009). The need for antidepressant therapy in as many as 10-12% of pregnant women (Bennett et al., 2004; Marcus, 2009), particularly during the third trimester (Bennett et al., 2004), is thought to carry potential risks to progeny as these drugs are capable of crossing the placenta (Hostetter et al., 2000).

The literature regarding the effects of early-life exposure to antidepressants is controversial. Human infants exposed to serotonin-selective reuptake inhibitors
(SSRIs) \textit{in utero} have been reported to display withdrawal effects (Nordeng et al., 2001) and delayed motor coordination (Casper et al., 2003). In some studies, children with prenatal SSRI exposure also showed increased internalizing behaviors and altered hypothalamic-pituitary-axis function, suggesting vulnerability to maladaptive mood states (Oberlander et al., 2008; Oberlander et al., 2009; Oberlander et al., 2010). By contrast, other studies found no association between prenatal exposure to SSRIs and long-term changes in mental development or internalizing behaviors (Casper et al., 2003; Misri et al., 2006). Some SSRIs have been suggested to provide safer alternatives than others for use during pregnancy, \textit{i.e.}, S-citalopram (escitalopram, S-CIT) vs paroxetine (Gentile, 2010), although caution is warranted since some SSRIs have been more thoroughly investigated than others.

In contrast to the relatively small amount of information on the ramifications of \textit{in utero} SSRI exposure, untreated maternal stress, anxiety, and depression have better understood consequences regarding the development and neurobehavioral outcome of children (Brand and Brennan, 2009). Specifically, low birth weights and increased risk for prematurity are linked to exposure to stress and anxiety (Wadhwa et al., 1993). Moreover, elevation of maternal cortisol directly influences cortisol levels in the developing fetus (Sarkar et al., 2007), likely contributing to impairments of hypothalamic-pituitary-adrenal (HPA) axis functioning, and in turn, stress responses in children (Charil et al., 2010). Maternal stress and anxiety affect other behaviors in children including increased hyperactivity, inattention, impulsivity and impairing cognition and emotion (O’Connor et al., 2002; Van den
Bergh et al., 2005; Mennes et al., 2006; Buss et al., 2011). Similar neurobehavioral outcomes have been correlated with untreated maternal depression where identified risks to offspring include increased internalizing behaviors and exaggerated stress responses (Misri et al., 2006; Weissman et al., 2006; Brand and Brennan, 2009; Hay et al., 2010).

Human SERT gene variants, including the serotonin-transporter linked polymorphic region (5-HTTLPR) and related single nucleotide polymorphisms (Hu et al., 2006; Wendland et al., 2006; Wendland et al., 2008; Murphy et al., 2013), are associated with differences in SERT expression and anxiety-related personality traits (Canli and Lesch, 2007; Murphy and Lesch, 2008). Analogous polymorphisms do not occur in rodents, however, mice and rats with constitutive changes in SERT expression have been engineered (Bengel et al., 1998; Ansorge et al., 2004; Jennings et al., 2006; Zhao et al., 2006; Homberg et al., 2007). Similar to humans, the most consistent phenotype associated with decreased SERT expression in rodents is increased anxiety-like behavior (Holmes et al., 2003c; Murphy and Lesch, 2008; Olivier et al., 2008; Kalueff et al., 2010). The finding that postnatal exposure to SSRIs is associated with changes in locomotor, depression-like, and exploratory behaviors in rodents, has led to the idea that the effects of constitutive alterations in SERT expression might be limited to sensitive postnatal periods (Sibille and Lewis, 2006; Homberg et al., 2010). However, given the complex developmental influence of SERT (Murphy and Lesch, 2008; Homberg et al., 2010; Trowbridge et al., 2011), in combination with our findings on the persistent effects of the 5-HTTLPR (Singh et al., 2010; Singh et al., 2011) and targeted gene deletion (Mathews et al., 2004; Perez
and Andrews, 2005; Perez et al., 2006) on SERT function in animal models, we questioned whether early exposure to SSRIs produces a behavioral phenotype identical to that of lifelong SERT deficiency (Ansorge et al., 2004; Popa et al., 2008).

Here, we directly compare behavioral, pharmacologic, and neurochemical responses to postnatal administration of S-(+)-citalopram (S-CIT) or fluoxetine (FLX) vs constitutive SERT deficiency. Mice were on a genetic background that enabled observation of changes in anxiety-related behavior in both directions (Holmes et al., 2003c). Our findings emphasize key differences between these two models of altered SERT function evidenced by contrasting changes in emotion-related behaviors and 5-HT1A-receptor responsiveness associated with opposing alterations in extracellular serotonin levels persisting late into adulthood.

3.2 Materials and Methods

3.2.1 Animals and treatments

Mice lacking one functional SERT allele (SERT+/− mice) on a congenic CD-1 × 129SvEv background (Bengel et al., 1998; Mathews et al., 2004; Perez and Andrews, 2005; Warden et al., 2012) were bred to produce SERT+/+, SERT+/−, and SERT−/− mice. For the postnatal antidepressant treatment portion of the study, SERT+/+ mice were bred for one generation to produce wildtype animals on the same genetic background as SERT deficient mice. Mice were maintained on a 12h-light/dark cycle with ad libitum access to food and water. Procedures involving animals were approved by the University of California, Los Angeles Chancellor’s
Animal Research Committee and were in accordance with National Institutes of Health Animal Care and Use Guidelines.

A total of 426 mice were studied. Group contained 13-28 mice per sex, age, and genotype or drug treatment. Individual group sizes are reported in Table 3-1. Wildtype neonates used for postnatal antidepressant treatment were weighed daily beginning on postnatal day (P)5 and ending on P21 to determine dosing solution volumes. Mice were injected subcutaneously (sc) with saline, S-CIT, or FLX. Drugs were administered at 10 mg/kg/d with doses calculated as the drug free base. Drugs were dissolved in saline at 1 mg/mL. Injection volumes were 10 mL/kg. These doses and regimens of postnatal S-CIT or FLX have been shown previously to alter emotion-related behaviors in adult mice (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008). During the brief, 5-min periods of maternal separation for injections, nests containing all pups were transferred to containers of warmed, dry rice to maintain pup body temperatures. Following weighing and injections, nests were immediately returned to their mothers in the home cages. Saline, S-CIT, and FLX treatment were randomized across pups in each litter. Injections were given 1 h before the onset of the dark cycle. Offspring of SERT+/- parents were not handled or injected because mice deficient in SERT display heightened sensitivity to stressful stimuli, including saline injection (Li et al., 1999; Tjurmina et al., 2002). Moreover, the overwhelming majority of previous studies on emotion-related behavior in SERT-deficient mice have not involved animals subjected to postnatal injection paradigms.
Postnatal antidepressant-treated and SERT-deficient mice were tested on measures of anxiety-, depression-, and locomotor-related behavior beginning during the 12th week of life (P78; 3-month time point) or the 40th week of life (P274; 10-month time point). All handling and behavior testing was carried out by a single investigator (S. C. A.) and took place during the dark phase under dim light. Behavior tests were conducted three days apart in the order listed below so that swim or injection stress did not influence behavior in the plus maze.

3.2.2 Elevated plus maze

The elevated plus maze was used to assess anxiety-related behavior (Walf and Frye, 2007). A single maze was used throughout the study having two opposing open arms (30 cm × 5 cm) with a 0.5 cm lip around the edges to prevent animals from falling (Lapiz-Bluhm et al., 2008), two opposing enclosed arms (30 cm × 5 cm × 15 cm), and a center platform (5 cm × 5 cm). The maze was raised 38.5 cm from the floor. The walls of the closed arms were constructed of clear Plexiglas to ensure even light levels across all arms. The floor of the maze was constructed of a continuous piece of opaque Plexiglas. Mice were placed on the center platform facing a closed arm and were allowed to explore the maze freely for 5 min. The maze was cleaned with 70% ethanol and dried between mice. Behavior was videotaped and scored by trained observers blind to postnatal treatment or genotype. Parameters quantified included numbers of open/closed arm four-paw arm entries, open/closed arm time, rears in the closed arms, and head dips from the
center platform or open arms. Time in the open/closed arms was analyzed as a percent of total arm time excluding time spent in the center platform of the maze.

3.2.3 Open field test

Locomotor activity in an open field was assessed using a 3-point digital video tracking system and Viewer²® software (BIOBSERVE GmbH, Bonn, Germany). Open field arenas were 50 cm × 50 cm × 40 cm. The center region was defined by a 22 cm × 22 cm central square. Animals were considered to be in the central square after the midpoint of the body entered the area. Mice were placed in a corner of the open field and were allowed to freely explore for 1 h. Total distance traveled (cm) and the ratio of center distance to total distance were measured. Additionally, time spent exploring the center region was analyzed. Data were collected in 5-min noncumulative intervals. Testing environments were thoroughly cleaned with 70% ethanol and allowed to dry between mice.

3.2.4 Forced swim test

This test was carried out as previously described with minor modifications (Porsolt et al., 1977; Shen et al., 2010). Briefly, each mouse was placed in a clear glass cylinder (27 cm high × 13 cm diameter) containing room temperature water (22 ± 2°C) at a depth of 15 cm. Behavior was videotaped during 6-min swim sessions. Afterwards, mice were transferred to a warm environment while their fur dried before being returned to their home cages. Mice were retested under the same conditions 24 h later to assess the effects of repeat swim stress (Porsolt et al., 1977;
Borsini et al., 1989), which is thought to alter depression-like behavior in SERT-deficient mice on a C57BL/6J background (Carroll et al., 2007). Cylinders were cleaned with 70% ethanol and refilled with fresh water between mice. Data were collected and analyzed in 1 min bins using FST High Throughput Forced Swim Test Analysis software (BIOBSERVE GmbH, Bonn, Germany). Time spent immobile during the last 4 min of each 6 min trial was summed (Porsolt et al., 2001).

3.2.5 Presynaptic 5-HT1A receptor function

We investigated responses of 5-HT1A autoreceptors because this receptor subtype is critical for controlling serotonergic neurotransmission (Richer et al., 2002) and is implicated in antidepressant and anti-anxiety responses (Sinton and Fallon, 1988; Hjorth and Auerbach, 1994; Holmes et al., 2003b; Rossi et al., 2008a; Rossi et al., 2008b; Popa et al., 2010; Richardson-Jones et al., 2010; Altieri et al., 2012; Altieri et al., 2013). Agonists at 5-HT1A receptors produce hypothermia in mice via a presynaptic mechanism (Martin et al., 1992; Li et al., 1999; Richardson-Jones et al., 2011). A randomized block design was used wherein all mice received three different treatments in different orders with a 3-day separation between testing days. Mice were injected with saline or the 5-HT1A receptor agonist 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT; 0.1 mg/kg or 0.4 mg/kg calculated as the free base and dissolved in saline at 0.1 mg/ml) (Li et al., 1999; Popa et al., 2008). Adult body weights were recorded at this time. Mice were habituated to a rectal temperature probe one day prior to the first testing day (Physitemp Instruments, Inc., Clifton, NJ). Two baseline temperatures were
recorded 20 min and 10 min prior to injection. Core body temperatures were recorded in 10 min increments post-injection for 1 h and then again at 90 min and 120 min after injection to verify that temperatures returned to baseline.

3.2.6 Guide cannula implantation surgery

At 10-14 months of age, each mouse was implanted unilaterally with a guide cannula for a CMA/7 microdialysis probe (CMA/Microdialysis, Solna, Sweden) under isoflurane anesthesia into the left ventral hippocampus (coordinates relative to Bregma: AP -2.8 mm, ML -3.5mm, DV -2.2mm). Each cannula was held in place with dental resin (Bosworth Trim II, from Bosworth Company, Skokie, IL), which was secured to the skull with a stainless steel screw (Eicom-USA, San Diego, CA). Microdialysis experiments described below were performed 3-20 days after cannula implantation.

3.2.7 Microdialysis and no net flux protocols

*Probe insertion.* During Zeitberger Time (ZT) 10-12, each subject was briefly (3-5 min) anesthetized using isoflurane for insertion of a CMA/7 microdialysis probe (active membrane length 2 mm; CMA/Microdialysis, Solna, Sweden) into the guide cannula. Immediately after insertion, an aCSF solution (147 mM NaCl, 3.5 mM KCl, 1.0 mM CaCl₂, 1.0 mM NaH₂PO₄, 2.5 mM NaHCO₃, 1.2 MgCl₂, pH 7.3 ± 0.03 at room temperature) was continuously infused through the probe at 3 μl/min for 30-60 min followed by a 0.3 μl/min flow rate for an additional 12-14 h to allow recovery from acute injury-related neurotransmitter release due to probe insertion.
Microdialysis. Male and female mice treated postnatally with saline, S-CIT, or FLX were used in experiments to determine the effects of 8-OH-DPAT on extracellular serotonin levels. At ZT 2-3 on the day after probe insertion, the flow rate of aCFS through the probe was increased to 3 μl/min for at least 30 min before collecting dialysate samples. Basal samples were collected in 6 min increments over the course of 2 h, after which 1.2 μM S-CIT was infused into the probe to increase local concentrations of extracellular serotonin (Hjorth et al., 1997; Tao et al., 2000). Samples were collected for another 2 h. Subsequently, mice received an intraperitoneal (ip) saline injection (1 mL/kg), and dialysate samples were collected over 1 h. Each mouse then received increasing doses of 8-OH-DPAT (0.025, 0.05, 0.1, 0.2, and 0.4 mg/kg, ip) and dialysate samples were collected for 1 h after each dose. Sampling times were 6 min throughout.

Dialysate samples were analyzed online continuously by high-performance liquid chromatography coupled to electrochemical detection (HTEC-500, Eicom-USA, San Diego, CA). Dialysates and standards were separated using an optimized mobile phase composed of 96 mM NaH₂PO₄, 3.8 mM Na₂HPO₄, pH 5.4, 2.8% MeOH, 50 mg/L EDTA-Na₂, and 500 mg/L sodium decanesulfonate in water purified by a Milli-Q Synthesis A10 system (EMD Millipore Corporation, Billerica, MA). The stationary phase was a PP-ODS II column (4.6 cm × 3 mm, Eicom-USA, San Diego, CA). There were no statistically significant differences between sexes, so microdialysis data from male and female mice within each postnatal treatment group were combined. Group sizes were N=11 for postnatal saline-treatment, N=12 for postnatal S-CIT treatment, and N=12 for postnatal FLX-treated mice.
No net flux. Male and female mice treated postnatally with SSRIs and male SERT-deficient mice were used in experiments to determine levels of extracellular serotonin in the ventral hippocampus. Zero net flux protocols were similar to previously published methods (Mathews et al., 2004; Szapacs et al., 2004; Luellen et al., 2007) with the following modifications. At ZT 2-3 on the day following probe insertion, the flow rate of aCSF was increased to 3 μl/min at least 30 min prior to dialysate sampling. Basal samples were taken over 2 h. Subsequently, four concentrations of serotonin ranging from 2.5 nM to 50 nM were infused via the microdialysis probe using a programmable gradient infusion CMA/102 pump (CMA/Microdialysis, Harvard Apparatus, Holliston, MA). Dialysates were collected for 90 min at each concentration of serotonin. Samples were collected in 6 min intervals and analyzed online continuously as described above.

Probe verification. After microdialysis experiments, mice were sacrificed by cervical dislocation and brains were rapidly removed and preserved in 7% paraformaldehyde phosphate buffer (PB), pH 7.3 for 48-72 h at room temperature on a laboratory rotator. Brains were then transferred to a 30% sucrose-PB solution and stored at -80°C. Preserved brains were sectioned at 50 μm using a cryostat and stained by cresyl violet. Probe positions was examined under a microscope. Only data from mice with correct probe placement are reported.

3.2.8 Chemicals

S-(+)-Citalopram oxalate and fluoxetine hydrochloride were obtained from BioTrend Chemicals (Destin, FL). S(-)-8-Hydroxy-DPAT hydrobromide, sodium
phosphate monobasic (NaH$_2$PO$_4$), sodium phosphate dibasic (Na$_2$HPO$_4$), and ethylenediaminetetraacetic acid disodium salt (EDTA-Na$_2$) were purchased from Sigma (St. Louis, MO). Methanol was from EMD Millipore (Billerica, MA) and sodium decanesulfonate was from TCI America (Portland, OR). Sterile endotoxin-free saline was purchased from Anawa Trading (Wangen, Switzerland).

3.2.9 Statistics

Body weight, temperature, and 8-OH-DPAT microdialysis challenge data were analyzed by two-way analysis of variance (ANOVA) with genotype or postnatal SSRI and age or 8-OH-DPAT dose as the independent variables. In the case of significant interaction terms, data were subsequently analyzed by ANOVA at each age or 8-OH-DPAT dose. Nonsignificant interaction terms are generally not reported and main effect analyses followed. Individual group comparisons were made using preplanned contrasts.

Behavior data from each parameter were normalized to same-sex controls (saline-treated mice for postnatal SSRI comparisons and SERT+/+ mice for genotype comparisons). ANOVA was used to assess differences between groups with subsequent individual group comparisons via least squares means for a priori hypotheses. Global emotionality was assessed by factor analysis using 10 preselected nonredundant parameter (see Table 1) using orthogonal rotation, or varimax. Scree plots were used to extract factor matrices (Eigenvalues >0.7). Factor loadings >0.3 were considered significant. Z-score normalization was applied using
the equation $Z=(\chi-\mu)/\sigma$ where $\chi$ is the individual score, $\mu$ represents the mean for the control group, and $\sigma$ is the population standard deviation (Guilloux et al., 2011).

For no net flux data, perfused serotonin ($C_{in}$) vs the difference between perfused serotonin and serotonin in the dialysate ($C_{in} - C_{out}$) was used to perform linear regression analyses. The $x$-intercept was used to determine levels of extracellular serotonin corrected for extraction fraction ($E_d$), the latter of which was obtained from the slope of the regression line. Data were analyzed using a nonparametric Kruskal-Wallis test due to non-Gaussian distributions followed by Dunn’s multiple comparisons.

Statistics were computed using GraphPad Prism (GraphPad Inc., La Jolla, CA) or SAS Statistical Software (SAS Institute Inc., Cary, NC). All data are expressed as means ± standard errors (SEMs) with $P<0.05$ considered statistically significant.

3.3 Results

3.3.1 Early life exposure to SSRIs is associated with long-term reductions in body weight

Mice receiving postnatal treatment with saline, S-CIT, or FLX from postnatal day (P) 5 through P21 were weighed daily just prior injections. To compensate for differences in body weights arising from litter size effects, data are reported as change in body weight from P5. Significant postnatal treatment × time interactions were observed in female $[F(2,32)=2.9, P<0.001]$ and male $[F(2,32)=9.8, P<0.001]$ mice. Significant differences between groups occurred on P6 and P20-P21 ($P<0.05$)
for female mice and P6-P21 ($P<0.01$) for male mice. Postnatal administration of FLX to female mice was associated with reduced body weight compared to saline-treated mice on P6 and P20-P21 ($P<0.05$) (Fig. 3-1a). Effects of SSRIs on postnatal body weights were more pronounced in male mice. Here, postnatal administration of S-CIT significantly reduced body weight during P7-P10 ($P<0.05$) and P16-P21 ($P<0.01$) (Fig. 3-2a). Male mice receiving FLX showed reductions in body weight throughout the treatment regimen ($P<0.01$) (Fig. 3-2a). Body weights were not recorded for SERT-deficient mice during the postnatal period to prevent effects of handling stress on the anticipated behavioral phenotypes of these mice (Li et al., 1999; Tjurmina et al., 2002).

At 10-months of age, reductions in body weight continued to be observed in female mice treated postnatally with FLX (Fig. 3-1b). By contrast, older female SERT-/ - mice showed increased body weight compared to wildtype mice (Fig. 3-1c). In male mice, both S-CIT and FLX exposure during early life were associated with reduced body weight at 3-months ($P<0.05$ and $P<0.001$, respectively) and 10-months of age ($P<0.001$ and $P<0.001$, respectively; Fig. 3-2b). No significant differences in body weights were observed in adult male SERT-deficient mice (Fig. 3-2c). Together, these data suggest that early life exposure to SSRIs causes long-term decreases in body weight, particularly in male mice.
3.3.2 Anxiety-related behavior is modulated differently by lifelong SERT deficiency vs transient postnatal SSRI exposure

Anxiety-like behavior was investigated in the elevated plus maze and open field. Each test assesses responses to novelty and conflict-avoidance behavior. To enable global analysis (vide infra), data were normalized to appropriate same sex control groups at each age. For the elevated plus maze, anxiety-like behavior is most commonly evaluated by investigating behavior in the aversive open arms of the maze (Handley and Mithani, 1984; Walf and Frye, 2007). Significant differences between groups were observed for latency to first open arm entry \( [F(5,414)=6.0, P<0.001] \) (Fig. 3-3a,e), percent open arm entries \( [F(5,414)=5.3, P<0.001] \) (Fig. 3-3b,f), and time spent in the open arms \( [F(5,414)=3.0, P<0.05] \) (Fig. 3-3c,g). Individual group comparisons revealed that S-CIT-treated mice spent more time and made more entries into the open arms \( (P<0.05) \) of the elevated plus maze compared to saline-treated mice indicative of reduced anxiety-like behavior (Fig. 3-3b,c). No changes in anxiety-like behaviors were associated with postnatal FLX administration. By contrast, time to first enter an open arm was prolonged for SERT+/- and SERT-/- mice \( (P<0.01) \) compared to SERT+/+ mice indicating increased anxiety-like behavior (Fig. 3-3c). A trend toward significant differences between groups was observed with respect to numbers of head dips \( [F(5,413)=2.0, P<0.079] \), an exploratory behavior associated with the open arms of the maze (Fig. 3-3d,h).
In the open field, anxiety-like behavior is commonly assessed by measuring time spent and distance traveled in the center region of the arena (Crawley, 1985; Prut and Belzung, 2003). Significant differences between groups were observed for center time during the first 30 min of the test \(F(5,395)=5.0, P<0.001\) and throughout the entire 1-h test period \(F(5,395)=3.4, P<0.01\). The first 30 min of the test is reflective of anxiety-like behavior associated with the novelty of the arena. Individual group comparisons indicated that postnatal SSRI exposure was not associated with alterations in center time during the first 30 min (Fig. 3-4a) or the entire time in the open field (data not shown.) By contrast, SERT-/- mice showed robust reductions in time spent in the center of the arena during the first 30 min \(P<0.001; \) Fig. 3-4c) and throughout the test \(P<0.01; \) data not shown). The ratio of center/total distance traveled was also significantly different across groups for the first half \(F(5,395)=4.0, P<0.01\) and entire length of the test \(F(5,395)=3.8, P<0.01\). Individual group comparisons during the first 30 min showed that whereas no changes were observed in postnatal SSRI-treated mice (Fig. 3-4b), SERT-/- mice traveled less in the center of the arena compared to SERT+/+ mice \(P<0.01; \) Fig. 3-4d).

To gain unbiased perspectives on how the entire group of animals (>400) performed across behavior tests, factor analysis was carried out (Table 3-1). Factor analysis is a statistical method that enables reduction of a set of observed variables into a small number of unobserved (latent) factors that account for correlations among observed variables (Holmes and Rodgers, 1998; Brigman et al., 2009). Here, factor analysis using data from all groups of mice identified 3 factors accounting for
the majority of the common variance shared by all variables included in the model. Individual variables were considered to be loaded onto a particular factor when standardized regression coefficients were >0.3 (Table 3-1). Open arm latency, open arm time, percent open arm entries, and head dips loaded onto Factor 1 (referred to as “Anxiety 1”), which accounted for 46% of the common variance (Table 3-1). Together, these variables reflect measures of anxiety-like behavior in the elevated plus maze. Interestingly, center time and center/total distance in the open field, which are likewise thought of as measures of anxiety-related behavior, loaded onto a separate factor, which accounted for 35% of the common variance and is referred to as “Anxiety 2” (Table 3-1). Total arm entries and number of rears in the closed arms in the elevated plus maze, and total distance traveled in the open field loaded onto Factor 3 (18% of the common variance). The latter measures are thought to reflect locomotor and exploratory behaviors.

We next applied Z-score normalization to each variable (Guilloux et al., 2011). Z-scores for variables that loaded together for each factor were weighted for standardized regression correlation coefficients and averaged. Z-scores for open arm latency, open arm time, percent open arm entries, and number of head dips (Factor 1) were combined to obtain an overall “Anxiety 1 Score”. When necessary, mathematical signs were reversed (e.g., reduced time/entries and head dips in the open arms reflect an increased anxiety). The results indicate that postnatal S-CIT-treatment caused an overall reduction in anxiety-related behavior in adult animals (P<0.05; Fig. 3-3i). Conversely, both SERT+/- and SERT-/- mice showed increased anxiety-related behavior in adulthood (P<0.05; Fig. 3-3j). Thus, these two different
models of early SERT disruption produce persistent and contrasting changes in anxiety-related behavior.

Variables loading onto Factor 2 were combined in a similar manner by transforming data from individual variables to Z-scores and combining weighted Z-scores to produce an overall “Anxiety 2” score. Postnatal treatment with S-CIT and FLX were not associated with changes in Anxiety 2 (Fig. 3-4e), however, robust increases in Anxiety 2 were observed selectively in SERT-/- mice compared to SERT+/+ and SERT+-/- mice (P<0.001; Fig. 3-4f). Similar to Anxiety 1, Anxiety 2 showed that SERT-/- mice exhibit increased anxiety-related behavior that is different from behavior observed in postnatal SSRI-treated mice.

3.3.3 Constitutive SERT deficiency and postnatal administration of FLX produce similar changes in activity/exploration

Activity-related behaviors were assessed using total arm entries in the elevated plus maze and total distance traveled in the open field. There were no significant differences across groups with respect to total arm entries in the plus maze (Fig. 3-5a,d), whereas a significant effect of group was noted for total distance traveled during the first 30 min [F(5,395)=5.6, P<0.001] and throughout the entirety [F(5,395)=4.3, P<0.001] of the open field test. Individual group comparisons indicated that postnatal FLX-treated mice (P<0.05) and SERT-deficient mice (P<0.001) traveled less distance during the first 30 min in the open field (Fig. 3-5b,e) and throughout the test (P<0.05 for FLX-treated mice and P<0.01 for SERT-/- mice vs respective controls; data not shown), indicative of hypolocomotive behavior.
Furthermore, significant differences between groups were observed for numbers of rears in the closed arms of the elevated plus maze \[F(5,413)=2.9, \ P<0.05\]. Here, both postnatal FLX-treated mice (Fig. 3-5c) and SERT-/- mice (Fig. 3-5f) showed reduced numbers of rears compared to respective control groups indicating decreased exploratory behavior.

Variables loading onto Factor 3 were converted to Z-scores and weighted averages were used to calculate an overall “Activity/Exploration Score”. The data show that postnatal FLX \(P<0.01\); Fig. 3-5g) and SERT-deficiency \(P<0.01\) SERT+/- mice; \(P<0.001\) SERT-/- mice; Fig. 3-5h) are associated with reduced activity. These findings are in agreement with previous literature indicating that both models show similar characteristics in terms of measures of locomotion and exploration (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008).

3.3.4 Depression-related behavior in the forced swim test does not differ with respect to postnatal SSRI exposure or lifelong SERT deficiency

Immobility behavior was measured during the last 4 min of two 6 min swim sessions carried out 24 h apart. No significant differences between groups were observed for trial 1 \([F(5,396)=0.6, \ P<0.72]\) or trial 2 \([F(5,396)=0.4, \ P<0.84]\) (data not shown). Immobility during the first trial was included as a variable in the factor analysis (Table 3-1), although it did not load onto any of the three identified factors indicating minimal contributions to the common variance associated depression-related behavior.
3.3.5 Postnatal SSRIs and SERT deficiency produce opposing changes in 5-HT1A autoreceptor function

An agonist-induced hypothermia challenge was used to assess 5-HT1A autoreceptor function. All mice received saline, 0.1 mg/kg, or 0.4 mg/kg 8-OH-DPAT in random order 3 days apart. Main effects of 8-OH-DPAT challenge [$F(2,122)=209.3, P<0.0001$] and postnatal antidepressant treatment [$F(2,122)=3.8, P<0.05$] were significant for 3-month old female mice. Both doses of 8-OH-DPAT significantly reduced body temperature compared to saline-injection ($P<0.001$). Female mice exposed during postnatal life to S-CIT showed potentiated responses to 0.4 mg/kg 8-OH-DPAT compared to mice given postnatal saline and challenged with the same dose of 8-OH-DPAT at 3-months of age ($P<0.01$; Fig. 3-6a,c). Similar effects were observed in 3-month-old male receiving postnatal SSRIs whereby significant main effects of 8-OH-DPAT challenge [$F(2,86)=172.4, P<0.0001$] and postnatal antidepressant treatment [$F(2,86)=3.9, P<0.05$] were observed. Male mice responded to both doses of 8-OH-DPAT showing significant reductions in body temperature compared to saline-injected mice ($P<0.001$). Similar to female mice, male S-CIT-treated mice receiving 0.4 mg/kg 8-OH-DPAT showed potentiated reductions in body temperature compared to S-CIT-treated mice challenged with saline ($P<0.01$) and FLX-treated mice ($P<0.05$) administered 0.4 mg/kg 8-OH-DPAT (Fig. 3-7a,c).

Significant 8-OH-DPAT $\times$ genotype interactions were observed for female [$F(4,120)=8.3, P<0.0001$] and male [$F(4,179)=14.1, P<0.0001$] SERT-deficient mice indicating a high degree of dissimilarity in responses to 8-OH-DPAT challenge.
across genotypes. Both doses of 8-OH-DPAT resulted in significant reductions in body temperature for female and male SERT+/+ and SERT+-/ mice compared to saline-challenged mice of the same genotype \( P<0.001 \). Conversely, female SERT-/- mice \( P<0.001; \) Fig. 3-6b,d) and male SERT-/- mice \( P<0.001; \) Fig. 3-7b,d) failed to respond to either dose of 8-OH-DPAT.

For 10-month-old female mice exposed postnatally to SSRIs, a main effect of 8-OH-DPAT occurred \[ F(2,120)=187.3, P<0.0001 \] with further analyses indicating that S-CIT and FLX were associated with significant reductions in body temperature \( P<0.001 \). Unlike younger female mice, 10-month-old female mice treated postnatally with S-CIT mice did not respond to 8-OH-DPAT in a hypersensitive manner (Fig. 3-8a,c). Female SERT-/- mice at 10-months of age continued to show no significant reductions in body temperature after either dose of 8-OH-DPAT (Fig. 3-8b,d). In male mice at 10-months of age, a significant 8-OH-DPAT × postnatal antidepressant interaction was observed \[ F(4,118)=3.0, P<0.05 \] indicating differential effects of 8-OH-DPAT with respect to postnatal treatment groups. Further analysis indicated that male 10-month-old mice treated postnatally with S-CIT responded to 0.4 mg/kg 8-OH-DPAT in a potentiated manner compared to saline- and FLX-treated mice receiving equivalent doses of 8-OH-DPAT (Fig. 3-9a,c). Male 10-month-old SERT-/- mice did not respond to either dose of 8-OH-DPAT compared to age-matched SERT+/+ mice (Fig. 3-9b,d). Taken together, these data indicate that physiological responses to a 5-HT1A agonist occur in opposing directions in mice administered S-CIT postnatally vs SERT-/- mice. The former is associated with a hypersensitive response to the higher dose of 8-OH-DPAT, while
the latter is associated with a completely blunted 5-HT1A thermic response to both doses of 8-OH-DPAT. Moreover, effects of postnatal S-CIT persist into late adulthood in male mice.

In addition to body temperature responses to 5-HT1A receptor activation, we employed microdialysis to investigate changes in hippocampal extracellular serotonin levels resulting from systemic administration of 8-OH-DPAT. Increasing doses of 8-OH-DPAT (0.025-0.4 mg/kg) were administered to mice that had been treated postnatally with S-CIT or FLX. Dialysate samples were collected and analyzed every 6-min for 1-h after each dose. Data were analyzed by comparing percent decreases in serotonin levels in 10-14 month-old mice. A main effect of postnatal antidepressant was observed [F(2,160)=13.7, P<0.001]. Postnatal S-CIT treatment was associated with potentiated reductions in serotonin levels after all doses of 8-OH-DPAT. These reductions were 30% greater than those occurring in mice receiving postnatal saline treatment (Fig. 3-9e). In mice receiving postnatal FLX, only the first/lowest dose of 8-OH-DPAT produced a significantly greater reduction extracellular serotonin compared to postnatal saline administration. These data provide further evidence that 5-HT1A receptors, which control extracellular serotonin levels via autoreceptor-mediated feedback (Richer et al., 2002; Altieri et al., 2013), are hyperresponsive in adult mice exposed to S-CIT during postnatal development.

A preliminary experiment was carried to determine if 5-HT1A expression and G-protein coupling might account for differences in 5-HT1A function in postnatal SSRI-
treated mice. Expression of 5-HT1A autoreceptors was analyzed by autoradiography using (3H)-8-OH-DPAT. No differences in 5-HT1A binding were observed in the dorsal raphe with respect to postnatal SSRI administration (Fig. 3-10a). Furthermore, 8-OH-DPAT-stimulated (35S)-GTPγS binding was not altered in mice treated postnatally with S-CIT or FLX indicating intact G-protein coupling (Fig. 3-10b). Thus, functional changes in 5-HT1A autoreceptors do not appear to be associated with increases in receptor expression or G-protein coupling particularly in postnatal S-CIT-exposed mice.

3.3.6 Hyper- vs hyposerotonergia is associated with constitutive loss of SERT compared to postnatal SERT inhibition by S-citalopram

We utilized microdialysis to quantify extracellular serotonin levels in the ventral hippocampus in the two models of serotonin transporter disruption. Extracellular serotonin levels were corrected for in vivo extraction fraction using no-net-flux microdialysis (Fig. 3-11a,b). At 10-14 months of age, significant reductions in extracellular serotonin were observed in mice treated postnatally with S-CIT compared to mice treated postnatally with saline (P<0.01; Fig. 3-11c). By contrast, SERT-/- mice showed significant increases in extracellular serotonin levels compared to SERT+/+ mice (P<0.001; Fig. 3-11d). Modest increases in hippocampal extracellular serotonin levels were also detected in SERT+/- mice (P<0.05; Fig. 3-11d). Basal dialysate serotonin levels were also different with respect to postnatal treatment [$\chi^2(2)=7.0$, $P<0.05$] and genotype [$\chi^2(2)=33.8$, $P<0.001$]. Mice receiving postnatal S-CIT showed reduced basal dialysate serotonin levels ($P<0.05$; Fig. 3-11e), whereas SERT+/- mice ($P<0.01$) and SERT-/- mice ($P<0.001$) showed
increased basal dialysate serotonin levels (Fig. 3-11f). Together, these data indicate that both models of early life SERT disruption permanently alter the trajectory of the serotonin system. Once again, however, changes occur in opposing directions such that postnatal S-CIT administration is associated with a hyposerotonergic state and lifelong SERT deficiency is defined by a hyperserotonergic state.

3.4 Discussion

Studies in rodents dating back to the early 1980’s identified a critical time frame in development when drugs that inhibit SERT generate lasting changes in emotion-related behavior (Mirmiran et al., 1981; Hilakivi and Hilakivi, 1987; Fernandez-Pardal and Hilakivi, 1989; Vogel et al., 1990; Velazquez-Moctezuma and Diaz Ruiz, 1992; Frank and Heller, 1997; Ansorge et al., 2004; Maciag et al., 2006b; Maciag et al., 2006a; Ansorge et al., 2008; Bhagya et al., 2008; Karpova et al., 2008; Popa et al., 2008; Harris et al., 2011). More recently, it has been postulated that reproducible and robust increases in anxiety-like behaviors manifested by SERT-deficient mice (Holmes et al., 2003c; Holmes et al., 2003a; Holmes et al., 2003b; Kalueff et al., 2007; Joeyen-Waldorf et al., 2009) are replicated when SERT is blocked in a limited manner during postnatal development (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008). However, we find that SERT inhibition from P5-P21 is insufficient to recapitulate changes in anxiety-related behaviors observed in SERT-deficient mice (Fig. 3-12).
We and others have shown that mice with reduced SERT expression consistently display reductions in open arm time/entries in the elevated plus maze (Holmes et al., 2003c; Holmes et al., 2003b; Kalueff et al., 2007; Joeyen-Waldorf et al., 2009) and center time/distance traveled in the open field (Holmes et al., 2003b; Kalueff et al., 2007; Joeyen-Waldorf et al., 2009), though this depends somewhat on background strain (Holmes et al., 2003c). By contrast, increases in anxiety-related parameters most often observed in SERT-deficient mice have not been reported in adult mice treated postnatally with SSRIs (Ansorge et al., 2004; Ansorge et al., 2008; Karpova et al., 2008; Popa et al., 2008; Harris et al., 2011). In fact, in the present study, using a background strain with lower baseline anxiety levels than the strain used by Ansorge et al., (CD-1 × 129S6SvEv vs 129SvEvTac × 129S6SvEv, respectively), mice treated postnatally with S-CIT show decreased anxiety in the elevated plus maze indicated by reduced exploration of the open arms and reduced time to first open arm entry (Ansorge et al., 2004; Ansorge et al., 2008). Notably, similar changes were not observed in mice treated during the postnatal period with FLX by us or Ansorge et al. This diametrically opposes increases in a broad range of anxiety-related behaviors in SERT-deficient mice.

Differences in interpretation regarding which indices constitute "emotion-related behaviors" lead to variable conclusions regarding similarities between postnatal and constitutive SERT disruption models (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008). In behavior tests where exposure to novelty and conflict/avoidance are used to assess anxiety-related behaviors, increased anxiety is most often associated with reduced time/entries in the open arms of the elevated
plus maze (Walf and Frye, 2007). or decreased time and distance traveled in the center of an open field. Behavior measuring locomotor activity and exploration is most often associated with total arm exploration on the plus maze, total distance traveled in the open field, and vertical activity (rears). The design of the current study wherein mice were exposed to a range of behavioral tests to investigate changes in emotion-related behavior allowed us to employ hierarchical statistical analyses. Moreover, behavior testing is associated with inherent inter-animal variability that is inevitable regardless of stringent controls for factors that influence behavior (Izidio et al., 2005; Belsky et al., 2009; Lewejohann et al., 2010; Lewejohann et al., 2011), which further necessitates examination of behavior across a number of variables and tests to gain global perspectives.

Here, we reproduce previously reported behavior changes including reductions in total arm entries, rearing, and total distance traveled in the open field in postnatal FLX-treated mice and SERT-/- mice (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008). Based on factor analysis integrating results across behavioral variables examined in the present study, we conclude that similarities between postnatal SSRI treatment and constitutive SERT deficiency are limited to a behavioral domain dominated by motor activity and exploration.

Postnatal administration of S-CIT or FLX caused reductions in early life body weight that persisted into adulthood. Effects were observed in male and female mice, though they were more pronounced in males. This is consistent with a previous report of reduced body weight at P21 and 3 months of age in male mice
exposed to the same postnatal dose regimen of FLX (Karpova et al., 2008). In rats, early life SSRI exposure leads to decreases in body weight during postnatal treatment, as does adult SSRI-treatment (Hansen et al., 1997; Hansen and Mikkelsen, 1998; Vijayakumar and Meti, 1999; Andersen et al., 2002; Maciag et al., 2006b). One study in mice reported that exposure to S-CIT during postnatal life produced brief increases in weight limited to the postnatal period (Popa et al., 2008). Other models of hyposerotonergia (i.e., mice deficient in vesicular monoamine transporter in SERT-expressing cells or mice deficient in tryptophan hydroxylase 2 under the control of Sert or Pet1 promoters) were also recently reported to have reduced postnatal body weight and growth retardation (Narboux-Neme et al., 2013).

Given the importance of 5-HT1A autoreceptors in controlling serotonergic tone (Gardier et al., 1996; Altieri et al., 2013), we hypothesized that differences in serotonergic circuitry involving 5-HT1A autoreceptors underlie contrasting phenotypes arising from postnatal SERT inhibition vs constitutive SERT deficiency. Previous work shows that administration of 0.1 mg/kg 8-OH-DPAT produces a decrease in body temperature in adult SERT+/+ and SERT+-/+ but not SERT-/- mice on C57BL/6 and 129S6/SvEv backgrounds (Li et al., 1999; Li et al., 2000; Holmes et al., 2003c). Potentiated hypothermic responses following administration of 0.4 mg/kg 8-OH-DPAT to adult mice exposed to postnatal S-CIT have also been previously shown (Popa et al., 2008). In the present study, we administered both doses of 8-OH-DPAT and likewise observed that 0.4 mg/kg induced hyperresponsive 5-HT1A responses in postnatal S-CIT-treated female and male
mice at 3-months of age. Furthermore, we extend previous findings and show hyperresponsive decreases in body temperature in 10-month-old male mice at both doses of 8-OH-DPAT.

These results support the idea that early S-CIT exposure generates lasting changes in serotonin system function. Similar effects did not occur in response to early exposure to FLX once again indicating differential effects of individual SSRIs. Male and female mice with constitutive loss of SERT expression did not respond to either dose of 8-OH-DPAT confirming previous reports at the lower dose (Li et al., 1999; Li et al., 2000). Using microdialysis to investigate the physiological status of 5-HT1A autoreceptors, 8-OH-DPAT-induced reductions in extracellular serotonin levels were exacerbated in postnatal S-CIT-treated mice across a range of doses, providing further evidence for hyperresponsivity of 5-HT1A receptors in postnatal S-CIT-exposed mice.

Adult serotonergic tone also differed between postnatal SSRI exposure and constitutive SERT deficiency models. We previously showed that SERT-/- mice have elevated extracellular serotonin levels in the frontal cortex and striatum (Mathews et al., 2004). We find similar gene-dose dependent increases in extracellular serotonin in the ventral hippocampus in the present study. By contrast, levels of extracellular serotonin were reduced in mice treated with S-CIT but not FLX during postnatal life. These data show that opposing changes in 5-HT1A responsiveness associated with postnatal S-CIT exposure vs constitutive SERT deficiency are correlated with lifelong altered serotonin system function. Continuing to discover
underlying causative factors in the serotonin system and emotion-related brain networks modulated by serotonin, *e.g.*, axonal innervation, receptor regulation, and membrane excitability, are important next steps for understanding how these two developmental paradigms produce differing behavioral phenotypes.

Importantly, the different SSRIs used here produced dissimilar outcomes in terms of their effects on adult behavior, physiology, and neurochemistry. While postnatal S-CIT was associated with reductions in anxiety behavior, hypersensitive 5-HT1A receptors, and reduced levels of extracellular serotonin, postnatal administration of FLX did not produce similar changes. Behaviorally, postnatal FLX was associated with reduced locomotor/exploratory behaviors similar to behavioral changes in SERT-/- mice and in agreement with previous literature on postnatal administration of this SSRI (Ansorge et al., 2004; Ansorge et al., 2008). Differences in postnatal exposure to FLX compared to S-CIT might be related to “off-target” effects. While the primary mechanism of action of SSRIs is to block SERT, secondary actions are remarkably different between SSRIs (Carrasco and Sandner, 2005) potentially accounting for their differential outcomes. Further work is required to tease apart mechanistic disparities between S-CIT and FLX that lead to their differential effects and to examine the effects of other SSRIs commonly used to treat depression and anxiety during pregnancy.

Several caveats regarding the interpretation of the current data are in order. We cannot rule out the possibility that behavioral differences are influenced by maternal genotype (Côté et al., 2007). Postnatal antidepressant-treated mice were
generated from SERT+/- dams, while SERT-deficient mice were from SERT+-/ dams. We selected this breeding strategy because it enabled comparisons between postnatal antidepressant-treated mice and *unhandled/uninjected* SERT-deficient mice. An alternative strategy involves injecting all offspring of SERT+/- pairings with saline or an SSRI during the postnatal period (Ansorge et al., 2004; Ansorge et al., 2008). Unlike previous studies, we selected the present experimental design to avoid potentially confounding effects arising from genotype-specific changes in behavior associated with injection stress (Li et al., 1999; Tjurmina et al., 2002). All data were normalized to respective control groups matched for maternal genotype, age, and sex prior to analysis to enable comparisons across study groups.

When viewed in their entirety, these findings lead us to conclude that these two models of early serotonin system disruption lead to divergent adult phenotypes; early exposure to the SSRI S-CIT is associated with a prolonged “hyposerotonergic” state, while constitutive SERT deficiency produces a persistent “hyperserotonergic” state. Furthermore, early life exposure to different SSRIs has variable long-term behavioral consequences, *i.e.*, reduced anxiety-related behavior associated with postnatal S-CIT vs reduced activity/exploration associated with postnatal FLX. These findings necessitate further research to determine the clinical benefits/risks of SSRI exposure *in utero* in humans, as opposed to untreated maternal depression wherein the latter has known consequences (Misri et al., 2006; Weissman et al., 2006; Brand and Brennan, 2009; Hay et al., 2010).
Figures

(a) 

Δ Weight from P5

(b) 

Adult Body Weight (g)

(c) 

Adult Body Weight (g)
Figure 3-1: Female body weights. Body weights were recorded daily during postnatal administration of saline, FLX or S-CIT. (a) Modest reductions in body weight were associated with FLX administration on P6 and P20-21. (b) Postnatal administration of FLX also resulted in long-term reductions in body weight in 10-month old female mice. (c) In contrast to postnatal FLX exposure, lifelong loss of SERT expression resulted in increased body weight in 10-month-old female mice. Data are means ± SEMs. *P<0.05 with respect to saline-treated mice and **P<0.01 vs SERT+/+ mice.
**Figure 3-2: Male body weights.** During postnatal drug administration, body weights were recorded daily from postnatal day (P) 5 through P21 prior to injections. (a) Postnatal treatment with antidepressants resulted in body weight reductions associated with S-citalopram (S-CIT) from P7-P10 and P16-P21 and with FLX from P7-P21. At the time of behavioral testing, body weights were recorded for (b) postnatal SRI-treated mice and (c) SERT-deficient mice. Male mice treated with S-CIT and FLX during the postnatal period continued to show reduced body weight during early and late adulthood. No changes in body weights were observed in male SERT-deficient mice. Data are represented as means ± SEMs. *P<0.05, **P<0.01, and ***P<0.001 vs age-matched saline-treated mice.
**Figure 3-3: Anxiety-related parameters from the elevated plus maze.** From the factor analysis (correlation coefficients >0.3), factor 1 (explaining 46% of the common variance) is comprised of open arm latency, open arm time, percent open arm entries (open arm entries/total arm entries), and head dips (exploratory behavior performed on the open arms). These four parameters are shown for mice exposed to SSRIs postnatally (a-d) and SERT-deficient mice (e-h). Mice treated postnatally with S-CIT show increased percent open arm entries (b) and open arm time (c), whereas SERT+/- and SERT-/- mice show increased latencies to first open arm entry (e). Analysis of weighted Z-scores revealed opposing changes in anxiety-related behavior such that postnatal S-CIT was associated with reduced anxiety and SERT deficiency was associated with increased anxiety (ij). All data are expressed as means ± SEMs. *P<0.05 vs saline-treated or SERT+/+ mice and **P<0.01 vs SERT+/+ mice.
**Figure 3-4: Anxiety-like parameters in the open field.** Accounting for 35% of the common variance, factor 2 is composed of center/total distance traveled and time spent in the central region of the open field. Although the main effects of drug treatment were significant, no significant differences were observed between individual groups for postnatal antidepressant groups (a,b). SERT-/- mice spent less time and travel shorter distances in the center of the arena (c,d). Analysis of weighted Z-scores indicates that SERT-/- mice but not postnatal SSRI-treated mice show increased anxiety-like behavior in the open field (e,f). Data are expressed as means ± SEMs. **P<0.01 and ***P<0.001 vs SERT+/+ mice.
Figure 3-5: Locomotor and exploratory behaviors. Factor analysis indicated that total arm entries and rears in the elevated plus maze, and total distance traveled in the open field load onto Factor 3, which is responsible for 18% of the common variance. Total distance traveled and numbers of rears were significantly reduced for FLX-treated mice and SERT-/- mice. In terms of the weighted activity/exploration Z-scores, both postnatal FLX-treated mice and SERT-deficient mice were associated with reductions in these variables. All data are expressed as means ± SEMs. *$P<0.05$ and **$P<0.01$ with respect to saline-treated mice or SERT+/- mice, ***$P<0.001$ vs SERT+/- mice.
**Figure 3-6: Body temperature responses of 3-month-old female mice to 8-OH-DPAT.**

Female postnatal SSRI-exposed and SERT-deficient mice were administered two doses of 8-OH-DPAT. Time-courses show changes in body temperature over the 2-h testing period following administration of 0.4 mg/kg 8-OH-DPAT in (a) female postnatal saline-treated and SSRI-treated mice and (b) female SERT-deficient mice. Analyses of areas under the curve (c,d) were carried out for both groups of mice. Potentiated reductions in body temperature were observed in mice treated postnatally with S-CIT compared to postnatal saline- and FLX-treated mice administered 0.4 mg/kg of 8-OH-DPAT. By contrast, female SERT-/- mice did not show changes in body temperature after either dose of 8-OH-DPAT. Data are means ± SEMs. **P<0.01 vs saline-treated mice or SERT+/+ mice administered 0.4 mg/kg 8-OH-DPAT, ***P<0.001 vs SERT+/+ mice administered 0.4 mg/kg 8-OH-DPAT.
Figure 3-7: Body temperature responses of 3-month-old male mice to 8-OH-DPAT. Time-courses showing changes in body temperature after 8-OH-DPAT are shown for male (a) postnatal saline-, FLX-, and S-CIT-treated mice and (b) SERT-deficient mice. Area under the curve analyses (c,d) indicated that male mice administered 0.4 mg/kg 8-OH-DPAT show hypersensitive 5-HT1A responses whereas male SERT-/− mice show no change in body temperature after either dose of 8-OH-DPAT. Data are means ± SEMs. **P<0.01 vs saline-treated mice administered 0.4 mg/kg 8-OH-DPAT, #P<0.05 vs FLX-treated mice administered 0.4 mg/kg 8-OH-DPAT, and ***P<0.001 vs SERT+/+ mice administered 0.4 mg/kg 8-OH-DPAT.
Figure 3-8: Hypothermic responses to 8-OH-DPAT in postnatal SSRI and SERT-deficient female mice at 10 months of age. Female postnatal SSRI-treated mice challenged with 8-OH-DPAT showed reductions in body temperature but potentiated responses were not observed in postnatal S-CIT-treated 10-month-old mice female mice. Conversely, aging SERT-deficient mice continued to exhibit a lack of response to both doses of 8-OH-DPAT. Data are as means ± SEMs. ***P<0.001 vs SERT+/+ mice administered 0.4 mg/kg 8-OH-DPAT.
**Figure 3-9: 5-HT1A autoreceptor responsiveness in postnatal SSRI-treated and SERT-deficient mice.** Time-course showing changes in body temperature associated with 0.4 mg/kg of 8-OH-DPAT over the 2-h test period for 10-month-old male (a) postnatal saline-treated and SSRI-treated mice and (b) SERT-deficient mice. (c,d) Administration of 0.1 mg/kg or 0.4 mg/kg 8-OH-DPAT was associated with potentiated hypothermia in mice receiving postnatal S-CIT indicated by significantly greater areas under the curve. By contrast, SERT-/- mice a complete loss of temperature response after both doses of 8-OH-DPAT. (e) Microdialysis data indicated that subcutaneous administration of increasing doses of 8-OH-DPAT was associated with potentiated decreases in serotonin levels in mice treated postnatally with S-CIT compared to postnatal saline-treated mice in ventral hippocampus. Data are means ± SEMs. For hypothermia data, \*P<0.05, \**P<0.01 vs saline-treated mice administered 0.4 mg/kg 8-OH-DPAT, \#\#P<0.01 vs FLX-treated mice administered 0.4 mg/kg 8-OH-DPAT, \***P<0.001 vs SERT+/+ mice administered 0.4 mg/kg 8-OH-DPAT. For microdialysis data, \*P<0.05, \**P<0.01, and \***P<0.001 vs postnatal saline-treated mice.
**Figure 3-10: 5-HT1A binding and G-protein coupling.** (a) No differences in (3H)-8-OH-DPAT binding were detected with respect to postnatal SSRI administration indicating that 5-HT1A receptor expression is similar across the groups [F(2,9)=0.3, P<0.95]. (b) An 8-OH-DPAT stimulated (35S)GTPγS binding assay was used to look for differences in G-protein coupling. Two-way ANOVA with 8-OH-DPAT concentration and postnatal treatment as independent variables revealed that higher doses of 8-OH-DPAT increased (35S)-GTPγS binding [F(1,22)=16.5, P<0.001], but no differences were associated with postnatal SSRI exposure [F(2,22)=1.3, P<0.29]. Data are as means ± SEMs, N=3-4 per group.
**Figure 3-11: No net flux determination of extracellular serotonin levels.** Linear regression analyses of the difference between the concentration of serotonin infused into microdialysis probe (Cin) and the dialysate serotonin concentration (Cout) vs Cin in the CA3 region (ventral) of the hippocampus. No-net-flux data are for (a) postnatal antidepressant-treated mice and (b) SERT-deficient mice. The x-intercept was used to determine extracellular serotonin levels corrected for extraction fraction. Extracellular serotonin determined by no-net-flux (c,d) were shown to be reduced in postnatal S-CIT-treated mice compared to postnatal saline-treated mice at 10-14 months of age. By contrast, extracellular serotonin was increased in SERT+/- and SERT-/− mice compared to SERT+/+ mice. (e,f) Similar changes were observed in basal dialysate serotonin levels. Data are expressed as means ± SEMs. *P<0.05 vs postnatal saline-treated mice or SERT+/+ mice, **P<0.01 and ***P<0.001 vs SERT+/+ mice.
Expression of serotonin transcription factors

Serotonergic phenotype markers and SERT expression

Transient SERT Expression

Increasing SERT function

Developmental Models of Serotonin Contributing to Adulthood Emotionality

Conception

Death

S-Citalopram Exposure

Presynaptic

Postsynaptic

HYPOSEROTONERGIC

HYPERSEROTONERGIC

SERT Reductions

Constitutive SERT

Anxiety

EPM

serotonin

serotonin transporter

5-HT1A receptor

P5

P21

E10

E12

E15

birth

P5

P21

Early Adulthood

Late Adulthood

P5

P21
Figure 3-12: Effects of developmental serotonin transporter disruptions on serotonergic circuitry and emotional phenotypes in mice. A role for serotonin in mediating several developmental processes has been established. Serotonin emerges early during embryonic development (~embryonic (E) day 12.5) and is preceded by transcription factors (e.g. Pet1, LmxB, and GATA3) necessary for serotonergic phenotype determination. Serotonin transporter (SERT) expression in nonserotonergic regions occurs briefly during development, predominately in thalamic glutamatergic neurons that establish sensory modalities. Furthermore, evidence for varied SERT function throughout adulthood exists. Here, we demonstrate that blocking SERT during postnatal (P) life (P5-P21) with the serotonin-reuptake inhibitor (SSRI) S-citalopram in mice produces behavioral and serotonergic circuitry phenotypes that are differentiated from those arising from constitutive SERT deficiency. Specifically, the former model of developmental SERT disruption results in hyposerotonemia accompanied by reduced anxiety on the elevated plus maze (EPM). This directly contrasts characteristics of a hyperserotonergic and enhanced anxiety-like state exhibited in mice that contain lifelong reductions in SERT.
### Tables

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Elevated Plus Maze (EPM)
Forced Swim Test (FST)
Open Field Test (OFT)

Factor loadings >0.3 displayed
Table 3-1. Factor analysis. Three major factors were found to account for the majority of the common variance in the overall behavior set consisting of 10 variables from the elevated plus maze, open field, and forced swim test. Standardized regression correlation >0.3 indicated variables with significant loading onto particular factors. The analysis revealed that open arm latency, open arm time, percent open arm entries (open/total arm entries) and numbers of head dips loaded onto Factor 1 (Anxiety 1 Behavior), which accounted for 46% of the variance. Factor 2 (36% of the variance), referred to as Anxiety 2 Behavior, was specified by center time and center/total distance traveled in the open field. Factor 3 (Activity/Exploratory Behavior) was associated with 18% of the common variance with loadings from total arm entries and rears in the elevated plus maze, and total distance traveled in the open field.
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Chapter 4

Rethinking 5-HT1A receptors: Emerging modes of inhibitory feedback of relevance to emotion-related behavior

4.1 Introduction and Historical Perspective

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter and neurohormone formed by the hydroxylation and subsequent decarboxylation of the essential dietary amino acid L-tryptophan. Serotonin is found primarily in the gastrointestinal tract, platelets, blood vessels, thyroid, pancreas, mammary glands, (Gershon, 2000; Walther et al., 2003; Cote et al., 2007; Berger et al., 2009; Paulmann et al., 2009) and central nervous system (CNS) (Hensler, 2006; Deneris and Wyler, 2012). In the brain, serotonin is thought to be a key modulatory neurotransmitter involved in the regulation of numerous physiological and behavioral processes including mood- and anxiety-related behavior, cognitive function, food intake, sexual behavior, sleep, cardiovascular function, blood pressure, pain, body temperature, and others (Veasey et al., 1995; Jonnakuty and Gragnoli, 2008; Lam et al., 2010; Watts et al., 2012).

Serotonin was first reported in 1937 by Vialli and Erspamer and named enteramine (Vialli, 1937, 1940). In 1948, it was identified as a vasoconstrictor in blood serum where it
was referred to as "serotonin" (Rapport et al., 1948). Afterwards, scientists realized that enteramine and serotonin were one in the same (Erspermer and Asero, 1952, 1953). Serotonin was recognized as a neurotransmitter when it was discovered in extracts from mammalian brain (Twarog and Page, 1953; Amin et al., 1954). In 1986, the pharmacology of serotonin was reviewed (Bradley et al., 1986) and for the first time, the existence of three receptor families (5-HT1–3) was described; additional families were suspected. It is now known that the effects of serotonin are mediated by at least 14 different receptors, which are grouped into subfamilies based on pharmacological responses to specific ligands, sequence similarities at the gene and amino acid levels, gene organization, and second messenger coupling pathways (Hannon and Hoyer, 2008; Nichols and Nichols, 2008; Filip and Bader, 2009; Pytliak et al., 2011). Serotonin receptors are assigned to one of seven families, 5-HT1–7, with individual subtypes further designated by letters.

Among serotonin receptors, much attention has been focused on the 1A subtype (5-HT1A). The human 5-HT1A receptor was cloned in 1987 as a single intronless gene (Kobilka et al., 1987) located on chromosome 5 (5q11.2-q13). In mice, the Htr1a gene resides on the distal part of chromosome 13. The 5-HT1A receptor protein consists of 422 amino acids. Evidence from human and rodent studies suggests that 5-HT1A receptors are implicated in a variety of physiological and pathological processes, such as learning, memory, schizophrenia, Parkinson disease, and notably in the etiology and treatment of mood and anxiety disorders (Strobel et al., 2003; Gordon and Hen, 2004; Lesch and Gutknecht, 2004; Hirvonen et al., 2008; Le Francois et al., 2008; Akimova et al., 2009; Polter and Li, 2010; Ohno, 2011).
4.2 Adult Expression and Function of 5-HT1A Receptors

4.2.1 Direct Autonomous Inhibition

In the mammalian brain, 5-HT1A receptors are divided into two distinct classes based on localization. The cell bodies of central serotonergic neurons are found in the raphe nuclei in the brain stem (Fig. 1A) (Sotelo et al., 1990; Jans et al., 2007), where 5-HT1A receptors are located on soma and dendrites (Pazos and Palacios, 1985; Kia et al., 1996; Riad et al., 2000). These 5-HT1A autoreceptors exert inhibitory feedback in response to local release of 5-HT in the raphe nuclei from axonal collaterals (Fig. 1B) (Wang and Aghajanian, 1977; Verge et al., 1985; Sprouse and Aghajanian, 1986; Meller et al., 1990; Hjorth and Sharp, 1991; Pineyro and Blier, 1999). Additionally, sources of local serotonin release have been proposed to arise from somatic and dendritic sites on serotonergic neurons (de Kock et al., 2006; Colgan et al., 2009; Colgan et al., 2012). Serotonin release in the cell body region results in reductions in serotonergic pacemaker activity and suppression of serotonin synthesis, turnover, and release in projection areas (Blier and de Montigny, 1987; Kennett et al., 1987; Sprouse and Aghajanian, 1988; Bohmaker et al., 1993; Jolas et al., 1993). In support of this, constitutive 5-HT1A knockout mice show increased rates of serotonin neuronal discharge (Richer et al., 2002) and elevated basal dialysate serotonin levels in frontal cortex and hippocampus (Parsons et al., 2001).

4.2.2 Indirect Inhibition from Medial Prefrontal Cortex

5-HT1A receptors are also expressed by non-serotonergic pyramidal, GABAergic, and cholinergic neurons (Cassel and Jeltsch, 1995; Azmitia et al., 1996; Santana et al., 2004; Palchaudhuri and Flugge, 2005; Artigas et al., 2006) in limbic regions such as prefrontal
cortex, hippocampus, lateral septum, and amygdala, as well as in several hypothalamic and thalamic nuclei (Fig. 4-1A) (Hamon et al., 1990; Beck et al., 1992; Riad et al., 2000). Activation of 5-HT1A heteroreceptors mediates hyperpolarizing responses to released serotonin, which typically reduces postsynaptic neuronal excitability and firing rates (Sprouse and Aghajanian, 1986; Hamon et al., 1990; Riad et al., 2000). As such, 5-HT1A heteroreceptors are involved in the modulation of other neurotransmission systems. For example, 5-HT1A receptors in the medial prefrontal cortex (mPFC) modulate dopamine cell firing and release (Sakaue et al., 2000; Diaz-Mataix et al., 2005; Diaz-Mataix et al., 2006).

In addition to direct/local 5-HT1A receptor-mediated autoregulation, evidence exists for an indirect negative feedback mechanism that involves 5-HT1A heteroreceptors in the mPFC (Fig. 4-1B) (Hajos et al., 1999; Celada et al., 2001; Sharp et al., 2007). The mPFC-dorsal raphe nucleus (DRN) pathway comprises glutamatergic descending projections that are hypothesized to decrease serotonin cell firing by activating DRN GABAergic interneurons to inhibit 5-HT release (Borsini et al., 1995; Hajos et al., 1998; Casanovas et al., 1999; Hajos et al., 1999; Celada et al., 2001; Stratz et al., 2001; Varga et al., 2001; Jankowski and Sesack, 2004). This pathway has been suggested as one of the neuroanatomical substrates controlling the effects of stress by preventing overactivation of DRN serotonergic neurons (Amat et al., 2005). A minor glutamatergic pathway may also exist from the mPFC that excites 5-HT cell firing directly in the DRN (Hajos et al., 1998; Jankowski and Sesack, 2004). It is still not well understood how inhibitory 5-HT1A heteroreceptors excite cortical glutamatergic neurons. The most plausible explanation is the disinhibition of glutamatergic neurons via mPFC GABA interneurons expressing 5-
HT1A heteroreceptors (Aznar et al., 2003; Santana et al., 2004). However, further studies are required to determine the specific mechanisms of 5-HT1A receptors in the mPFC.

4.2.3 Intracellular Signaling

5-HT1A receptors are inhibitory G-protein coupled receptors (GPCRs). Early studies identified that 5-HT1A receptors function by coupling to Gi/Go proteins in most cells (De Vivo and Maayani, 1986). Extracellular receptor binding of serotonin or 5-HT1A agonists leads to intracellular exchange of GDP for GTP on Gi/Go alpha subunits. This, in turn, inhibits adenylyl cyclase, which reduces cAMP levels and protein kinase A activity (Cooper and Londos, 1982). Furthermore, agonist-induced activation of 5-HT1A receptors results in potassium channel activation and calcium channel inhibition (Hamon et al., 1990; Haj-Dahmane et al., 1991; Barnes and Sharp, 1999; Lanfumey and Hamon, 2000; Albert and Robillard, 2002; Polter and Li, 2010). 5-HT1A receptors are coupled to different GPCR pathways based on localization (Mannoury la Cour et al., 2006). 5-HT1A autoreceptors couple exclusively to Gαi3 leading to partial inhibition of adenylyl cyclase (Liu et al., 1999; Palego et al., 1999; Marazziti et al., 2002; Valdizan et al., 2010), an effect that may depend on specific agonists (Clarke et al., 1996; Johnson et al., 1997; Marazziti et al., 2002). By contrast, 5-HT1A heteroreceptors couple mainly to Gαo in the hippocampus, and equally to Gαo and Gαi3 in cerebral cortex (Mannoury la Cour et al., 2001). Differences in 5-HT1A Gα subunit coupling might explain regional differences in activation vs inhibition of intracellular signaling pathways. 5-HT1A autoreceptor desensitization is more pronounced compared to 5-HT1A heteroreceptor desensitization, which also could be related to differential Gα coupling (Chaput et al., 1986; Mannoury la Cour et al., 2001). Stimulation of
5-HT1A receptors also leads to activation of G-protein-coupled inward rectifying potassium channels (GIRKs) (Luscher et al., 1997) in raphe neurons (Clarke et al., 1987; Kelly et al., 1991; Clarke et al., 1996; Bayliss et al., 1997; Katayama et al., 1997; Loucif et al., 2006) and hippocampus (Andrade and Nicoll, 1987; Colino and Halliwell, 1987; Oleskevich, 1995). Whether 5-HT1A receptors fully couple to inhibit adenylyl cyclase remains controversial, as it has been suggested that the hyperpolarizing response mediated by 5-HT1A autoreceptors is due to the activation of GIRK channels via G-protein βγ subunits (Kovoor and Lester, 2002).

In addition to their canonical function, 5-HT1A receptors activate growth factor-regulated signaling pathways, such as mitogen-activated protein kinases (MAPK) and Akt signaling pathways (Polter and Li, 2010). In MAPK signaling, ERK is preferentially affected by 5-HT1A receptors. For example, in RN46A cells, a model of serotonergic raphe neurons that express endogenous 5-HT1A receptors, adenylyl cyclase and ERK1/2 phosphorylation were inhibited by 5-HT1A-receptor activation (Kushwaha and Albert, 2005). However, in hippocampal-derived differentiated HN2-5 cells, 5-HT1A agonists increased ERK phosphorylation and activity (Adayev et al., 1999). These and other studies suggest that the modulation of ERK may depend on neuronal origin, as well as maturation states (Albert and Tiberi, 2001; Cowen et al., 2005; Druse et al., 2005; Chang et al., 2009; Newman-Tancredi et al., 2009).

The Akt signaling pathway is also activated in 5-HT1A receptor-expressing cells and primary hippocampal neurons (Cowen et al., 2005; Hsiung et al., 2005; Hsiung et al., 2008). Activation of Akt by 5-HT1A receptors led to inactivation of GSK3β in hippocampal cultures, (Cowen et al., 2005; Chen et al., 2007; Talbot et al., 2010) an effect also observed
in raphe cultures (Druse et al., 2005). Recently, it has been suggested that the GSK3-regulating effects of 5-HT1A receptors are mediated by the PI3K/Akt signaling pathway (Polter et al., 2012). Taken together, 5-HT1A autoreceptors and heteroreceptors have diverse intracellular signaling capabilities contributing to the complex regulation of the serotonin system, as well as neuronal networks modulated by serotonin.

### 4.3 5-HT1A Receptors in Development

Serotonin is a morphogenic factor (Lauder et al., 1982) and alteration of serotonin levels during early developmental windows has been shown to influence mood- and anxiety-related behavior in adult animals (Oberlander et al., 2009; Trowbridge et al., 2011; van Kleef et al., 2012). Thus, mapping and understanding factors that regulate the developmental trajectory of 5-HT1A receptor expression are important given that autoreceptors modulate serotonin release and heteroreceptors are widely expressed in emotion-related circuits, where they mediate the effects of released serotonin. In rodents, 5-HT1A receptors appear in early embryonic development. Using *in situ* hybridization and immunocytochemistry, 5-HT1A mRNA has been detected as soon as embryonic day (E) 12 and 5-HT1A receptor protein by E14 in neuronal cultures prepared from brain stem (Hillion et al., 1994). A surge in 5-HT1A mRNA levels in the brain stem occurs beginning on E13, with peak levels occurring at E15-16 (Hillion et al., 1993). Developmentally related changes in 5-HT1A-receptor expression also occur at postsynaptic sites. Transient expression is evident in the septum and preoptic region during embryonic development (Bonnin et al., 2006). 5-HT1A expression occurs at high levels in the cerebellum during the first two postnatal weeks tapering off to near undetectable levels by P21 (Daval et al., 1987;
Miquel et al., 1994). In brain regions associated with the regulation of mood and anxiety, 5-HT1A-receptor expression likewise exhibits complex patterns during development. *In situ* hybridization and [3H]-8-OH-DPAT binding both indicate low 5-HT1A expression in the dentate gyrus granule cell layer of the hippocampus at E14.5, with levels gradually increasing during the first few postnatal weeks and reaching near adult levels by P13 (Daval et al., 1987; Miquel et al., 1994; Patel and Zhou, 2005; Bonnin et al., 2006).

Reports on the ontogeny of 5-HT1A receptors in the developing human brain, albeit fewer are in agreement with those on rodents. In one study, [3H]-8-OH-DPAT binding was used to examine 5-HT1A receptor expression from tissue acquired at different stages of gestation (Bar-Peled et al., 1991). Although expression levels varied with respect to gestational age (16-22 weeks), the relative regional distribution of 5-HT1A receptors was similar across time points. The highest receptor densities occurred in subregions of the hippocampus and frontal cortex, where a surge in 5-HT1A expression was observed at 18-22 weeks of gestation resulting in levels 3-4 times higher than those reported in adults (Bar-Peled et al., 1991). Others have used *in situ* hybridization to compare 5-HT1A receptor mRNA levels in human tissue collected at ~28 weeks of gestation compared to 6-7 years of age (del Olmo et al., 1998). Human fetal brain contained the highest 5-HT1A mRNA levels in raphe nuclei, cerebellum, and the CA1 and dentate gyrus regions of the hippocampus. When compared to later ages, only expression in the cerebellum showed dramatic changes, with mRNA levels being lower in children and undetectable in adults (del Olmo et al., 1994; del Olmo et al., 1998). The latter is similar to the cerebellar expression pattern reported in rodents. Thus, 5-HT1A-receptor expression undergoes tight temporal regulation and in
some regions, brief emergence and disappearance, suggesting morphogenic influences, especially in areas of the brain involved in modulating anxiety levels in adults.

A few studies have investigated functional aspects of 5-HT1A receptors during development by means of electrophysiology or pharmacologic challenge. Electrophysiological recordings in the prefrontal cortex of rats indicated that 5-HT1A receptor-mediated hyperpolarization appears late in postnatal development between P16-P19 corresponding temporally with increases in receptor protein expression (Daval et al., 1987; Miquel et al., 1994; Beique et al., 2004; Bonnin et al., 2006). In mice, 5-HT1A-receptor mediated outward currents were not evident at P4 but were observed by P12 (Calizo et al., 2010). 5-HT1A responses developed after the appearance of physiology characteristic of serotonergic neurons, which appeared to be fully developed by P12. 5-HT1A receptor-signaling pathways important for mediating developmental processes are complex. For example, 8-OH-DPAT-induced activation of the MAPK pathway, and specifically ERK1/2 kinases, requires different PKC isozymes to regulate distinct developmental processes (e.g., cell division vs synaptic activity/strengthening) at P6 vs P15 (Mehta et al., 2007). More work will be needed to understand functional changes in 5-HT1A mediated pre- and post-synaptic responses during postnatal development, particularly in terms of critical developmental windows important for shaping and determining anxiety-related behavior later in life.
4.4 5-HT1A Receptors in Mood and Anxiety

4.4.1 Mechanisms of Antidepressants

5-HT1A autoreceptors play an important role in regulating serotonergic activity through feedback inhibition pathways. As such, the involvement of 5-HT1A receptors in the mechanism of action of antidepressants has been widely investigated, and literature supporting a role for this receptor population in the delayed efficacy of antidepressants has evolved. 5-HT1A autoreceptors (and possibly heteroreceptors) limit increases in extracellular 5-HT levels induced by SSRIs. Even after chronic SSRI treatment, 5-HT1A autoreceptors maintain some control over serotonin release (Gardier et al., 1996; Hjorth et al., 1997; Trillat et al., 1998; Hervas et al., 2000; Popa et al., 2010).

The delayed efficacy of SSRIs has been partially attributed to the need for desensitization of 5-HT1A autoreceptors in the raphe nuclei, enabling firing rates of serotonergic neurons to overcome inhibition (Blier and de Montigny, 1983b, a; Artigas et al., 1996; Gardier et al., 1996; Dawson et al., 2000; El Mansari et al., 2005). However, only ~50% of studies in rodents show increased extracellular serotonin after chronic administration of serotonin reuptake inhibiting antidepressants (Luellen et al., 2010). In this context, concomitant administration of 5-HT1A receptor antagonists with SSRIs has been hypothesized to hasten or potentiate changes in serotonin levels to improve clinical efficacy (Artigas et al., 1994; Artigas et al., 2006). The ability of the partial 5-HT1A antagonist, pindolol, to accelerate antidepressant clinical efficacy has been investigated in patients with major depressive disorder. Meta-analyses suggest that pindolol administered in combination with SSRIs augments and accelerates symptomatic improvement after 2
weeks of treatment specifically in patients without prior history of treatment (Ballesteros and Callado, 2004; Whale et al., 2010; Portella et al., 2011).

However, not all findings support a role for 5-HT1A autoreceptor desensitization and its effects to accelerate SSRI treatment. For example, administration of pindolol in combination with SSRIs to patients with treatment resistant depression or extensive treatment histories does not hasten or produce symptom amelioration (Segrave and Nathan, 2005; Portella et al., 2009). Human studies examining the role of 5-HT1A receptors in the treatment and etiology of major depressive disorder indicate that sensitivity to receptor signaling after administration of agonists are complicated based on reports of reduced receptor number and binding, as well as genetic influences (Savitz et al., 2009). Additionally, mechanisms of autoreceptor desensitization are not clear and may differ depending on the antidepressant administered. For example, chronic fluoxetine results in desensitization of autoreceptors, which is associated with a reduction in receptor stimulated [35S]GTPγS binding (Pejchal et al., 2002; Shen et al., 2002; Castro et al., 2003; Rossi et al., 2008). However, G-protein coupling is not altered in association with administration of the SSRI sertraline, indicating that antidepressants differ with regard to mechanisms of 5-HT1A desensitization (Rossi et al., 2008).

In addition to conflicting findings regarding 5-HT1A autoreceptor desensitization in clinical studies, desensitization occurs in animal models of depression generated via chronic stress paradigms (Lanfumey et al., 1999; Grippo et al., 2005; Evrard et al., 2006; Bambico et al., 2009; Rozeske et al., 2011). 5-HT1A autoreceptors and heteroreceptors are also desensitized in serotonin transporter knockout mice in association with a phenotype
characterized by enhanced anxiety (Li et al., 1999; Li et al., 2000; Holmes et al., 2003; Kalueff et al., 2010). Together, these studies highlight the complex regulation of 5-HT1A pathways, such that desensitization may be common to both the treatment and precipitating etiological factors associated with major depressive disorder.

4.4.2 Animal Models of 5-HT1A Disruption

In light of data suggesting a role for 5-HT1A receptors in mediating antidepressant responses in humans, it is logical to suspect that these receptors also function in the neurocircuitry and pathophysiology of emotion-related behavior. Genetic manipulation of the murine Htr1A gene has been carried out to study the behavioral effects of receptor under- or overexpression. In both cases, the majority of data point to a role for 5-HT1A receptors in anxiety-related behavior (see Table B-1, Appendix B) provides a comprehensive summary of the literature to date focused on anxiety- and depression-like behaviors, and learning and memory in animal models of altered 5-HT1A expression. Taken together, these studies provide convincing evidence that global reductions in 5-HT1A receptor expression lead to increased anxiety-related behavior in adult animals across different strains of mice and behavior test paradigms.

The first reports on mice with reduced 5-HT1A expression appeared simultaneously in 1998. Three separate laboratories produced constitutive 5-HT1A knockout mice, each in a different background strain (See Table B-1, Appendix B) (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Consistent changes in behavior were observed in tests for anxiety-related behavior associated with conflict in novel environments, including exploration in the center of the open field or the open arms of the elevated plus maze.
Changes in behavior were more consistently replicated across tests in male vs female mice. In terms of depression-like behaviors, mice with reduced 5-HT1A expression displayed reduced immobility in the forced swim (Parks et al., 1998; Ramboz et al., 1998) and tail suspension tests (Heisler et al., 1998). However, interpretations of these behavioral changes were not in agreement. Some authors perceived decreased immobility as an increased stress response, (Parks et al., 1998) while others took a more traditional view of reduced immobility (Porsolt et al., 1979) as indicative of decreased learned helplessness/behavioral despair or an increased antidepressant-like response (Heisler et al., 1998; Parks et al., 1998).

Following these initial reports, others confirmed increased anxiety-like behavior in 5-HT1A knockout mice when presented with conflict situations (see Table B-1, Appendix B for a full list of references). Findings also included modestly enhanced fear responses to stressful stimuli such as foot shock (Gross et al., 2000; Groenink et al., 2003; Klemenhagen et al., 2006; Tsetsenis et al., 2007). In terms of learning and memory, mice with reduced 5-HT1A expression displayed deficits in hippocampal-dependent learning indicated by increased latencies and path lengths to find the hidden platform in the Morris water maze (Sarnyai et al., 2000; Wolff et al., 2004). As opposed to mice with constitutive deletion of 5-HT1A receptors, mice with developmentally limited overexpression of 5-HT1A heteroreceptors in dentate gyrus and cortex also displayed reduced anxiety in novel, conflict environments (Kusserow et al., 2004). By contrast, mice with permanent overexpression failed to show changes in anxiety-like behaviors (Bert et al., 2006). Similar to 5-HT1A knockout mice, transient 5-HT1A overexpression resulted in deficits in hippocampal-dependent learning and memory in the Morris water maze (Bert et al., 2005).
Deficits were less pronounced or absent in permanent overexpressing mice (Bert et al., 2008). Information pertaining to changes in learning and memory after 5-HT1A receptor manipulation is reviewed in detail elsewhere (Bert et al., 2008; Ogren et al., 2008).

Recently, studies have been carried out to delete specific subpopulations of 5-HT1A receptors enabling the differentiation of pre- vs postsynaptic behavioral effects (Richardson-Jones et al., 2011). When 5-HT1A autoreceptors were selectively inactivated, mice exhibited increased anxiety-like behavior similar to constitutive knockout mice. These findings highlight the importance of raphe 5-HT1A receptors in mediating anxiety responses. By contrast, when 5-HT1A heteroreceptors were specifically targeted, normal anxiety-like behavior was observed, however, increased depression-like behavior was observed in the forced swim test (Richardson-Jones et al., 2011). Increased anhedonic behavior related to reduced consumption of palatable food has also been observed in mice with 5-HT1A heteroreceptor knockout. The latter findings on depression-related behavior illustrate an interesting dichotomy since mice with constitutive deletion of 5-HT1A auto- and heteroreceptors show decreases in depression-like behavior (Appendix B, Table B-1). Thus, the synergistic effects of genetically inactivating auto- and hetero 5-HT1A receptors cannot be predicted by studying knockout of either receptor population in isolation.

In addition to mice with complete loss of 5-HT1A autoreceptors, mice with 30% reductions in 5-HT1A autoreceptor expression have been generated (Richardson-Jones et al., 2010). These mice display potentiated responses to the administration of fluoxetine as indicated by increased dialysate serotonin levels. These findings are important in the context of human *HTR1A* gene polymorphisms, including the C(-1019)G *HTR1A* single
nucleotide polymorphism, (Lemonde et al., 2003; Lemonde et al., 2004; Blier, 2010) which is hypothesized to affect the expression and function of 5-HT1A receptors and to contribute to the high numbers of antidepressant nonresponders (Rush et al., 2009).

4.5 New Perspectives on 5-HT1A Autoreceptors

4.5.1 Networks and 5-HT1A Inhibitory Function

It is widely accepted that 5-HT1A receptors located on serotonergic neurons participate in autoinhibition. However, a more complex picture of inhibitory function than simple one-to-one regulation of serotonin cell firing is emerging. Serotonin neurons are anatomically heterogeneous and their projections to the forebrain are topographically organized (Jensen et al., 2008; Sperling and Commons, 2011; Waselus et al., 2011; Bang et al., 2012). Furthermore, distinct, reproducible patterns of serotonin neuron activation within the raphe nuclei can be seen in response to specific external stimuli, suggesting that anatomical topography underlies functional topography (Vasudeva et al., 2011; Waselus et al., 2011). Additionally, microdialysis studies examining extracellular serotonin levels after the application of various stressors have supported the idea of defined network patterns of serotonin neurons such that stress-induced changes in serotonin levels are stressor- and region specific (Adell et al., 1997). These observations suggest that feedback inhibitory pathways might work to control serotonin-network activity. In one model, raphe serotonin neurons exhibit autonomous feedback such that groups of functionally similar serotonergic neurons regulate themselves in a homeostatic manner (Fig. 4-1C) (Bang et al., 2012).

Non-autonomous feedback has also been suggested in which crosstalk between distinct groups of serotonin neurons provides “lateral inhibition” influencing patterns of
activation (Fig. 4-1C) (Bang et al., 2012). Along these lines, recent work by Commons and colleagues has demonstrated complementary patterns of activation of serotonergic neurons after exposure and withdrawal to nicotine, which are different and reciprocally switched after 5-HT1A receptor blockade (Sperling and Commons, 2011). These findings suggest that endogenous feedback inhibition provided by 5-HT1A receptors might be regionally organized and depend on behavioral states. While these observations do not exclude an autoregulatory function, they raise the possibility that 5-HT1A receptors might also operate in a non-autonomous fashion to mediate communication between different groups of serotonin neurons, thus suggesting that 5-HT1A receptors are involved in a regional form of autoregulation between different raphe subfields. These studies are consistent with anatomical studies showing that there are many interconnections between the different raphe nuclei (Bang et al., 2012) and axon collaterals of serotonin neurons travel for some distance within the dorsal raphe nuclei (Li et al., 2001).

There are also a number of distinct serotonin subsystems with unique genetic programming and functions (Gaspar and Lillesaar, 2012). Recent studies have shown that in raphe nuclei, there are at least three different serotonin cell-types grouped by anatomical, physiological, and molecular characteristics, and their distribution transcends the traditional anatomical classification of raphe subfields. Furthermore, forebrain regions receiving serotonergic projections are innervated by serotonin neurons with distinct characteristics, forming a highly organized circuit (Kiyasova et al., 2012). Therefore, understanding and identifying functionally specific axon collaterals within the raphe that are involved in controlling emotional behaviors is likely to be an important future direction of research.
4.6 Future Prospects

4.6.1 Targeting Specific Receptor Populations

The evolving distinctions between the specific and diverse roles for 5-HT1A auto- vs heteroreceptors and the manner in which different populations of neurons expressing 5-HT1A receptors elicit control over serotonergic tone is now shifting away from simplified views centered on autonomous autoreceptor-mediated feedback inhibition. Classical definitions of autoreceptors, which have long been associated with controlling serotonergic firing rates via feedback inhibition, remain an important functional property of these receptors. Yet, rather than one-to-one associations, emerging findings point to the idea that inhibitory regulation is also more complex and might operate at several levels that involve multiple pathways and/or networks that influence neighboring circuits.

As the rich complexity of 5-HT1A receptor organization and function continues to be uncovered, optimizing treatment strategies for mood and anxiety disorders by preferentially targeting different 5-HT1A receptor populations or networks to produce therapeutic effects should become possible. For example, the novel 5-HT1A agonist, F15599, has been shown to reduce immobility in the forced swim test (Assie et al., 2010) and to promote these effects primarily through activation of 5-HT1A heteroreceptors localized in the frontal cortex (Newman-Tancredi et al., 2009). These findings support the idea that in addition to 5-HT1A autoreceptor desensitization following antidepressant treatment, increased 5-HT1A heteroreceptor activity occurs to promote antidepressant efficacy (Blier et al., 1997; Haddjeri et al., 1998; Haddjeri et al., 1999a; Haddjeri et al., 1999b). Furthermore, in support of the roles of 5-HT1A autoreceptors in mood and
anxiety-related behavior, recent advances using siRNA to silence 5-HT1A autoreceptor expression have been shown to ameliorate immobility associated with learned helplessness and to augment fluoxetine-induced increases in serotonin levels in postsynaptic regions (Bortolozzi et al., 2012; Ferres-Coy et al., 2012).

4.6.2 5-HT1A Receptors: A View of the Future

The serotonin system, and 5-HT1A receptors in particular, exhibit properties that continue to challenge and enlarge our understanding of receptor function. In addition to direct autonomous inhibition, evidence now exists for indirect inhibition of raphe serotonergic neurons arising from a mPFC-DRN pathway. It will be informative to sort out whether direct and indirect pathways work independently or synergistically to limit serotonin release in the mPFC. Moreover, the actions of serotonin might be coordinated across different brain regions as part of a global circuitry via lateral inhibition within and across subpopulations of raphe neurons, some of which have different developmental origins and genetic lineages. Thus, a picture of 5-HT1A receptor-mediated control of emotion-related behavior via coordination of brain-wide networks is coming to light. Still, additional work will be needed to uncover precise interactions between different parts of this network, hierarchical principles of 5-HT1A receptor function, and early-life sequencing with regard to lasting consequences for network development.

Many animal models of altered 5-HT1A receptor expression have been created. Taken together, studies on these models highlight two important aspects of 5-HT1A receptors in regard to anxiety-related behavior. First, most studies point to 5-HT1A receptors, particularly during postnatal development, as key regulators of anxiety (see
supplemental Table 1). By contrast, pharmacologic inhibition or genetic manipulation of 5-HT1A receptors in adult animals fails to produce anxiogenic behavior (Gross et al., 2002; Lo Iacono and Gross, 2008). Second, unraveling the significance of 5-HT1A auto- vs heteroreceptors in regard to establishing baseline anxiety behavior is important. Null 5-HT1A mutant mice in which 5-HT1A heteroreceptors were ectopically overexpressed during development (Kusserow et al., 2004) or where 5-HT1A receptor expression was restored in forebrain regions, e.g., hippocampus, cortex, striatum, (Gross et al., 2002) exhibit reversal or rescue of increased anxiety behavior, respectively. However, the importance of heteroreceptors in gain-of-function experiments has been questioned by recent tissue-specific conditional knockout strategies where 5-HT1A autoreceptors, but not heteroreceptors, have been shown to be critical components for establishing normal levels of anxiety (Richardson-Jones et al., 2011). As additional information on the specific roles of different 5-HT1A receptor subpopulations and their influence over emotion-related behaviors during various timeframes become clear, avenues for novel treatment strategies for mood and anxiety disorder should become evident.

Further, as we sort out which 5-HT1A receptor inhibitory circuits play key roles in shaping specific types of behavior, these are also expected to become targets for the development of more selective and hopefully, more effective therapeutics. Recent findings have shown that different populations of 5-HT1A receptors are coupled to different/multiple intracellular signaling pathways. This suggests the possibility of developing allosteric modulators or other types of small-molecule drugs that modify specific intracellular signaling pathways in cases where a particular type of receptor is
coupled to more than one pathway in the same cell type or to different $G\alpha$ subunits in different cells types.

Finally, the serotonin system remains unique among known neurotransmitter systems in that it is the only system identified as having two molecularly distinct autoreceptors. In addition to 5-HT1A receptors, serotonergic function is regulated by 5-HT1B autoreceptors, which are expressed presynaptically on serotonin axon terminals, in addition to postsynaptic axonal localization associated with dopaminergic, GABAergic, and glutamatergic systems (Sari, 2004). Thus, both 5-HT1A and 5-HT1B receptors function as autoreceptors and heteroreceptors. It is tempting to think that 5-HT1B receptors might exhibit complexity similar to 5-HT1A receptors in terms of multiple inhibitory circuits or hierarchical organization. Perhaps, continued investigation of 5-HT1A (and 5-HT1B) receptors, as well as other receptor subtypes that function both as auto- and heteroreceptors, e.g., dopamine D2 and noradrenergic alpha2 receptors, will reveal additional modes of regulatory feedback and network organization. Nonetheless, it appears that recent advances in understanding 5-HT1A receptor function lead the way in terms of extending and expanding our thinking as to how behavioral circuits are organized and controlled by a specific receptor subtype. They also provide exciting new opportunities for drug discovery and development, particularly in the treatment of mood and anxiety disorders.
**Figure 4-1: Inhibitory mechanisms of 5-HT$_{1A}$ autoreceptors.** (A) Serotonergic cell bodies expressing 5-HT$_{1A}$ autoreceptors are located deep within the brainstem. Serotonin neurons projecting to the forebrain are organized into two main clusters designated as dorsal and median raphe nuclei with distinct subpopulations within these primary nuclei. 5-HT$_{1A}$ heteroreceptors are localized postsynaptically in various brain regions including the hippocampus (HIP), prefrontal cortex (PFC), thalamus (TH), lateral septum (SEP), amygdala (AMYG), and hypothalamic nuclei (HYP). Many of these regions have been associated with the pathophysiology of mood and anxiety disorders. (B) The top panel depicts a “conventional” serotonin synapse. Here, activation of 5-HT$_{1A}$ autoreceptors controls serotonergic tone via one-to-one autoinhibitory feedback to reduce firing rates of serotonin neurons. 5-HT$_{1A}$ heteroreceptors also regulate serotonergic activity through descending glutamatergic projections originating in the medial prefrontal cortex (mPFC). This pathway makes connections with serotonin neurons via brainstem GABAergic inhibitory interneurons. (C) Current hypotheses regarding inhibitory mechanisms of 5-HT$_{1A}$ autoreceptor activation paint a more complex picture. For instance, emerging evidence suggests that serotonin neurons within a subpopulation or even across subpopulations affect each other via “lateral inhibition”. Taken together, new perspectives on the functional aspects of 5-HT$_{1A}$ receptors associated with regulation of serotonergic activity are important avenues for future investigation, particularly regarding increased understanding of the roles of 5-HT$_{1A}$ receptors in the etiology and treatment of psychiatric disorders.
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Chapter 5

Real-time quantitative PCR to measure BDNF and SERT mRNA levels in models of SERT deficiency

BDNF mRNA Levels after Genetic and Pharmacologic SERT Inhibition

5.1 Background

Since the discovery of BDNF 25 years ago as a neurotrophin promoting growth and survival of specific neuronal populations during development, its known functions in the brain have rapidly expanded to include roles in synaptic plasticity and various neurological and psychiatric disorders (Barde et al., 1982; Alfonso et al., 2005; Wong et al., 2010). BDNF is highly conserved in vertebrates and shares sequence homology with nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4). The precursor form of BDNF (proBDNF) is typically anterogradely transported to nerve terminals in large dense core vesicles where its release occurs by regulated and constitutive mechanisms, followed by proteolytic cleavage to generate the mature homodimer (Altar et al., 1997; Mowla et al., 1999). Both forms of BDNF are expressed in the central and peripheral nervous systems during development and throughout adulthood with the highest brain levels found in the hippocampus (Ivanova and Beyer, 2001; Donovan et al., 2008). The actions of the precursor
and mature forms of BDNF are mediated through pan-neurotrophin receptor (p75NTR) and tropomyosin receptor kinase (TrkB) families, respectively (Chen et al., 2005). The proBDNF-p75NTR complex initiates apoptotic cascades, while the mature BDNF-TrkB complex stimulates growth and survival via mitogen-activated protein (MAP) kinase and phosphoinositide 3 (PI3)-kinase pathways (Feng et al., 2001; Teng et al., 2005).

A hypothesis suggesting a role for BDNF in major depressive disorder arose from studies reporting stress-related decreases and antidepressant-induced increases in BDNF levels. In rodents, single or repeated immobilization stress has been associated with reductions in BDNF levels (Nibuya et al., 1999; Xu et al., 2004; Fuchikami et al., 2008). By contrast, release of endogenous BDNF and subsequent TrkB receptor activation has been associated with therapeutic responses in animal models of depression-related behaviors (Saarelainen et al., 2003). Moreover, exogenous BDNF infusion into the hippocampus produces antidepressant-like effects in learned helplessness and behavioral despair models in rodents (Siuciak et al., 1997; Shirayama et al., 2002). In situ hybridization studies have been carried out to determine region-specific alterations in BDNF mRNA in response to both antidepressant treatments and stress. These studies revealed that stress negatively affects affect BDNF levels, while antidepressants positively affect BDNF levels in the granule cell layer and dentate gyrus subregions of the hippocampus. By contrast, changes in the CA1 and CA3 subregions are difficult to discern based on conflicting results (Nibuya et al., 1996; Coppell et al., 2003; De Foubert et al., 2004; Jacobsen and Mørk, 2004; Larsen et al., 2008). Subregion specific changes in BDNF mRNA levels in the hippocampus using genetic approaches to knockout BDNF in specific regions have also highlighted the importance of the dentate gyrus in mediating the effects of antidepressant treatments.
In humans, robust associations between untreated major depressive disorder and reduced serum BDNF levels have been reported (Brunoni et al., 2008; Sen et al., 2008; Molendijk et al., 2011). In postmortem issue, BDNF mRNA is reduced in the frontal cortex and hippocampus of suicide victims (Dwivedi et al., 2003), while recent studies have additionally shown decreases in BDNF mRNA and protein in female major depressive disorder patients (Guilloux et al., 2012). Similar to rodent studies, reports and meta-analyses suggest that antidepressant treatment in humans with MDD is associated with increases in BDNF levels (Sen et al., 2008; Molendijk et al., 2011). Taken together, preclinical and clinical evidence support the idea that BDNF plays a modulatory role in both the pathogenesis and treatment of mood disorders.

The human BDNF gene contains eleven 5′ noncoding exons, which are alternately spliced to a common 3′ coding exon. Nine functional promoters and alternative polyadenylation sites have also been identified giving rise to a large repertoire of diverse transcripts that are expressed in a tissue- and cell-specific manner (Liu et al., 2006; Pruunsild et al., 2007). A noncoding antisense strand (antiBDNF) has also recently been identified in the human BDNF locus that is not present in rodents and is suggested to further regulate human BDNF transcription (Pruunsild et al., 2007). Complex transcriptional regulation also occurs within the mouse BDNF gene (Fig. 5-1), which contains multiple upstream noncoding exons with distinct promoters and a common downstream coding region, producing nine different transcripts (Timmusk et al., 1993; Liu et al., 2006; Aid et al., 2007). Evidence for the modulation of exon-specific BDNF mRNAs came from initial studies showing that induced seizure activity rapidly upregulates
transcripts containing exons I, II, and III in rodents (Metsis et al., 1993). Expression patterns in the hippocampus likewise show differential temporal recruitment with exon I- and II-containing BDNF mRNAs regulated postnatally, while exon III and IV expression is enhanced in adults (Sathanoori et al., 2004).

A number of studies have now demonstrated that individual transcripts are regulated in response to antidepressant treatment and stress in rodents, suggesting additional biological importance of this transcriptional variation (Dias et al., 2003; Dwivedi et al., 2006; Hansson et al., 2006; Khundakar and Zetterström, 2006; Tsankova et al., 2006; Calabrese et al., 2007; Nair et al., 2007). Studies have revealed that acute immobilization stress downregulates total BDNF mRNA and specifically transcripts containing exons I, II, III, and V in the DG hippocampal subfield (Dias et al., 2003; Khundakar and Zetterström, 2006; Fuchikami et al., 2008). Recently, Tsankova and coworkers (2006) reported that exon III/IV-containing BDNF mRNA is reduced in response to chronic social defeat stress in mice and downregulation is reversed after administration of imipramine. However, it is difficult to conclude from these studies which are the key transcripts that are influenced by antidepressants and in particular, by serotonin, based on conflicting results arising from different antidepressant treatments and methods employed to quantify transcript levels. Moreover, few studies have reported on the influence of stress in regulating the newly identified BDNF transcripts and how these transcripts might account for alterations in total BDNF levels after stress (nomenclature corresponding to that used by Liu et al., 2006) (Fuchikami et al., 2008). Furthermore, while previous studies support the idea that stressors differentially recruit BDNF transcripts, disparities in techniques used to measure mRNA levels make it difficult to infer which are the key transcripts altered by stress.
Our goal was to gain a more comprehensive understanding of the interactions between the expression patterns of the diverse BDNF mRNA transcripts and serotonin. Mice with constitutive reductions in SERT have aided in our understanding of the developmental role of serotonin and how altered SERT function modulates mood and anxiety in adults (Bengel et al., 1998). Disruption of SERT in mice results in neurochemical and behavioral changes including increased extracellular serotonin levels, hyperresponsivity to stress, and an increased anxiety-like phenotype (Tjurmina et al., 2002; Holmes et al., 2003; Mathews et al., 2004; Szapacs et al., 2004; Kim et al., 2005; Perez and Andrews, 2005; Kalueff et al., 2007). This is in contrast to pharmacological agents used in adults to block SERT to produce therapeutic effects in treating depression, indicating that SERT blockade during development and adulthood may have opposing effects to mediate mood and anxiety (Ansorge et al., 2004; Ansorge et al., 2008). Importantly, SERT-deficient mice show parallels to a polymorphism in the promoter region of the human SERT gene, the 5-HTTLPR (Lesch et al., 1996; Lesch and Mössner, 1998). Allelic variation produced by this polymorphism gives rise to long and short alleles, and the short variant has been associated with reduced SERT expression and enhanced susceptibility to stress-associated depression (Caspi et al., 2003; Canli and Lesch, 2007). Thus, changes in brain biochemistry and/or anatomy arising during development in response to reduced SERT expression might lead to altered adult behaviors.

A number of studies have begun to explore the relationship between BDNF and serotonin using both SERT knockout mice and conditional BDNF knockout mice (Luellen et al., 2006; Daws et al., 2007; Deltheil et al., 2008; Guiard et al., 2008). In addition, a double mutant mouse model heterozygous for inactivation of the BDNF allele and lacking the
serotonin transporter indicates a variety of abnormalities including decreased body size, decreased serotonin content, reduced axonal sprouting and dendritic arborization within the hippocampus, and an exacerbated anxiety-related phenotype (Ren-Patterson et al., 2005). In addition to this, one study reported on the effects of constitutive SERT deficiency and chronic serotonin reuptake inhibitor (SRI) treatment with duloxetine in rats (Calabrese et al., 2010). These investigators reported that total BDNF levels were reduced in the hippocampus and prefrontal cortex of SERT−/− rats and that duloxetine treatment significantly increased these levels in SERT−/− rats compared to SERT+/+ rats receiving duloxetine. They also showed that BDNF isoforms III, IV, VI and IX were important for these effects (Calabrese et al., 2010). Here, we investigated BDNF transcription after osmotic minipump administration of imipramine (10 mg/kg) and fluoxetine (10 mg/kg) in serotonin transporter (SERT)-deficient mice. A real-time quantitative PCR (RT-qPCR) assay was developed to detect changes in the originally identified BDNF transcripts. Our results highlight that BDNF levels are decreased in SERT-deficient mice most prominently in the frontal cortex. However, antidepressant treatments showed no overall effects. Taken together, these studies support the combined role of serotonin and BDNF in the pathology of mood and anxiety disorders and the need to further explore their relationship.

5.2 Materials and Methods

5.2.1 Animals and Treatment

Wildtype and SERT-deficient male mice on a congenic CD1 × 129SvEv background bred by heterozygous brother-sister matings or wildtype mice on an outbred CD-1
background (Charles River, Wilmington, MA) were used for investigations. Mice were maintained on a 12:12 light:dark schedule with access to water and food ad libitum. Tail clips were taken at weaning (21 days of age) for genotyping the SERT allele. At eight weeks of age, mice were implanted subcutaneously with osmotic minipumps (Alzet Model 2004, Cupertino, CA) to deliver saline, fluoxetine (10 mg/kg/d), or imipramine (10 mg/kg/d). Mice were minimally handled post-surgery except to monitor body weight 10 days after the procedure. Since SERT-/- mice display stress responses to single saline injections (Tjurmina et al., 2002), this method of drug delivery was chosen specifically to avoid potential changes in behavior or BDNF levels that may be related to daily injections or handling. Behavior experiments were conducted ~3 weeks after minipump implantation with at least 3 days in between each test as described below. Group sizes are found in Table 5-1. All experiments were approved by the Pennsylvania State University Institutional Animal Care and Use Committee, and were in accordance with the National Institutes of Health Animal Care and Use Guidelines.

5.2.2 Elevated Plus Maze

Anxiety levels in SERT-deficient mice were assessed using the elevated plus maze (physical maze descriptions can be found in Chapters 2 and 3 and Appendix Fig. A-1A). Mice were placed in the central platform facing a closed arm and allowed to freely explore the maze for 5 min. Anxiety-related behavior was analyzed by measuring entries and time spent on the open, aversive arms of the maze. Total arm entries, and rears and dips provided measures of locomotor and exploratory behaviors, respectively. The maze was thoroughly cleaned with ethanol in between mice.
5.2.3 Marble Burying Test

Traditionally, marble burying has been viewed as a way to measure compulsive-like behavior in mice. Recent literature also suggests that this test might be used as a measure of anxiety-like behavior wherein mice with higher levels of anxiety avoid novel objects (marbles) presented to them in a novel environment. Mice were placed in a novel cage containing ~6 cm of sawdust with 15 marbles aligned in a 3 × 5 pattern (Appendix A, Figure A-3B). After 30 min in this environment, mice were returned to their home cages and the number of marbles buried (>75% covered in sawdust) or not buried was counted.

5.2.4 RNA Isolation and cDNA Preparation

One day after the last behavior test (on day 25 of antidepressant treatment), mice were euthanized by cervical dislocation. Brains were rapidly removed and the frontal cortex, brain stem, and hippocampus were collected from each mouse and rapidly placed into RNAlater (Qiagen, Valencia, CA) and stored at 4°C for 24 h to prevent RNA degradation. Excess RNAlater solution was then removed and tissue was transferred to -80°C for storage. For RNA isolation from each brain region, 150 µL QIAzol lysis reagent was added to tissue samples, which were homogenized using a hand-held, motorized homogenizer. Once completely homogenized, an additional 850 µL of QIAzol lysis reagent was added to each tube. Homogenates were incubated for 5 min after which 200 µL of chloroform was added. Samples were shaken vigorously for 15 sec and allowed to incubate for an additional 2-3 min. Samples were then centrifuged at 12000 × g for 15 min at 4°C. The upper aqueous layer containing RNA was transferred to a new collection tube to which 1 volume of 70% ethanol was added. Tubes were quickly vortexed. The RNA samples were
further purified using RNeasy Lipid Tissue Minikits as per the manufacturer’s protocols (Qiagen, Valencia, CA). A speed vacuum concentrator was used to pellet 5 µg of RNA, and DNase treatment was used to further purify samples using a TURBO DNase-Free Kit using the directions of the manufacturer (Life Technologies Ambion, Austin, TX). RNA concentrations and purity were obtained using a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific, Logan, UT). Samples were then reverse transcribed into cDNA using High Capacity Reverse Transcription Kits (Life Technologies Applied Biosystems, Foster City, CA). Samples were stored temporarily at -20°C until real-time qPCR experiments were carried out.

5.2.5 Real-time Quantitative PCR with SYBR Green

Extensive assay validation experiments used to detect reliably unique BDNF transcripts or total mRNA levels have been carried out and are detailed below. After validation, amplifications of cDNA from individual brain region samples were carried out using cDNA (12 ng), primers specific for BDNF exons I and V and total BDNF (300 nM) and SYBR Green PCR Mastermix (Life Technologies Applied Biosystems, Foster City, CA) in triplicate in 15 µl reaction volumes using an ABI 7900HT Fast Real Time PCR System (Life Technologies Applied Biosystems, Foster City, CA; Genomics Core Facility, Pennsylvania State University). Data were normalized to a control gene (glyceraldehyde phosphate dehydrongenase, GAPDH) and were analyzed using a relative quantification ΔCt method described in Fig. 5-2.
5.3 Results and Discussion

5.3.1 BDNF qPCR Assay Validation

Primer pairs were designed using Primer3 to amplify the nine mouse BDNF mRNA transcripts. These primers amplify the junction region between the respective noncoding exons and the common coding region to eliminate amplification of genomic DNA. We have established specificity (determined by efficiency) and the absence of primer dimers through melt curve analyses and gel electrophoresis. To carry out experiments to measure individual BDNF transcripts in mice with reduced SERT expression, work was completed to optimize primer pairs for BDNF Exons I and V and for total BDNF. Representative amplification plots of Exons I and V demonstrate that the concentrations of 90 ng/μl – 1.41 ng/μl of input cDNA are evenly distributed (i.e. exponential increase between each dilution relative to the dilution factor) (Fig. 5-3A). Melt curves and gel electrophoresis using PCR products indicate selectivity for BDNF or the control gene (Fig. 5-3B). Furthermore, linear regression analyses were conducted to identify the range of cDNA required to achieve dynamic range and to determine efficiency of the reactions (Efficiency = -1+10(-1/slope)) (Fig. 5-4). Primer sequences and efficiencies for BDNF and control genes can be found in Table 5-2.

Low copy numbers of BDNF transcripts have necessitated extensive work to demonstrate that amplification by RT-qPCR and optimized primer design produced reliable and replicable results. To test further the sensitivity of our assay, we analyzed levels of total BDNF mRNA in mice lacking one copy of the BDNF gene (BDNF+/-) compared to wildtype mice (BDNF+/+). Our results showed an approximate 50% reduction in total
BDNF mRNA in BDNF+/− mice compared to BDNF+/+ mice in the frontal cortex, hippocampus, and brain stem (Fig. 5-5).

5.3.2 Total BDNF and Exons I and V mRNA levels in SERT-deficient mice

In addition to this, mice with reduced SERT expression exhibit altered basal levels of BDNF mRNA, particularly in the frontal cortex (Fig. 5-6). Total BDNF mRNA levels are significantly reduced in SERT+/− mice ($P<0.05$) and SERT−/− mice ($P<0.01$) compared to SERT+/+ mice (Fig. 5-6A). Furthermore, BDNF exon-specific reductions were observed with a significant downregulation in Exon I ($P<0.05$) and Exon V ($P<0.01$) in SERT−/− mice compared to SERT+/+ mice (Fig. 5-6B,C). Similar reductions in BDNF mRNA were observed in brain stem in these mice, although preliminary data in the hippocampus do not reflect such alterations (data not shown). These changes in BDNF mRNA levels in response to reductions in SERT expression support our hypothesis that interactions occur between the serotonin and BDNF systems, although these changes appear to be region specific. These results are also in support of previous findings in SERT−/− rats showing total and isofrom-specific reductions in BDNF levels (Calabrese et al., 2010). Furthermore, this suggests that reduced levels of BDNF may be associated with the anxiety-like phenotype exhibited in SERT-deficient mice.

The effects of chronic antidepressant administration in mice vs reduced SERT expression on levels of BDNF mRNA were explored. Osmotic minipumps were implanted to deliver the serotonin reuptake inhibitor fluoxetine (10 mg/kg/d) or the tricyclic antidepressant imipramine (10 mg/kg/d) over the course of 25 days. We hypothesized that antidepressants would increase key BDNF transcripts in a gene dose-dependent manner.
Specifically, imipramine would increase BDNF levels in SERT wild-type mice and to a lesser extent in SERT-deficient mice. Similar changes were anticipated in SERT+/+ and SERT+-/ mice receiving fluoxetine, but we hypothesized that this effect would be attenuated in SERT-/- mice. Our data indicate that antidepressant treatment with fluoxetine or imipramine did not have any effects on BDNF transcripts or total mRNA levels in the frontal cortex, although a main effect of genotype was observed in these mice (Fig. 5-7). Similar results with respect to drug treatment were found in the brain stem and hippocampus (data not shown). Although our data do not support our hypothesis, these findings parallel reports by others whereby fluoxetine had no effects on BDNF Exons I or V in the hippocampus (Dias et al., 2003; Altieri et al., 2004; Dwivedi et al., 2006). Conflicting evidence, however, indicates that Exon I is increased by repeated fluoxetine treatment in rodents (Khundakar and Zetterström, 2006).

BDNF transcript containing Exon IV has gained attention due to its purported role in activity-dependent transcription of BDNF (Timmusk et al., 1993; Shieh et al., 1998; Tao et al., 1998) and because of studies indicating that this isoform is reduced after immobilization stress and chronic social defeat (Tsankova et al., 2006; Fuchikami et al., 2008). Mice with a specific knockdown of promotor IV have been generated (Sakata et al., 2009) and studies have indicated that these mice display hypolocomotion, increased anhedonia and learned helplessness behaviors but unaltered anxiety-like behavior (Sakata et al., 2010; Jha et al., 2011). Similar to the RT-qPCR optimization carried out for Exons I and V above, linear regression analyses and confirmatory melt curves and gel electrophoresis were performed for BDNF transcript containing promoter IV (Fig. 5-8). The efficiency and sequences of the primers used are listed in Table 5-2. Following assay
optimization, preliminary data collected in hippocampus revealed that there were no changes in Exon IV in response to antidepressant treatment, although there was a trend toward a reduction in BDNF transcript IV in SERT-/- mice treated with saline (Fig. 5-9). More experiments are required to obtain a more comprehensive profile of this transcript with respect to SERT deficiency and chronic antidepressants.

5.3.3 Anxiety-related and locomotor behavior after SERT deficiency and antidepressant administration

Anxiety-like behavior was assessed using the elevated plus maze and marble burying tests. SERT-deficient mice displayed increased anxiety as indicated by a trend toward increased latency to enter an aversive, open arm of the maze (Fig. 5-10A) and significant reductions in time spent ($P<0.05$) and entries ($P<0.05$) made into the open arms (Fig. 5-10B,C). Locomotor behavior was unchanged as indicated by arm entries (5-11A). In terms of exploratory behaviors, SERT-/- showed reduced number of rears but no changes in the number of head dips (Fig. 5-11B,C). These results are in line with previous reports in SERT-/- mice and rats (Holmes et al., 2003; Olivier et al., 2008; Kalueff et al., 2010). Marble burying in a novel environment was also assessed as a potential indicator of anxiety-like behavior (Njung'e and Handley, 1991). Defensive marble burying has been suggested to be a model of obsessive-compulsive behaviors that are alleviated by the administration of SRIs (Njung'e and Handley, 1991; Gyertyan, 1995). In the current study, marble burying behavior in a novel environment was reduced in SERT-/- mice ($P<0.05$) (Fig. 5-12) in line with a previous report on these mice (Kalueff et al., 2006). Avoidance of marbles might indicate increased anxiety-like behavior in response to novel objects in a novel
environment. No effects of antidepressant treatment were observed with respect to marble burying.

5.4 Conclusions and Future Prospects

This study was designed to ascertain region- and BDNF transcript-specific alterations occurring in response to genetic and pharmacologic manipulations in the serotonin system. The serotonin system has been associated with the etiology of mood and anxiety disorders, and recent evidence has established that changes in neurotrophic factors such as BDNF may also play a role. Ultimately, this work may have implications for targeting therapeutics to alter levels of BDNF for improved treatment of mood and anxiety disorders. We discovered that basal levels of total BDNF and various BDNF transcripts are downregulated in mice with reduced SERT expression, and this downregulation is brain region dependent. Future lines of investigation are needed to ascertain the mechanisms by which these molecular modifications may be related to the anxious phenotype of SERT-deficient mice and how this may correlate in clinical settings.

Rhesus and Human SERT mRNA levels

5.5 Background

As described in detail in Chapter 1, the serotonin system plays an important role in the pathogenesis and treatment of mood and anxiety disorders. Of particular relevance is the serotonin transporter (SERT), which is the protein responsible for clearance of serotonin from the extracellular space. SERT is also the major substrate for common
antidepressant drugs such as SRIs (Kanner and Schuldiner, 1987; White et al., 2005). A number of polymorphisms within the human serotonin transporter gene have been identified that are hypothesized to influence its expression and function (Lesch et al., 1996; Hu et al., 2006a; Wendland et al., 2006; Wendland et al., 2008). Of these, the serotonin transporter-linked polymorphic region (5-HTTLPR) has generated interest based on its hypothesized role in stress-associated depression (Caspi et al., 2003; Schinka et al., 2004; Sen et al., 2004). This polymorphism is found in humans and higher order nonhuman primates (Lesch et al., 1997; Bennett et al., 2002; Murphy et al., 2008). It occurs within the promoter region, giving rise to short ‘s’ and long ‘l’ allelic variants. The short variant is proposed to result in impaired transcriptional efficiency and increased susceptibility to depression (Lesch et al., 1996; Caspi et al., 2003; Kendler et al., 2005). We have previously shown that in peripheral blood cells from rhesus macaques, which also express the 5-HTTLPR, the ‘s’ allele is associated with reduced SERT uptake (Singh et al., 2010). Reports on SERT mRNA levels with respect to 5-HTTLPR genotype have been conflicting, with some suggesting that mRNA levels are reduced in blood cells (Lesch et al., 1996; Murphy and Lesch, 2008), while others report no change in postmortem raphe tissue (Lim et al., 2006). In addition to rhesus macaque peripheral blood cells, we also obtained blood samples from depressed human patients and human lymphoblast cell lines. Using modifications of the RT-qPCR assay described above, we developed an assay to measure SERT mRNA levels applicable to both rhesus and human peripheral blood cells. These mRNA studies parallel and compliment the work of colleagues who established methods to examine uptake and SERT protein levels in peripheral blood cells. Determining potential correlations between
mRNA levels and protein expression are important mechanistic avenues to explore to understand functional changes that occur in these complex models.

5.6 Materials and Methods

5.6.1 RNA Isolation from Peripheral Blood Cells

Frozen rhesus macaque peripheral blood cells genotyped for the 5-HTTLPR were generously provided by the laboratory of Dr. Michael Murphy-Corb, University of Pittsburgh. Pooled samples of mixed genotype were also supplied and used for developing the protocol to obtain pure RNA from frozen blood samples. For rhesus peripheral blood cells, frozen stocks containing ~17 million cells were rapidly thawed in a 37°C water bath and diluted with 4 ml of lymphoblast assay buffer (150 mM NaCl, 5 mM KCl, 1.2 mM MgCl$_2$.6H$_2$O, 5 mM glucose, 10 mM HEPES, 2 mM CaCl$_2$.2H$_2$O; pH 7.4). Samples were aliquoted such that each contained 8-9 million cells and were pelleted at 12000 rpm for 10 min at 4°C. Supernatants were removed and 1 ml of QIAzol lysis reagent was added. Cell suspensions were passed 12 times through a 23-gauge needle on ice and then vortexed. Subsequently, 200 μl of chloroform was added and samples were vigorously shaken for 15 s followed by incubation on ice for an additional 10 min. Samples were centrifuged at 14000 rpm for 15 min at 4°C. The upper aqueous layer was collected and GlycoBlue (150 μg/ml final concentration; Life Technologies Ambion, Austin, TX) and 1 volume of isopropanol were added. Samples were stored overnight at -20°C. The next day, samples were centrifuged at 14000 rpm for 15 min at 4°C, washed with 1 ml of 75% ethanol, and centrifuged again using the same conditions. The supernatant was removed and the pellets
were allowed to dry for 10 min before resuspending in 80 µl nuclease-free water. RNA concentrations and purity were measured using a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific, Wilmington, DE) before and after DNase-treatment of 5 µg of RNA (TURBO DNase free kit, Life Technologies Ambion, Austin, TX). Samples were reverse transcribed into cDNA (High Capacity cDNA Reverse Transcription kit, Life Technologies Applied Biosystems, Grand Island, NY) according to manufacturer’s protocols. For samples obtained from human cell culture lines, some samples came from frozen stocks, while others were prepared fresh to compare RNA yields in fresh vs frozen cells. All other protocols adhered to those used for rhesus peripheral blood cells.

5.6.2 Real-time Quantitative PCR with Taqman Probes

A listing of the primer and probe sequences used to amplify human SERT and two control genes, GAPDH and β-actin can be found in Table 5-3. These sequences were obtained from previous literature (Kinnally et al., 2010; Yu et al., 2010). Similar to the optimization and validation experiments presented above for BDNF, primer pairs were tested and validated by running serial dilutions of cDNA and plotting semi-log plots of the standard curves to obtain slopes and efficiencies (Fig. 5-13). After confirmation, cDNA samples from genotyped rhesus macaque peripheral blood cells of human lymphoblast cell lines were analyzed in quadruplicate using 80 ng cNDA, primers (900 nM), probes (250 nM), and Fast Universal PCR Mastermix (Life Technologies Applied Biosystems, Foster City, CA). Samples were amplified using an ABI 7900HT Fast Real Time PCR System (Life Technologies Applied Biosystems, Foster City, CA). Data were analyzed by a relative quantification method whereby the geometric mean cycle threshold (Ct) values of both control genes
were combined across samples and subtracted from the samples containing primers and probes for SERT to get ΔCt. A final equation of $2^{\Delta Ct}$ was applied.

5.7 Results and Discussion

5.7.1 SERT mRNA levels with respect to rhesus 5-HTTLPR

In accordance with previous findings by our laboratory, we observed that 5-HTTLPR is associated with differential serotonin uptake whereby carriers of the ‘s’ allele showed reduced serotonin uptake (Singh et al., 2010; Singh et al., 2011). In the current study, we also showwws that these changes in uptake are associated with reduced surface expression of SERT (Singh et al., 2011). However, neither changes in SERT mRNA (Fig. 5-14) nor correlations with surface SERT binding (Singh et al., 2011) with respect to the rhesus 5-HTTLPR were observed. These results are in line with a previous study showing no changes in SERT mRNA levels in relationship to genotype using a large cohort of monkeys (Yu et al., 2010). These data also highlight the large within-group variability that inherently occurs in these samples. This variability is consistent with studies in humans were variability exists with upwards of 5-fold differences in SERT mRNA (Little et al., 1998; Hu et al., 2006b). However, it is not the case that differences in mRNA levels account for the observed alterations in surface SERT expression, and ultimately, SERT function.

5.7.2 SERT mRNA levels in human lymphoblast cell lines

Preliminary work in human lymphoblast cell lines has been carried out to determine whether these cells express SERT and whether functional differences can be determined
based on the complex genotype of *SERT* in these individuals. Previous work by others in our laboratory has demonstrated that the peripheral blood cells responsible for serotonin uptake are platelets with minimal uptake of SERT by lymphoblasts (Beikmann et al., 2012).

To begin, we first sought to investigate whether freezing samples affects RNA yields and compared this to the RNA yields acquired from frozen rhesus peripheral blood cells. These experiments confirmed that similar amounts of RNA were achieved from fresh vs frozen samples (Fig. 5-15a). Next, RT-qPCR was used to assess SERT mRNA levels to determine whether low SERT expression levels might contribute to difficulties obtaining functional measurements in these cell lines. RNA was isolated from cell lines established from 10 separate individuals designated by a 3-digit identification code. Results were compared to a positive control comprised of HEK cells overexpressing SERT. Relative quantification of SERT mRNA indicated that lymphoblast cell lines contain very low levels of SERT mRNA (Fig. 5-15b). These low quantities might explain the difficulties associated with obtaining functional measurements from lymphoblast cell lines. Further work, including measuring SERT protein levels, will be required before solid conclusions can be made.
Figure 5-1: Representation of the mouse BDNF gene. Original descriptions of the mouse BDNF gene identified 5 noncoding exons that are alternatively spliced to encode the same mature BDNF protein (upper panel). Subsequent studies revealed a more complex picture of the mouse BDNF gene, which contains nine noncoding exons upstream of the coding region (lower panel). Sequence alignment allowed parallels to be drawn between the original nomenclature and the new nomenclature.
Figure 5-2: The ΔΔCt method of relative quantification. To quantify levels of individual BDNF transcripts in the hippocampus, frontal cortex, and brain stem, data were analyzed by means of relative quantification. A calibrator sample was identified (saline-treated wildtype mice) and was normalized to a reference or control gene (GAPDH) by obtaining the difference in Ct values (ΔCt, denoted as “A” in the diagram). Similarly, ΔCt was calculated for the remainder of the samples (saline, imipramine, and fluoxetine-treated mice of all genotypes, denoted as “B” in the diagram). The ΔΔCt values were obtained by calculating the difference between the target sample (“B”) and the calibrator sample (“A”) and applying the overall equation of $2^{-\Delta\Delta C_t}$. 
Figure 5-3: Optimization of primer pairs. Total BDNF and BDNF transcripts containing Exons I and V were optimized. Serial dilutions of cDNA were added as templates to mixtures containing SYBR green and the respective primer pairs for the target of interest. (a) Even spacing between dilutions observed in the amplification plots demonstrates an efficient reaction with an approximate doubling with each cycle. (b) Gel electrophoresis of PCR products run in duplicate indicate single bands for Exon I (179 bp), Exon V (278 bp), total BDNF (72 bp), and GAPDH (176 bp). Specificity is also indicated by single peak melt curves.
Figure 5-4: Standard curve linear regression analyses. Representative semi-logarithmic plots of serial dilutions of cDNA vs cycle threshold are shown for (a) GAPDH (11.3 ng – 0.7 ng) and total BDNF (45 ng – 0.7 ng) and (b) Exons I and V (45 ng – 2.8 ng). The efficiency of the primer pairs was determined using the slope of the line \((1+10^{-1/slope})\).
Figure 5-5: SYBR green real-time quantitative PCR assay validation. BDNF wildtype (BDNF+/-) and heterozygous (BDNF+/-) mice were used to validate assay sensitivity for detecting changes in the levels of total BDNF mRNA. A significant reduction in total BDNF mRNA (P<0.01) of ~50% in BDNF+/- mice compared to BDNF+/+ mice was observed. N=4-7 mice/genotype/brain region. FC=frontal cortex, HIP=hippocampus, and BS=brain stem.
Figure 5-6: Total and exon-specific reductions in BDNF with respect to SERT deficiency. RT-qPCR analysis was used to determine of total BDNF mRNA (a), Exon I mRNA (b), and Exon V mRNA (c) in the frontal cortex. A one-way analysis of variance revealed that significant reductions occurred in total BDNF mRNA levels and levels of BNF Exons I and V in mice with reduced SERT expression. *P<0.05 and **P<0.01 with respect to SERT+/+ mice. N=4-6 mice/genotype/drug treatment.
(a) Total BDNF

(b) Exon I

(c) Exon V
Figure 5-7: BDNF mRNA levels in SERT-deficient mice after chronic administration of antidepressants. RT-qPCR analysis of total BDNF mRNA (a), Exon I mRNA (b), and Exon V mRNA (c) in the frontal cortex of SERT-deficient mice treated with antidepressants is shown. A significant main effect of genotype was observed in total BDNF mRNA (P<0.001), BDNF Exon I transcript (P<0.01), and BDNF Exon V transcript (P<0.001). Overall, no changes in BDNF mRNA were observed as a result of treatment with antidepressants. N=4-6 mice/genotype/drug treatment. IMI=imipramine, FLX=fluoxetine.
Figure 5-8: Optimization of RT-qPCR assay for BDNF Exon IV. Similar to previous experiments, detailed work was carried out to ensure efficient and reliable amplification of BDNF transcripts containing Exon IV. (a) A representative standard curve using a semi-logarithmic scale for input cDNA vs cycle threshold values is shown. The slope of the line was used to calculate the efficiency. (b) Gel electrophoresis and melt curves are shown to indicate specificity of primers used to amplify Exon IV (177 bp).
Figure 5-9: BDNF IV variant expression in hippocampus after antidepressants in SERT-deficient mice. Preliminary experiments conducted to examine levels of BDNF transcript variant IV were performed in the hippocampus. These results indicate that antidepressant treatment had no effect on Exon IV expression. N=3 mice/genotype/group. IMI=imipramine, FLX=fluoxetine.
Figure 5-10. Anxiety-related behavior in the elevated plus maze. Mice were allowed free exploration of the plus maze for 5 min after which time and entries spent exploring the open and closed arms of the maze were measured. Parameters obtained from the aversive, open arms of the maze were used to assess anxiety-like behaviors. (a) A trend toward an increased latency to enter an open arm was observed for saline-treated SERT-/- mice ($P<0.08$). Additionally, saline-treated SERT-/- mice spent less time (a) and made fewer entries (c) into the open arms of the elevated plus maze. No effects of drug treatment were observed. *$P<0.05$ with respect to saline SERT+/+ mice.
**Figure 5-11. Locomotor and exploratory behaviors measured using the elevated plus maze.** (a) No changes in locomotor behavior were observed with respect to total arm entries. Rears (b) and head dips (c) were considered exploratory behaviors. SERT+/+ and SERT-/- mice showed reductions in number of rears compared to SERT+/- mice. There were no genotype-related changes in numbers of head dips. No effects of drug treatment were observed on any parameters measured. **P<0.01 compared to SERT+/- mice within the same drug treatment.**
Figure 5-12: Marble burying test. Marble burying in a novel cage was used to test for differences in anxiety-like behavior. Mice were placed in a novel cage containing 15 marbles for 30 min. After the test, the number of marbles buried was counted. SERT-/- mice buried fewer marbles than SERT+/+ counterparts indicating that the novelty of both the cage and the objects was associated with increased anxiety-like behavior in these mice.

*P<0.05 with respect to SERT+/+ mice of the same drug treatment.
Fig. 5-13: Standard curves for SERT and control genes. TaqMan assays were used to amplify serotonin transporter (SERT) and control genes (β-actin and GAPDH). Representative semi-log plots of serial dilutions of cDNA vs cycle threshold (Ct) are shown. Linear regression analyses were performed to obtain the slope and efficiencies of the primer and probe sets. These are listed in Table 5-3.
Fig. 5-14. SERT mRNA levels with respect to 5-HTTLPR genotype. RT-qPCR was carried out to measure SERT mRNA levels in rhesus peripheral blood cells. There are no differences in SERT mRNA levels with respect to 5-HTTLPR genotype. Overlays of dot plots are shown to depict within-group variability that exists in mRNA. This figure has been modified from Figure 4 in (Singh et al., 2012).
Fig 5-15. SERT mRNA levels in human peripheral blood cells. (a) To determine the viability of RNA in frozen human peripheral blood samples, RNA yields obtained from these samples were compared to fresh human peripheral blood cells and to frozen cells isolated from rhesus monkeys. These results indicate no major effects of freezing on samples as similar yields were obtained in frozen samples compared to fresh samples. (b) SERT mRNA levels in individual human lymphoblast cell lines were compared with control HEK cells transfected with SERT. Relative quantification of SERT revealed that these cell lines only contained very small amounts of SERT mRNA that approach the limits of detection of the assay.
### Table 5-1: Group Sizes

Numbers of wildtype (CD-1 or SERT+/+) and SERT-deficient (SERT+/- and SERT-/-) mice implanted with osmotic minipumps containing saline, imipramine (IMI), or fluoxetine (FLX) are shown.

<table>
<thead>
<tr>
<th></th>
<th>CD-1</th>
<th>SERT+/+</th>
<th>SERT+/-</th>
<th>SERT-/-</th>
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<td>Saline</td>
<td>13</td>
<td>10</td>
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<td>17</td>
</tr>
<tr>
<td>IMI</td>
<td>10</td>
<td>8</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td>FLX</td>
<td>9</td>
<td>2</td>
<td>16</td>
<td>10</td>
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<tr>
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<td>Primer Sequences</td>
<td>Efficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------------------------------------------</td>
<td>------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Forward 5’ TGCACCACCAACTGCTTAG 3’  Reverse 5’ GATGCAGGGATGATGTTTC 3’</td>
<td>97%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total BDNF</strong></td>
<td>Forward 5’ AAGAAAACCATAAGGACCGGACTTGT 3’  Reverse 5’ GAGGCTCCAAAGGCACTTGACT 3’</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BDNF Exon I</strong></td>
<td>Forward 5’ CACATTACCTTCTCAGATCTCTGTGGG 3’  Reverse 5’ CCGTGACGTTTACTCTTTCATGGG 3’</td>
<td>101%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BDNF Exon IV</strong></td>
<td>Forward 5’ TACATATCGGCCACAAAGACCTCG 3’  Reverse 5’ GGATGGTCATCAGCTTCTCACCT 3’</td>
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<tr>
<td><strong>BDNF Exon V</strong></td>
<td>*Forward 5’ CCGCTGGCTGGCTGGCTGCAAGCTTTC 3’  Reverse 5’ CCGTGACGTCTTTTACTCTCATGGG 3’</td>
<td>96%</td>
<td></td>
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Table 5-2: Primer sequences and efficiencies for the BDNF study in mice.

Forward and reverse primer pairs for the control gene (GAPDH), total BDNF, and BDNF Exons I, IV, or V and their respective efficiencies are listed. NCBI accession numbers are provided for murine gene information.
<table>
<thead>
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<th>Gene</th>
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<th>Eff.</th>
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<td>β-actin</td>
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<td>Forward 5’ TGAGCGCGGCTACAGCTT 3’ Reverse 5’ CCTTAATGTCACGCACGATT 3’ Probe 5’ FAM - ACCACCACGGCCGAGCGG – TAMRA 3’</td>
<td>97%</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Yu 2010</td>
<td>Forward 5’ GACCAC AGTCCATGCCAC 3’ Reverse 5’ CATCACGCCACAGTTTCC C 3’ Probe 5’ FAM - ACCCAGAAGACTGTGGATGGCCC – TAMRA 3’</td>
<td>82%</td>
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<tr>
<td>SERT</td>
<td>Yu 2010</td>
<td>Forward 5’ CTCTTGGTGCCGGCTTGTGG 3’ Reverse 5’ TGGTCACCAGGGCATCTTG 3’ Probe 5’ FAM-TTTCGCTAGCTACAACAAAGTTCAACAACAACACTGCTAC-TAMRA 3’</td>
<td>98%</td>
</tr>
</tbody>
</table>
Table 5-3. Primer sequences and efficiencies for SERT studies in rhesus macaques and humans. Primer pair sequences used in these studies were obtained from previously published literature as designated in the table. Sequences for the primers and TaqMan probes and their respective efficiencies are listed. The contents of this table are adapted from (Singh et al., 2012).


Brunoni AR, Lopes M, Fregni F (2008) A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the


Jha S, Dong B, Sakata K (2011) Enriched environment treatment reverses depression-like behavior and restores reduced hippocampal neurogenesis and protein levels of brain-derived neurotrophic factor in mice lacking its expression through promoter IV. Translational psychiatry 1:e40.


Xu H, Luo C, Richardson JS, Li XM (2004) Recovery of hippocampal cell proliferation and BDNF levels, both of which are reduced by repeated restraint stress, is accelerated by chronic venlafaxine. Pharmacogenomics J 4:322-331.

Chapter 6

Global conclusions and future prospects

6.1 Importance of Identifying and Understanding Differences in Models of Developmental SERT Disruption

A primary purpose of this thesis research was to acquire a clearer understanding of the developmental influences of the serotonin neurotransmitter system and how deviations from what is considered normal development lead to lifelong altered neurochemical trajectories that manifest as altered emotionality in adulthood. Two studies (Chapters 2 and 3) were undertaken to compare directly two models of early serotonin transporter (SERT) disruption. Serotonin transporters are primary targets of antidepressant drugs used to treat symptoms of major depression. The first model is a mouse line with constitutive deletions in SERT, which were generated more than a decade ago (Bengel et al., 1998). These mice show robust increases in anxiety-like behavior (Joeyen-Waldorf et al., 2009). The second model involves pharmacologic inhibition of SERT by antidepressants during postnatal development. This model was first reported by Mirmiran et al. using the tricyclic antidepressant clomipramine in rats during early postnatal life to
study changes in rapid eye movement (REM) sleep (Mirmiran et al., 1981). Another group re-interpreted the original findings as indicative of “endogenous depression” (Vogel et al., 1988; Vogel et al., 1990); disruption of REM sleep is a common feature of depression. Subsequently, findings that clomipramine produced changes in REM sleep, sexual dysfunction, and behavioral despair in rats were reported (Hilakivi and Hilakivi, 1987; Hilakivi et al., 1988; Fernandez-Pardal and Hilakivi, 1989; Neill et al., 1990; Velazquez-Moctezuma and Diaz Ruiz, 1992; Frank and Heller, 1997; Hansen et al., 1997; Maciag et al., 2006b; Maciag et al., 2006a; Bhagya et al., 2008).

Fluoxetine was the first serotonin-selective reuptake inhibitor (SSRI) antidepressant developed and it remains one of the most highly prescribed medications for the treatment of mood and anxiety disorders (Lindsley, 2012). Gingrich and coworkers have compared postnatal fluoxetine administration to genetic SERT deficiency regarding altered behavioral states in adulthood (Ansorge et al., 2004; Ansorge et al., 2008). They concluded that brief postnatal SERT inhibition produced behavioral changes similar to the phenotype observed in SERT-deficient mice. Behavior changes included increased emotionality as indicated by increased anxiety in tests using a novel environment and conflict, and altered fear in tests of inescapable shock. This group, as well as another, extended these findings to include other SSRIs, including S-citalopram (S-CIT; escitalopram) (Ansorge et al., 2004; Ansorge et al., 2008; Popa et al., 2008). Indeed, mice exposed to SSRIs during postnatal life were different from saline-injected mice. However, behavioral changes occurred mainly in locomotor or exploratory behaviors (e.g., total distance traveled
in a novel open field or numbers of rears). No differences were detected in parameters that are most often thought to reflect anxiety-related behavior (e.g., time and entries in the open aversive arms of the elevated plus maze and time spent in the center of an open field arena) (Walf and Frye, 2007), in postnatal SSRI-exposed mice. By contrast, the latter are reduced in SERT-deficient indicative of an increased anxiety-like phenotype. The dissimilarities between these two models of early life SERT disruption with respect to anxiety-like behaviors was the major impetus for the experiments that comprise part of this thesis due to the clinical relevance of both animal models.

Two major conclusions can be derived from the results of experiments presented in Chapters 2 and 3. (1) Administration of antidepressants to block SERT during early postnatal life in rodents produces adult behaviors that are different, and sometimes opposite those observed in mice that lack SERT throughout life. And (2), differential secondary (off-target) actions of SSRIs likely cause divergent behaviors observed in mice treated with different SSRI, i.e., FLX vs S-CIT. As will be discussed in greater detail below, the first conclusion is clinically important in terms of how we regard the treatment of depression during pregnancy vs the ramifications of untreated maternal depression. Moreover, lifelong SERT deficiency can be used to model, to some extent, polymorphisms occurring in the human SERT gene (e.g. 5-HTTLPR), which are hypothesized to alter SERT expression and have been shown to be associated with differences in anxiety-like personality traits, particularly neuroticism (Canli and Lesch, 2007; Murphy and Lesch, 2008). The second
conclusion focuses on differences between antidepressants. It is not a new concept that SSRIs, which generally have the same primary mechanism of action, also have diverse secondary mechanisms of action (Carrasco and Sandner, 2005). The latter are likely to contribute to variations in efficacies of these drugs in humans and they underlie why responses to one SSRI may differ on an individual basis.

6.2 Antidepressants During Pregnancy vs Untreated Maternal Depression and Differential Effects of SSRIs: Weighing the Benefits and Risks

The safety of antidepressant treatment during pregnancy is an ongoing controversial issue. Conclusions from studies in Chapters 2 and 3, along with the work of others, support the idea that consideration of antidepressant use during pregnancy should be weighted heavily when compared to the alternative of neglecting to treat major depressive disorder. Many preclinical and clinical studies have monitored the effects of maternal stress, anxiety, and depression during pregnancy and the effects on general health, emotionality, and learning in offspring from these mothers. In rodents, the application of stressors during pregnancy increases the production of glucocorticoids, which have the ability to cross the placenta and permanently alter HPA axis function and stress responses in offspring (Lupien et al., 2009). Similarly, children of mothers suffering from major depression show similar profiles of altered stress responsiveness (e.g., increased cortisol levels) and learning and behavior impairments (reviewed in (Field et al., 2006; Nulman et al., 2012). Additionally, depression during pregnancy is a predictor of postpartum
depression (Skouteris et al., 2009) and continued exposure of infants and children to mothers with ongoing struggles with depression can lead to poorer outcomes on development (e.g. behavioral or cognitive) (Brand and Brennan, 2009). Overall, it can be generally concluded that the effects of failing to address depressive symptoms during pregnancy negatively impact children. Nevertheless, the first line of treatment for major depressive disorder, SSRIs, are not without effects in terms of safety during pregnancy, as well as efficacy across depressed individuals, as described in detail below.

Despite similar primary mechanisms of action of SSRIs to block SERT, secondary actions of these drugs are a means by which their actions can produce differential effects. The results in Chapter 3 support the idea that some SSRIs might have safer profiles during pregnancy than others regarding outcomes for children exposed to these agents in utero (e.g., FLX treatment was not associated with long term alterations in 5-HT1A autoreceptor function thereby leading to normal serotonergic state in adulthood). However, the issue of which antidepressant might be best suited for treatment of depression during pregnancy is complicated by evidence that overall efficacy of SSRIs is low; many patients fail to respond to antidepressant treatment and often, serial treatment with different types of SSRIs are required, each of which takes weeks before benefits can be observed in cases where alleviation of symptoms is actually achieved (reviewed in Chapter 1; (Rush, 2007; Sinyor et al., 2010). Furthermore, despite SERT being the primary target of antidepressants, not all SSRIs or dose regimens succeed in producing increases in
levels of extracellular serotonin levels as determined by microdialysis experiments in preclinical studies (Andrews, 2009; Luellen et al., 2010).

Serotonin-selective reuptake inhibitors, tricyclic antidepressants (TCAs), and mixed serotonin and norepinephrine reuptake inhibitors (SNRIs) have been among the psychotropic agents prescribed during pregnancy, with SSRIs most often reported on in terms of safety of use (reviewed in (Gentile, 2011; Patil et al., 2011). In addition to differences in secondary effects of SSRIs discussed in Chapter 3, cytochromes that metabolize these drugs differ. Whereas S-CIT is metabolized by cytochrome P-450 (CYP) 3A4 and CYP 2CA19, fluoxetine is metabolized by CYP 2D6 (Patil et al., 2011). The metabolism of fluoxetine by CYP 2D6 has been associated with a mechanism for the observance of trough levels of fluoxetine and its metabolite during pregnancy (Heikkinen et al., 2003; Sit et al., 2010; Patil et al., 2011). Changes in serum concentrations of these drugs due to variable mechanisms of metabolism might be one explanation for how their safety profiles differ (i.e., less drug available to cross the placenta and influence development of the fetus).

For two antidepressants, the SSRI paroxetine and the TCA clomipramine, clinical studies conclude that these drugs are generally unsafe for pregnant women. A number of studies have correlated negative outcomes for children from mothers taking paroxetine beginning in the first trimester including cardiac malformations and septal defects, suggesting that this drug should not be prescribed during pregnancy (Bar-Oz et al., 2007; Cole et al., 2007). As described previously,
administration of clomipramine to rodents during postnatal life has been shown to produce many adverse effects in offspring that resemble features of depression-like behavior (Hilakivi and Hilakivi, 1987; Hilakivi et al., 1988; Fernandez-Pardal and Hilakivi, 1989; Neill et al., 1990; Velazquez-Moctezuma and Diaz Ruiz, 1992; Frank and Heller, 1997; Hansen et al., 1997; Maciag et al., 2006b; Maciag et al., 2006a; Bhagya et al., 2008). Similarly, cardiac malformations have been observed in children of mothers who were prescribed Clomipramine providing one instance where preclinical studies support clinical findings to advocate against prescribing this TCA (Kallen, 2007; Gentile, 2011). While these two drugs can be ruled out as safe antidepressants during the antenatal period, none of the most commonly prescribed SSRIs (e.g., sertraline, citalopram, escitalopram, fluoxetine, fluvoxamine) have provided clear evidence for safe outcomes for children exposed in utero (Gentile, 2011; Gentile and Galbally, 2011) although some are reportedly better than others (e.g., sertraline) (Nielsen and Damkier, 2012).

### 6.3 Overcoming Variability and Obtaining Reproducibility in mRNA Studies

In Chapter 5, two methods of real-time quantitative PCR were employed, one using SYBR green chemistry and the other using Taqman probe chemistry. The SYBR green method is inexpensive compared to TaqMan once the assays are designed and reproducible. However, SYBR green assays require time-consuming validation to ensure that primer pairs are amplifying genes of interest reliably given
that SYBR green assays are nonspecific. Validation steps required for the BDNF study included (1) redesigning primer pairs and testing previously published, pairs which often times were not reliable; (2) optimizing forward and reverse primer concentrations; and (3) defining the dynamic range by running various serial dilutions of cDNA and with different dilution factors. Even once specificity was established, it remained difficult to maintain the consistency of the reaction efficiency of the primer sets from one experiment to the next. Thus, it became a lengthy process of designing and re-designing primer sets until a pair was working well.

After switching to TaqMan probes and primers for the SERT studies in rhesus and humans, assay reproducibility between experiments was maintained consistently and there was no question of specificity in terms of the sequence amplified. Additionally, the dynamic range for the gene of interest and the control genes was the same. Given the importance of the finding that BDNF levels were reduced in SERT-deficient mice and how this correlates with their anxiogenic phenotype, future studies that reliably detect BDNF-specific mRNAs should be undertaken using Taqman chemistries. Identifying BDNF-specific exons that are reduced in mice with constitutive SERT deletions can provide avenues for therapeutic targets for combating anxiety disorders. The use of RT-qPCR to measure mRNA levels of genes of interest is an invaluable tool. Once established and reproducible, these assays can be applied to ongoing projects where detail at the molecular level is needed.
6.4 Future Prospects

In the work presented in the primary project of this thesis, we supported our hypothesis that transient SERT disruption using pharmacological agents during postnatal life versus lifelong reductions in SERT differed in terms of emotionality associated with alterations in anxiety in both during late adolescence and throughout different stages in adult life. Investigations were extended to identify mechanistic differences that result in these divergent behaviors including physiological and in vivo microdialysis and no net flux data pointing to 5-HT1A autoreceptors. We have shown that postnatal S-CIT administration induces hypersensitivity of 5-HT1A autoreceptors, whereas constitutive SERT deficiency results in desensitized 5-HT1A receptors. Opposing changes in functional aspects of 5-HT1A autoreceptors were then correlated with contrasting extracellular serotonin levels that produced a “hyposerotonergic” state in S-CIT-treated mice and a “hyperserotonergic” state in SERT-deficient mice.

Understanding what precisely occurs during development that leads to persistent changes in the serotonin system are the next important steps in this project. Identifying new modes of network regulation by 5-HT1A autoreceptors (Chapter 4) using electrophysiology to investigate differences in firing rates of serotonin neurons will be important. Parsimonious explanations, such as reductions in the number of serotonin neurons, might explain hyposerotonergic states associated with postnatal S-CIT treatment. This idea is supported by evidence that
neonatal administration of citalopram and clomipramine to rats causes reductions in tryptophan hydroxylase immunoreactivity, which is the rate-limiting enzyme in serotonin synthesis (Maciag et al., 2006b). Alterations in other serotonin receptor subtypes, including 5-HT7 receptors (which are also targeted by the serotonin receptor agonist 8-OH-DPAT) might provide additional information on changes that underlie phenotypic differences.

Unraveling the alterations that occur after S-CIT exposure that are not associated with FLX might help to answer the question of why some antidepressants are safer than others during pregnancy. A study to administer other commonly prescribed SSRIs (e.g., sertraline, fluvoxamine and the racemic form of citalopram) to observe whether the effects of these drugs more closely resemble those of S-CIT or FLX might further our understanding of differential mechanisms of action that lead to potentially altered safety profiles. Overarching conclusions regarding the use of antidepressants during pregnancy are not possible at this stage, in large part due to the heterogeneity of methods used to assess patients and their children. Furthermore, negative effects associated with SSRI use during the antenatal period have only become evident in recent years, and literature is sparse, especially in children monitored beyond adolescence. Regardless, examining antidepressant safety with respect to individual SSRIs to elucidate those with minimal risks remains a better option than leaving depressive symptoms untreated.
References


Appendix A

Figures

(a) Elevated Plus Maze

- Anxiety measures
- Locomotor measure
- Exploration measures

- Open Arm Time
- Open Arm Entries
- Open Arm Latency
- Closed Arm Time
- Closed Arm Entries
- Total Arm Entries
- Rears
- Head Dips

(b) Open Field Test

- Anxiety measures
- Locomotor measure

- Center distance traveled
- Center time
- Total distance traveled

(c) Forced Swim Test

- Acclimation period
- 2 min
- Last 4 min
- Immobile vs active behaviors
- 24 hour retest session
Figure A-1: Representation of behavior apparatuses and respective measures.

Graphical depictions of the elevated plus maze (EPM) (a), open field test (OFT) (b) and forced swim test (FST) (c) are shown. Both the elevated plus maze and open field tests assess anxiety like behaviors using parameters of open time/entries and center distance:total distance/time, respectively. Additionally, these tests can provide locomotor behavior information by measuring total arm entries in the EPM and total distance traveled in the OFT. Exploratory behaviors including rears and head dips can also be measured in the EPM. Behavioral despair or depression-like behavior is measured by assessing immobility times during two trials of the FST.
Figure A-2: Elevated plus maze data across testing environments. Male SERT-deficient mice approximately 3 months of age (postnatal day 81; \( N=15 \) for SERT+/-, \( N=31 \) for SERT+-, and \( N=13 \) for SERT-/- mice) were tested in the elevated plus maze at the Pennsylvania State University (PSU). Male 3-month-old SERT-/- mice (PSU) spent significantly less time in the open arms (a) and made fewer open arm entries (b) than male SERT+/- mice. For reference, 2-month old SERT+/- mice that served as unhandled controls in the postnatal antidepressant cohort tested at PSU and SERT+/- mice tested at the University of California, Los Angeles (UCLA) are shown. Activity levels are consistent among wildtype mice at PSU regardless of age. Despite the higher activity levels of SERT+/- mice tested at UCLA, genotype effects reflecting an increased anxiety-related phenotype remain consistent across test locations (see main text; Figure 2). The shaded gray bars indicate mean percent time or entries into the open arms ± SEMs for 3 month-old SERT+/- mice. *\( P<0.05 \) with respect to SERT+/- mice; †\( P<0.05 \) with respect to SERT+/- mice.
## Appendix B

### Tables

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<th>PAPER</th>
<th>BACKGROUND STRAIN</th>
<th>AGE</th>
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<td>Open Field</td>
<td>time/entries/distance in center of arena</td>
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<td>é ANXIETY Anxiety more pronounced in males</td>
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<td></td>
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<td>Elevate Zero Maze</td>
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<td>Open Field</td>
<td>crosses/time/entries in center</td>
<td>é anxiety</td>
<td>é ANXIETY Anxiety more pronounced in males</td>
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<td>Forced Swim</td>
<td>percent immobility</td>
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<td>Ramboz, S. et al. 1998</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>Adult (specific age not reported)</td>
<td>♀ and ♂ (separate analysis)</td>
<td>Open Field</td>
<td>time/distance in center of arena, total distance, rearings &amp; nose pokes</td>
<td>é anxiety é exploratory behavior &amp; locomotor activity</td>
<td>é ANXIETY Anxiety more pronounced in males</td>
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<td>Elevated Plus Maze</td>
<td>time/entries into open arms, total arm entries, rearings &amp; head dips</td>
<td>é anxiety é head dips</td>
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<td>Dulawa, S.C. et al. 2000</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>16 18 weeks</td>
<td>♀</td>
<td>Prepulse Inhibition</td>
<td>Prepulse Inhibition</td>
<td>NC in PPI</td>
<td>Normal Sensorimotor gating</td>
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<td>Novelty Suppressed Feeding</td>
<td>latency to feed following 24 h food deprivation</td>
<td>é anxiety</td>
<td>é ANXIETY &amp; FEAR RESPONSE</td>
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<td></td>
<td>Foot Shock</td>
<td>percent freezing</td>
<td>é freezing</td>
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</table>

| Gross, C. et al. 2000 | 129 Sv CONSTITUTIVE KNOCKOUT | Adult (specific age not reported) | ♀ and ♂ (combined analysis) | Novelty Suppressed Feeding | latency to feed following 24 h food deprivation | é anxiety | é ANXIETY & FEAR RESPONSE |
|                        |                   |                      |          | Foot Shock       | percent freezing                                          | é freezing                                   |                                         |

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<th>PAPER</th>
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<th>GENDER</th>
<th>BEHAVIOR TEST</th>
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<tr>
<td>Sarnyai, Z. et al. 2000</td>
<td>Swiss Webster CONSTITUTIVE KNOCKOUT</td>
<td>Adult (specific age not reported)</td>
<td>N.S.</td>
<td>Morris Water Maze</td>
<td>latency to reach hidden platform/visible platform</td>
<td>é latency to reach hidden but not visible platform</td>
<td>ALTERED HIPPOCAMPAL DEPENDENT LEARNING AND MEMORY</td>
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<td>Y Maze</td>
<td>entries/time in each arm, first choice novel arm</td>
<td>No preference for novel arms</td>
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<td>Spontaneous Alternation</td>
<td># arms entered and # alternations between arms of Y maze</td>
<td>NC in entries or alternations</td>
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<td>Dirks, A. et al. 2001</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>8 14 weeks</td>
<td>♂</td>
<td>Acoustic Startle Reactivity</td>
<td>startle magnitude</td>
<td>NC in startle magnitude</td>
<td>INTACT STARTLE REACTIVITY</td>
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<td>8 10 weeks (combined analysis)</td>
<td>♂ and ♀ (combined analysis)</td>
<td>Open Field</td>
<td>distance in center &amp; total distance in arena</td>
<td>éanxiety é locomotor activity</td>
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<td>Elevated Plus Maze</td>
<td>percent entries into open arms</td>
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<td>Novelty Suppressed Feeding</td>
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<td>éanxiety</td>
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<td>Pattij, T. et al. 2002</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>8 weeks</td>
<td>♂</td>
<td>Elevated Plus Maze</td>
<td>percent open arm time, # of open arm entries</td>
<td>NC in anxiety</td>
<td>= ANXIETY</td>
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<td>Pattij et al. 2002</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>12 weeks</td>
<td>♂</td>
<td>Novel Environment</td>
<td>locomotion, rearing, grooming, exploration, stretched approach, posture, immobility &amp; burying behaviors</td>
<td>ê time locomotion, stretched approach, rearing &amp; burying ê # rearings, stretched approach, burying</td>
<td>ê EXPLORATORY ACTIVITY</td>
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<td>Groenink, L. et al. 2003</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>8 12 weeks</td>
<td>N.S.</td>
<td>Open Field</td>
<td>time/entries/distance in center of arena, total distance</td>
<td>NC anxiety or locomotor activity</td>
<td>= ANXIETY (explained as potential ceiling effect given increased anxiety of strain)</td>
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<td>Light Dark Test</td>
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<td>distance/entries/percent time in light compartment, total distance</td>
<td>NC anxiety</td>
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<td>Elevated Plus Maze</td>
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<td>distance/entries and percent time in open arms, total distance</td>
<td>NC anxiety or locomotor activity</td>
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<td>Fear Conditioning</td>
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<td>percent freezing</td>
<td>NC freezing behavior</td>
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<td>Pattij, T. et al. 2003</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>8 weeks</td>
<td>♂</td>
<td>Operant Conditioning</td>
<td>autoshaping, acquisition/reversal learning, extinction</td>
<td>ê autoshaping, NC acquisition/reversal learning or extinction</td>
<td>= REINFORCEMENT LEARNING</td>
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<td>Bailey, S.J. et al. 2004</td>
<td>Swiss Webster and C57BL/6 CONSTITUTIVE KNOCKOUT</td>
<td>8 20 weeks</td>
<td>♂</td>
<td>Elevated Plus Maze</td>
<td>percent time/entries in open arms, total arm entries</td>
<td>éxanxiety both strains</td>
<td>é ANXIETY &amp; FEAR RESPONSE é EXPLORATORY ACTIVITY (strain dependent)</td>
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<td>Open Field</td>
<td>percent time/entries in center of arena</td>
<td>éxanxiety both strains é locomotor activity in B6 only</td>
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<td>Wolff, M. et al. 2004</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>12 or 88 weeks</td>
<td>♂</td>
<td>Morris Water Maze</td>
<td>path length to reach hidden platform</td>
<td>é path length to reach hidden platform on younger but not older mice</td>
<td>ALTERED AGE AND HIPPOCAMPAL DEPENDENT LEARNING AND MEMORY</td>
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<td>Jones, M.D. &amp; Luck,i J. 2005</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>12 15 weeks</td>
<td>♀ and ♂ (separate analysis)</td>
<td>Tail Suspension</td>
<td>immobility time</td>
<td>é immobility</td>
<td>é IMMObILITY</td>
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<td>Klemenhagen, K.C. et al. 2006</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>Adult (specific age not reported)</td>
<td>♂</td>
<td>Light Dark Test</td>
<td>time in light compartment and # of ambulations</td>
<td>é anxiety NC in locomotor activity</td>
<td>é ANXIETY &amp; FEAR RESPONSE</td>
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<td>percent freezing</td>
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<td>Tsetsenis T &amp; Gross et al. 2007</td>
<td>mixed C57BL/6j, CBA/J, 129S6/SvEvTac CONSTITUTIVE KNOCKOUT</td>
<td>Adult (specific age not reported)</td>
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<td>Fear Conditioning</td>
<td>percent freezing</td>
<td>ë freezing behavior (cue dependent)</td>
<td>ë FEAR RESPONSE</td>
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<td>Bechtholt, A.J. et al. 2008</td>
<td>129 Sv CONSTITUTIVE KNOCKOUT</td>
<td>12-24 weeks</td>
<td>♀ and ♂ (separate analysis)</td>
<td>Sucrose Consumption</td>
<td>Intake and preference for sucrose over water</td>
<td>ë consumption and preference in females only (1% concentration)</td>
<td>ë ANHEDONIA</td>
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<td>Zanettini, C. et al. 2009</td>
<td>mixed C57BL/6j, CBA/J, 129S6/SvEvTac CONSTITUTIVE KNOCKOUT</td>
<td>12-20 weeks</td>
<td>♂</td>
<td>Open Field</td>
<td>time in center of arena and total distance</td>
<td>ë anxiety ë locomotor activity</td>
<td>ë ANXIETY &amp; ë SOCIAL INTERACTION</td>
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<td>Richardson Jones J.W. et al. 2010</td>
<td>mixed C57Bl/6J, CBA/J, 129S6/SvEvTac POPULATION SPECIFIC KNOCKOUT</td>
<td>11-13 weeks</td>
<td>♂</td>
<td>Open Field</td>
<td>time in center of arena and total distance</td>
<td>NC in anxiety or locomotor activity</td>
<td>= ANXIETY IN MICE WITH ~30% REDUCTIONS IN 5 HT1A AUTORECEPTORS</td>
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<td>latency to feed following 24 h food deprivation and in presence of fluoxetine</td>
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<td>Open Field</td>
<td>percent center distance and total distance</td>
<td>é anxiety in auto KO</td>
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<td>Light Dark Test</td>
<td>percent distance/entries in light compartment and total distance</td>
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<td>Forced Swim</td>
<td>percent mobility</td>
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<td>Bortolozzi, A. et al. 2012</td>
<td>C57BL/6 POPULATION SPECIFIC MODELS</td>
<td>10 15 weeks</td>
<td>♂️</td>
<td>Elevated Plus Maze</td>
<td>percent time/entries into open arms</td>
<td>é anxiety in KO restored in C-1A siRNA</td>
<td>é ANXIETY IN KO THAT IS RESTORED IN MICE WHERE C-1A siRNA IS INFUSED INTO THE DORSAL RAPHE</td>
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<td>Tail Suspension</td>
<td>immobility time</td>
<td>é immobility in KO and C</td>
<td>é IMMObILITY IN BOTH MODELS</td>
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<td>Elevated Plus Maze</td>
<td>percent time/entries into open arms</td>
<td>é anxiety in KO restored in C-1A siRNA</td>
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<td>Iacono, L.L. &amp; Gross, C. 2008</td>
<td>mixed C57BL/6J, CBA/J, 129S6/SvEvTac PHARMACOLOGIC INHIBITION</td>
<td>14 16 weeks</td>
<td>♂</td>
<td>Open Field</td>
<td>time/distance in center &amp; total distance in arena</td>
<td>ê anxiety</td>
<td>ê ANXIETY IN WILDTYPE MICE TREATED WAY100635 VIA OSMOTIC MINIPUMP FROM P13 P34 (0.15 mg/h per kg body weight)</td>
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<td>Elevate Plus Maze</td>
<td>time/entries into open arms</td>
<td>= anxiety</td>
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<td>Novelty Suppressed Feeding</td>
<td>latency to feed following 24 h food deprivation</td>
<td>ê anxiety</td>
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<td>Vinkers, C.H. et al. 2010</td>
<td>Swiss Webster PHARMACOLOGIC INHIBITION</td>
<td>P7, P12, P21</td>
<td>♂</td>
<td>Elevated Plus Maze</td>
<td>percent open arm entries &amp; total arm entries</td>
<td>ê anxiety</td>
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<td>Open Field</td>
<td># entries, percent distance and time spent in center of arena</td>
<td>ê anxiety and ê locomotor activity</td>
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<td># of ultrasonic vocalizations (USVs)</td>
<td>ê USVs</td>
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<td>NMRI outbred OVEREXPRESSION</td>
<td>15 22 weeks (separate analysis)</td>
<td>♂ and ♀</td>
<td>Elevated Plus Maze</td>
<td>time/entries in open/closed arms, total arm entries, rearings &amp; head dips</td>
<td>ê anxiety and ê locomotor activity</td>
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<td>Open Field</td>
<td>entries/time spent in center of arena, total distance &amp; rearings</td>
<td>ê locomotor activity (male only) and = anxiety</td>
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<td>NMRI outbred OVEREXPRESSION</td>
<td>12 13 weeks</td>
<td>♀ and ♂ (separate analysis)</td>
<td>Hole Board Morris Water Maze</td>
<td># nose pokes &amp; distance traveled latency &amp; path length to find hidden platform</td>
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<td>rearings &amp; total distance in arena time/entries into open arms, closed/total arm entries, rearings &amp; head dips</td>
<td>é locomotor activity &amp; exploration = anxiety &amp; locomotor activity</td>
<td>= ANXIETY &amp; é LOCOMOTOR AND EXPLORATORY BEHAVIORS IN MICE PERMANENTLY OVEREXPRESSION Changes more prominent in males</td>
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<td>♀ and ♂ (separate analysis)</td>
<td>Forced Swim</td>
<td>Immobility Time</td>
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Table B-1: Behavior phenotypes of animal models of 5-HT1A receptor disruption. This table supplies detailed information pertaining to models of 5-HT1A constitutive knockout, overexpression, and pharmacological inhibition and the outcome of these models on tests of emotion-related behavior including anxiety and depression parameters as well as learning and memory models.
References


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Bechtholt, A. J., Smith, K., Gaughan, S., and Lucki, I. (2008) Sucrose intake and fasting glucose levels in 5-HT(1A) and 5-HT(1B) receptor mutant mice, Physiol Behav 93, 659-665.


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