Developmental Monoamine Signaling Impacts Adult Affective and Aggressive behaviors

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ABSTRACT

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Most neuropsychiatric disorders have developmental origins and an emerging model postulates that such developmental vulnerability is often restricted to sensitive periods. The concept of sensitive developmental periods for the indelible modulation of complex behaviors is similar to that described for sensory systems (e.g. visual cortex, ocular dominance plasticity), but effected behaviors, modulating factors, and underlying mechanisms are much less well understood. Furthering our knowledge of sensitive periods that determine the developmental trajectory of complex behaviors is a necessary step towards improving diagnosis, prevention and treatment approaches for neuropsychiatric disorders. To fulfill this mission, I here investigate how genetic and environmental risk factors act during sensitive periods of brain development to alter adult behavior and thereby confer vulnerability to neuropsychiatric disorders.

My thesis is divided into four chapters. Chapter I provides general background and significance information relevant to chapters II-IV.

Chapter II focuses on elucidating and comparing the consequences of developmental serotonin (5-HT) transporter (5-HTT) and monoamine oxidase A (MAOA) blockade. Pharmacologic MAOA or 5-HTT blockade in adulthood has antidepressant and anxiolytic efficacy. Yet, genetically conferred MAOA or 5-HTT hypo-activity is associated with altered aggression and increased anxiety/depression. Here I test the hypothesis that increased monoamine signaling during development

causes these paradoxical aggressive and affective phenotypes. I find that pharmacologic MAOA blockade during early postnatal development (P2-P21) increases anxiety- and depression-like behavior in mice, mimicking the effect of P2-21 5-HTT inhibition. Moreover, MAOA or dopamine transporter (but not norepinephrine transporter) blockade during peri-adolescence (P22-P41) increases adult aggressive behavior. 5-HTT blockade from P2-P21 or P22-P41 reduces adult aggressive behavior. Altered aggression correlates positively with locomotor response to amphetamine challenge in adulthood and striatal dopamine and DOPAC content is increased while brainstem 5-HIAA content is decreased in high aggression. Taken together, these data suggest that genetic and pharmacologic factors impacting dopamine and serotonin signaling during sensitive developmental periods confer risk for aggressive and emotional dysfunction in humans.

Chapter III focuses on refining the 5-HT sensitive period affecting anxiety and depression-like behavior. Specifically, I hypothesized that the identified P2-21 period, which encompasses many developmental processes, contains a narrower critical period, affecting fewer developmental processes but having the same impact on adult behavior. This experiment serves two purposes: First, I seek to gain insight into the neural substrates and possible developmental processes underlying developmental programing of anxiety- and depression-like behaviors through 5-HT signaling. Second, I aim at providing translationally relevant data, informing clinical and epidemiological studies as to which developmental window might be sensitive to 5-HT altering factors in humans. This thesis research shows that postnatal fluoxetine (PN-FLX) treatment from P2-11 leads to increased adult anxiety- and depressive-like behavior in mice, while PN-FLX treatment from P12-21 or P22-41 has no effect in adult anxiety- and depressive-like

behavior. In addition, adult chronic FLX treatment could not rescue the behavioral phenotype produced by P2-11 5-HTT blockade.

Chapter IV focuses on the role of 5-HT_{2A} receptor signaling in mediating the effect of P2-11 5-HTT blockade on adult behavior. Htr2a-/- mice display reduced conflict anxiety. Because 5-HT_{2A} receptor antagonists do not reduce conflict anxiety in adulthood, I hypothesized that the behavioral htr2a-/- phenotype is at least partially of developmental origin, which would further indicate that increased 5-HT_{2A} receptor signaling during development could increase conflict anxiety. To investigate this hypothesis, I analyzed the effect of P2-11 5-HTT blockade on anxiety and depression-like behaviors in htr2a+/+, +/-, and -/- mice. Supporting my hypothesis, I find that absence of htr2a improved performance of PN-FLX treated mice in the novelty suppressed feeding task, by decreasing the latency to feed to control levels. Absence of htr2a, however, did not have ameliorative effects on PN-FLX phenotypes in the open field and shock escape tests. In summary, these data demonstrate that 5-HT_{2A} receptor signaling mediates some but not all consequences of increased P2-11 5-HT signaling.

Taken together, in my thesis work I identified and characterized two sensitive developmental periods whereupon early-life perturbation of monoamine signaling alters adult behavior: an early postnatal (P2-P11) 5-HT-sensitive period that affects anxiety and depression-related behaviors and a later peri-adolescent (P22-P41) DA- and 5-HT-sensitive period altering aggression and behavioral sensitivity amphetamine. These data give insight into the etiology of neuropsychiatric disorders and should ultimately help improving diagnosis, prevention and treatment approaches.

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Chapter I General Introduction

I.I Depression and anxiety

I.I.I Clinical presentation and treatments

Depression is a chronic, recurring illness that is among the most prevalent psychiatric disorders worldwide. Recent studies estimate that one in six adults in the United States will succumb to depression or anxiety in their lifetime (Kessler et al., 2005), and it is the third leading contributor to the global disease financial burden (Collins et al., 2011). Depression has been categorized into several classes including major depressive disorder (MDD), dysthymic disorder, and bipolar disprder. MDD is associated with negative mood, pessimism, hopelessness, a loss of interest in formally pleasurable activities (anhedonia), fatigue or loss of energy, significant weight loss or gain, changes in appetite, and sleep patterns (*The Diagnostic and Statistical Manual of Mental Disorders, 4th Edition*; DSM-IV-TR, 2000).

Anxiety is a natural adaptive consequence of stress that helps to cope with stressors. Anxiety disorders are maladaptive, dysfunctional, chronic and persistent, and can grow progressively worse if not treated (Lesch et al., 2003). Anxiety disorders include generalized anxiety disorder (GAD), social anxiety disorder (also known as social phobia), specific phobia, panic disorder with and without agoraphobia, obsessive-compulsive disorder (OCD), posttraumatic stress disorder (PTSD), anxiety secondary to medical condition, acute stress disorder (ASD), and substance-induced anxiety disorder. Anxiety is associated with worry, fatigue, difficulty concentrating, irritability, muscle tension, and sleep disturbance (*The Diagnostic and Statistical Manual of Mental Disorders, 4th Edition*; DSM-IV-TR, 2000). Anxiety disorders have a lifetime prevalence

rate of ~28% (Kessler et al., 2005). Depressive and anxiety disorders display high comorbidity rates, with at least 50–60% of individuals with depression reporting a lifetime history of one or more anxiety disorders (Kaufman and Charney, 2000).

There are many different treatments for depression such as psychotherapy, antidepressant medication, and electroconvulsive therapy (ECT). The first generation antidepressant drugs were the tricyclic antidepressants and the monoamine oxidase inhibitors. Tricyclic antidepressants act to inhibit serotonin or norepinephrine reuptake transporters, whereas monoamine oxidase inhibitors inhibit monoamine oxidase (a major catabolic enzyme for monoamine neurotransmitters). The discovery of the acute mechanisms of antidepressant drugs led to development of numerous second-generation medications including serotonin-selective reuptake inhibitors (SSRIs) and norepinephrine-selective reuptake inhibitors (NSRIs). The availability of clinically active antidepressants also made it possible to develop and validate a wide range of behavioral tests with which to study depression-like phenotypes in animal models (Nestler et al., 2002). However, current medications have their limitations. Some patients respond well to the treatments, while others only show partial responses or complete non-response (Fava and Davidson, 1996; Rush et al., 2006). In addition, current antidepressant medications require weeks of drug administration before achieving therapeutic benefits. SSRIs are also used as the first line treatment for anxiety disorders. The overlapping disease symptoms and treatment efficacies suggest that depression and anxiety might result from common pathophyiological conditions. The mechanisms of treatment and etiology are poorly understood. Elucidating the underlying pathophysiology of anxiety

and depression disorders as well as mechanisms of therapeutic treatments remains an unmet need.

I.I.II Genetic and environmental factors on depression/anxiety

Epidemiological and genetic studies demonstrate that both genetic and environmental factors contribute to depression and anxiety disorders. The prime example for genetic risk is the common functional polymorphism in the promoter region of the human serotonin transporter gene (*5-htt*), the *5HTTLPR*, which affects *5-htt* transcription and has been associated with neuroticism, elevated trait anxiety, and increased vulnerability to affective disorders in many studies (Collier et al., 1996; Lesch et al., 1996; Levinson, 2006). Environmental stressors such as stressful life events (job loss, marital problems, major health problems and loss of close personal relationships) and adverse childhood experiences have also been associated with increased risk for anxiety and depression (Bernet and Stein, 1999; Mundt et al., 2000; Caspi et al., 2002; Arnow, 2004).

The notion that environmental factors interact with genetic predisposition to affect psychiatric disease outcome has found great support in recent years. In this specific form the diathesis-stress model, genetic factors influence the risk of major depression in part by influencing the susceptibility of individuals to the depressive effects of stressful life events (Kendler, 1995; Silberg et al., 2001; Kendler et al., 2005; Karg et al., 2011). A landmark study carried out by Caspi and colleagues demonstrated that low expressing variants of the *5-htt* interact with either early life stress (childhood maltreatment) or adult stresses to modulate the likelihood of depressive symptoms, diagnosed major depression,

and suicidal behavior in the study subjects (Caspi et al., 2003). Many but not all studies could confirm these early findings (Kaufman et al., 2004; Kendler et al., 2005; Willis-Owen et al., 2005; Chipman et al., 2007; Scheid et al., 2007; Power et al., 2010; Fergusson et al., 2011a; Karg et al., 2011), leading to the general consensus that individuals carrying the low expressing "*s*" allele are more prone to depressive symptoms in response to stressful events during adulthood and childhood than individuals carrying the higher expressing "*l*" allele (Caspi et al., 2003; Kendler et al., 2005; Taylor et al., 2006; Mandelli et al., 2007; Aguilera et al., 2009). In addition, polymorphysm/mutations in genes encoding 5-HT receptors or tryptophan hydroxylase-2 (*tph2*) enzyme critical for synthesis of serotonin have been shown to interact with environmental factors to predispose individuals to depression and anxiety disorders (Strobel et al., 2003; Waider et al., 2011). Collectively, these underscore gene by environment (G×E) interaction.

I.I.III Neural circuitry of depression and anxiety

Neuroimaging studies of subjects diagnosed with major depression have identified abnormalities in multiple areas of the brain including the prefrontal cortex (PFC), the amygdala, the hippocampus, the amygdala, and parts of the striatum and thalamus as well as the functional connectivity within neural circuitry (Drevets, 2003; Hulvershorn et al., 2011). For instance, a comprehensive meta-analysis compiling 225 individual studies on brain changes revealed decreased volumes of the hippocampus, thalamus, frontal lobe and orbitofrontal cortex in major depressive disorder (MDD) patients in comparison to healthy controls (Kempton et al., 2011). In addition, resting cerebral blood flow (CBF) and glucose metabolism are abnormally elevated in the

amygdala, and CBF response to a negative emotional valence is abnormal between depressive groups and controls (Drevets, 2003). Relative to controls, individuals with depressive disorder show reduced rostral anterior cingulate cortex (rACC) volume or gray matter (Frodl et al., 2008; Leung et al., 2009; Mak et al., 2009; Abe et al., 2010). The reduced grey matter volume in the prefrontal cortex is also revealed in neuroimaging studies (Drevets et al., 1997). These alterations might underlie the emotional, behavioral and cognitive manifestations of major depression. For example, changes in orbital and medial PFC areas are hypothesized to cause abnormal emotional behavior and stress responses. The striatum (particularly the ventral striatum or the nucleus accumbens [NAc]) and amygdala, and related brain areas, are important in emotional memory and stress response and could as a result mediate the anhedonia, anxiety, and reduced motivation (Nestler et al., 2002; Tye et al., 2011). The hypothalamus has been postulated to play a role in neurovegetative symptoms of depression, such as changes in sleep, appetite, and energy (Nestler et al., 2002). The hippocampus is involved in the regulation of mood and in mediation of antidepressant response (Samuels and Hen, 2011). These different brain regions implicated in depression form a series of interacting circuits (Drevets, 2001; Liotti and Mayberg, 2001; Nestler et al., 2002). Since the regions implicated in depression and anxiety undergo rapid changes during postnatal development, developmental events could potentially affect the maturation of these structures and ultimately lead to anxiety and depression related behavior changes.

I.II Aggression

I.II.I Description of aggression

Aggression is a complex social behavior evolved in the context of defending and obtaining resources. Traditionally, aggression has been defined as overt behavior that has the intention of inflicting physical damage on another individual and the potential for aggressive behavior exists whenever the interests of two or more individuals conflict (Nelson and Trainor, 2007). The most widely utilized classification of aggression is that of premeditated aggression versus reactive aggression. Premeditated aggression, also referred to as proactive aggression, represents a planned behavior that is not typically associated with frustration or response to immediate threat and is more goal-oriented and purpose-full (Siever, 2008). In contrast, reactive aggression is considered to be more impulsive and characterized by high levels of autonomic arousal and precipitation by provocation associated with negative emotions (Feilhauer et al., 2011). Premeditated aggression is associated with cognitive control while reactive aggression is associated with little cognitive processing (Blair, 2001; Feilhauer et al., 2011). Reactive aggression becomes pathological if the aggressive responses are exaggerated in relation to the emotional provocation that occurs. Human aggression as a trait is assessed using psychometric measures including inventories, questionnaires and scales. Aggression figures prominently as a positive symptom in many neuropsychiatric disorders (Steiner et al., 2003; Chaplin, 2006; Haller and Kruk, 2006). Attention-deficit hyperactivity disorder (ADHD), mood disorders, and in particular bipolar disorders/pediatric mania, schizophrenia, conduct disorder, and borderline personality disorders are most notably characterized by aggressive behavior (Hollander, 1999; Barkley, 2003; Connor et al.,

2003; Perroud et al., 2011; Soyka, 2011). Thus understanding the neurobiology of aggression holds therapeutic promise for a range of neuropsychiatric diseases.

I.II.II Genetic and environmental factors on aggression

Both genetic and environmental factors contribute to aggression and violence. Twin and adoption studies have suggested the presence of substantial genetic influence on aggression, especially impulsive aggression (Coccaro et al., 1993; Coccaro et al., 1997; Miles and Carey, 1997). Genetic factors such as susceptibility genes contribute vulnerability towards the acquisition of abnormal aggressive behaviors. One prominent example of a single genetic factor contributing risk to aggression is a loss of function mutation in the monoamine oxidase A gene (*maoa*). This *maoa* null allele has been linked to male antisocial behavior and borderline mental retardation, as affected individuals display mild mental retardation, an increase in aggressive outbursts, anger, and fear, and violent impulsive behavior (e.g. rape, assault, arson, and exhibitionism) (Brunner et al., 1993b; Brunner, 1996). A low-expressing polymorphism, the *maoa-VNTR*, is also associated with aggressive behavior (Manuck et al., 2000; Zalsman et al., 2005; Jabbi et al., 2007). Other genes have also been associated with aggressiveness. The human polymorphism of low catechol-*O*-methyltransferase (COMT) activity points to the risk for increased aggressive behavior preferentially in males (Strous et al., 1997; Volavka et al., 2004). Furthermore, the tryptophan hydroxylase 1 (*tph1*) A779C polymorphism, which affects serotonin biosynthesis and influences 5-hydroxyindoleacetic acid concentrations (5HIAA) in cerebrospinal fluid (CSF), may predispose to suicidality in violent subjects (Nielsen et al., 1994; Nielsen et al., 1998).

Environmental factors such as stress, childhood maltreatment, and even socioeconomic factors also interact with different genetic variants to predispose to aggression (Kim-Cohen et al., 2005; Moffitt, 2005; Craig, 2007). A landmark study carried out by Caspi et al. showed that the *maoa* polymorphism interacts with childhood maltreatment and adversity to predispose to violence (Caspi et al., 2002; Kim-Cohen et al., 2006). Later many but not all studies replicated these findings showing that there is a positive interaction between *maoa* low expression alleles and childhood adversity for abnormal behavior including adult anti-social behavior (Foley et al., 2004; Haberstick et al., 2005; Huizinga et al., 2006; Kim-Cohen et al., 2006; Young et al., 2006; Prichard et al., 2008; Fergusson et al., 2011b; Philibert et al., 2011). In summary, human aggressive behavior is complex and multi dimensional, and genetic factors and lifetime experiences interact with each other to confer risk for pathological aggression. To adequately diagnose, treat, and/or prevent pathological aggression, one needs to understand the underlying pathophysiology, and the following chapters will summarize the current knowledge about the involvement of monoamine neurotransmitters and insight into the underlying circuitry. Another important and central factor for understanding how genetic and environmental factors confer risk is timing. In my thesis, I explored the hypothesis that the early life disruption of monoamine signaling would lead to altered adult behaviors. Understanding of the relationship between genetic factors that predispose an individual to display aggressive behaviors, life experiences, and timing promises a better understanding of the etiology of aggression.

I.II.III Monoamine and aggression

Abnormal monoamine signaling has been implicated in aggression. The most prominent monoamine neurotransmitter alterations in aggression have been identified for 5-HT, norepinephrine, and dopamine.

5-HT.

5-HT can affect aggressiveness and impulsivity. In general, low activity of the 5-HT system has been associated with impulsive aggression in both human and animal studies (Coccaro et al., 1989; Linnoila and Virkkunen, 1992; Miczek et al., 1994). In humans, a low concentration of the 5-HT metabolite (5-HIAA) is associated with impulsivity and aggression (Brown et al., 1979; Virkkunen et al., 1994). In animal studies, low levels of 5-HIAA are linked to heightened impulsive and aggressive behaviors (Fairbanks et al., 2001). Manipulations that lower 5-HT signaling such as p-chlorophenylalanine (PCPA) injection increase impulsivity and aggression; increasing serotonin activity with serotonin precursors or SSRIs can reduce aggression behavior in rodents (Chiavegatto et al., 2001; Miczek et al., 2001).

In terms of a role for 5-HT receptors in mediating aggressive behaviors, the anti-aggressive effects of drugs activating 5-HT_{1A} and 5-HT_{1B} receptors have been extensively documented (Miczek et al., 1989; Sijbesma et al., 1991; Centenaro et al., 2008). 5-HT_{1A} receptor agonists decrease several types of aggressivity (Olivier et al., 1995). Male mice that lack 5-HT_{1B} receptors (*htr1b-/-*) are more aggressive than wild type mice, suggesting that 5-HT1B receptor signaling exerts inhibitory control on aggressive behavior (Saudou et al., 1994). Mice lacking neuronal nitric oxide synthase (NOS) exhibit impulsive

aggressive behavior and brain serotonin dysfunction (Chiavegatto et al., 2001). 5-HTergic neurons in mice lacking the ETS transcription factor Pet1 are depleted by 80%, and this hypo-5-HTergic mouse model exhibits increased aggression in the resident-intruder test (Hendricks et al., 2003). In addition, mice lacking 5-HTT exhibited reduced aggressive behavior, which could be linked to the fact that 5-HTT deficient mice show significant reduction in tissue content of 5-HT measured in multiple brain regions (brainstem, frontal cortex, hippocampus, hypothalamus and striatum) (Bengel et al., 1998; Holmes et al., 2002a; Mathews et al., 2004; Kim et al., 2005). In addition, social approach behavior is strongly reduced in *5-htt* -/- mice as demonstrated by reduced time spent in a compartment containing a stimulus mouse (Page et al., 2009). Taken together, these various studies support the notion that aggression is associated with serotonin dysfunction and changes in the metabolism of serotonin could predict aggressiveness. Traditionally, studies in humans and animals indicate that 5-HT activity (such as CSF 5-HIAA) is inversely related to certain kinds of aggression such as hostile aggressive behavior.

Dopamine (DA).

The DAergic system is involved in behavioral activation, motivation, and reward processing (Ikemoto and Panksepp, 1999; Everitt and Robbins, 2000; Volkow et al., 2011). It also plays an active role in the modulation of aggressive behaviors. The D_2-receptor antagonist haloperidol is used effectively to treat aggressive patients who are also psychotic (de Almeida et al., 2005). In animal studies, hyperactivity in the dopamine system is associated with increases in impulsive aggression (Harrison et al., 1997;

Soderstrom et al., 2001). Systematic administration of the DA agonists methamphetamine or apomorphine decreased the threshold for defensive attack behavior elicited by electrical stimulation of the ventromedial hypothalamic nucleus (Maeda et al., 1985; Maeda and Maki, 1986). Increased accumbal DA release was observed in anticipation of the aggressive episodes in rats (Ferrari et al., 2003) as well as during and following aggressive encounters (Tidey and Miczek, 1996; van Erp and Miczek, 2000). These neurochemical studies link elevated DA and its metabolites in the prefrontal cortex and nucleus accumbens not only to the initiation of attacks and threats, but also to the defensive and submissive responses in reaction to being attacked (Puglisi-Allegra et al., 1990; Tidey and Miczek, 1996). Blockade of dopamine D_1 or D_2 receptors in the nuclear accumbens attenuate aggressiveness in mice (Couppis and Kennedy, 2008).

Genetic studies have been done to characterize the molecular aspects of dopamine function in aggression. Disruption of the DAT increases extracellular dopamine concentrations in the striatum causing hyperdopamine tone, and DAT knockout mice show increased reactivity and aggression in some behavioral tests (Rodriguiz et al., 2004). Heterozygous COMT-deficient male mice exhibited increased aggressive behavior and increased frontal cortex dopamine levels (Gogos et al., 1998). In summary, studies focused on elucidating the association of DA activity in appropriate expression of aggressive behavior. In general, aggressiveness is associated with hyper-DA activity.

Norepinenephrine(NE)

Desipramine , which is a NE transporter inhibitor, dose-dependently increases the duration of aggressive behavior in isolated mice and pretreatment of isolated mice with

DSP-4, a selective NE neurotoxin, significantly attenuates the enhancing effect of desipramine on aggressive behavior (Matsumoto et al., 1991; Matsumoto et al., 1995; Haller et al., 1998). In addition, mice that lack α_{2C}-adrenergic autoreceptors consequently have increased NE levels and show enhanced isolation-induced aggression toward an unfamiliar intruder, whereas mice that over-express these receptors show the opposite effects (Sallinen et al., 1998). Dopamine beta-hydroxylase knockout (*Dbh* -/-) mice lacking NE do not exhibit aggression in resident-intruder test (Marino et al., 2005). Taken together these findings demonstrate that NE signaling is positively correlated with aggressiveness. Mice lacking MAOA are hyper-aggressive and show significantly elevated levels of NE and 5-HT in hippocampus, the frontal cortex, striatum and the cerebellum as well as elevated levels of DA in striatum, which indicates that abnormal NE levels might be casually involved in the aggressive phenotype of these mice (Kim et al., 1997; Chen et al., 2007).

In summary, the NE, 5-HT and DA systems show substantial changes associated with aggressive phenotype. Perturbations in monoamine systems might produce profound changes in circuitry, structure and function of the brain, which contribute to the abnormal aggressive behaviors. However, the detailed manner in which different monoamine systems regulate aggressive behaviors is not clear. A lot of questions remain unanswered. For example, to which extent do monoamines and their metabolite level reflect an aggressive trait? Can transient perturbations produce aggressive states/traits? Can different monoamines interact with each other to potentiate aggressive behaviors? Answers to these questions are needed to advance the monoamine hypothesis of

aggression.

I.II.IV The neural circuitry of aggression

There are structural and functional brain changes implicated in aggressiveness. In humans, reductions in prefrontal gray matter have been reported in individuals with antisocial personality disorders (Raine et al., 2000). Healthy subjects demonstrated blood flow reductions in the orbital frontal cortex in an unrestrained aggressive scenario in imaging studies (Pietrini et al., 2000). Patients with a history of violent behaviors displayed significantly decreased prefrontal activity compared to non-aggressive patient controls (Amen et al., 1996). Positron emission tomography (PET) studies in subjects with impulsive personality disorders or psychiatric patients with a history of violence show relative hypo-metabolism compared with healthy control subjects in areas of frontal and temporal cortex (Goyer et al., 1994; Volkow et al., 1995; Soloff et al., 2003). Patients with orbital and medial prefrontal cortex lesions exhibit impulsive and aggressive behavior (Davidson et al., 2000). In rodents, lesions of the orbitofrontal cortex increase aggression in rats (de Bruin et al., 1983). Since prefrontal cortex modulates subcortical circuitry to influence emotional response, it is postulated that impaired regulatory control of the prefrontal cortex may lead to excessive negative emotional reactivity and consequent violent behaviors.

The amygdala, hypothalamus and hippocampus have also been implicated in aggression. Borderline personality disorder patients exhibit increased activation of the amygdala in response to negative pictures versus a resting condition (Minzenberg et al., 2007; New et al., 2007). Abnormal hippocampal asymmetry consisting of relatively

reduced left hippocampal activity and greater right activity has been reported in anti-social subjects (Raine et al., 1997; Raine et al., 2004). Hypothalamic activation has also been associated with aggression in domestic violence perpetrators (George et al., 2004). Animal studies have also been conducted to investigate the brain regions involved in aggression. Experiments in cats have shown that attack behavior can be elicited by electrical stimulation of the hypothalamus (Siegel et al., 1999). Chemical (cholinergic) stimulation of the septum elicits rage behavior in the cat (Siegel and Skog, 1970). Amygdala lesions attenuated the facilitatory influences produced by the provocation on thresholds for hypothalamic defensive attack (Maeda and Maki, 1986). In rats, electric stimulation of the hypothalamus also induces aggression (Kruk et al., 1984). Recent technical innovation has advanced the understanding of circuitry of aggression. Optogenetic stimulation of neurons in the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) causes male mice to initiate attacks and pharmacogenetic silencing of the VMHvl reversibly inhibits inter-male aggression (Lin et al.). Taken together, an imbalance between limbic "drives" and prefrontal control mechanisms might be very important for the regulation of aggression episodes. However, research towards the circuitry underlying aggression is still very sporadic and primitive, which presents an unmet need to elucidate the changes in brain structure and circuitry underlying different forms of aggression.

I.III The 5-HT and the DA system in the brain

I.III.I 5-HT function and signaling

5-HT regulates a variety of physiological functions, including thermoregulation, respiration, circadian rhythm, sleep, appetite, aggression, sexual behavior, pain modulation, neuroendocrine modulation, and cognition (Halford et al., 2005; Popova, 2008; Hilaire et al., 2010; Ravindran and Stein, 2010; Bardin, 2011; Monti, 2011). It is involved in a variety of pathological conditions such as depression, anxiety (general anxiety disorder, obsessive compulsive disorder, social phobia), schizophrenia, and impulsive disorders (Lucki, 1998; Stahl, 1998).

5-HT is synthesized from the amino acid tryptophan through the action of two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC). The first step in the synthesis of 5-HT is the TPH-mediated reaction converting tryptophan to 5-Hydroxy-L-tryptophan (5-HTP). This is the rate-limiting step in the pathway. In the second step, 5-HTP is converted to serotonin by the DDC (Walther et al., 2003). The vesicular monoamine transporter type 2 (VMAT2) then transports 5-HT from the neuronal cytoplasm into vesicles (Takahashi et al., 1997). 5-HT is released into the synaptic cleft in an activity-dependent manner. Once serotonin is removed from synaptic terminals via 5-HTT, it is repackaged into vesicles or degraded by monoamine oxidase A to be converted into 5-HIAA (Sangkuhl et al., 2009).

5-HT signals through at least 14 receptors in the central nervous system. 5-HT receptors are seven putative transmembrane spanning G-protein coupled metabotropic receptors, except for one member of the family, the 5-HT_3 receptor, which is a ligand-gated ion channel. 5-HT receptors have been classified into seven receptor families (5-

HT_{1-7}) on the basis of their structural, functional and to some extent pharmacological characteristics. In the intact brain the function of many 5-HT receptors can be unequivocally associated with specific physiological responses, ranging from modulation of neuronal activity and neurotransmitter release to behavioral changes (Berg et al., 1998; Barnes and Sharp, 1999).

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I.III.II DA function and signaling

DA has long been know to important in emotional regulation, reward, motivation and cognitive processes (Bromberg-Martin et al., 2010; Koob and Volkow, 2010; Cools and D'Esposito, 2011). The DA system has also been implicated in a variety of neuropsychiatric disorders such as Parkinson’s disease, depression, anxiety, schizophrenia, ADHD and addictive disorders (Drevets, 2001; Wise, 2002; Ross and Peselow, 2009; Heinz and Schlagenhauf, 2010; Koob and Volkow, 2010). The DAT regulates presynaptic dopamine homeostasis, and controls the activity of released dopamine by rapid uptake into presynaptic terminals (Giros and Caron, 1993; Jones et al., 1998). The DAT is the main target for psychoactive drugs, such as amphetamine and methylphenidate. Pharmacological agents that block the DAT display efficacy in treating attention dysfunction, hyperactivity, and impulsivity of ADHD.

The amino acids phenylalanine and tyrosine are precursors for catecholamines (dopamine, norepinephrine and epinephrine). The sequence of enzymatic steps starts with the enzyme tyrosine hydroxylase (TH), which converts the amino acid L-tyrosine into 3,4-dihydroxyphenilalanine (L-DOPA). L-DOPA is then converted into DA. The conversion of tyrosine to L-DOPA and L-DOPA to DA occurs in the cytosol. The

Vesicular Monoamine Transporter (VMAT-2) then takes up dopamine into the storage vesicles. The vesicles release their content through Ca-activated fusion with the neuronal membrane. Afterwards, the action of DA released at the synapse is terminated by diffusion or reuptake into presynaptic nerve terminals by the DAT (Jones et al., 1998; Viggiano et al., 2003).

I.IV Monoamine Oxidase and Serotonin Transporter

In the following paragraphs, I will introduce the fundamental properties of the genes coding for the MAOA and 5-HTT, which will shed light in understanding the relationship between these genes and their functions during development as well as in adulthood.

I.IV.I. General description of MAO and 5-HTT

MAOs catalyze the oxidative deamination of a number of biogenic amines in the brain and peripheral tissues (Shih, 1991). MAOs maintain low cytosolic and extra-cellular levels of monoamines and prevent various natural substrates from accumulating in monoaminergic neurons as false neurotransmitters. On the basis of substrate selectivity and inhibitor sensitivity, two forms of MAO were proposed and designated MAOA and MAOB. MAOA mainly metabolizes monoaminergic neurotransmitters, such as 5-HT, dopamine (DA), NE, and epinephrine (E), whereas MAOB mainly metabolize trace amines, such as tyramine and phenylethylamine (Strolin Benedetti et al., 1992). Both MAOA and MAOB are located throughout the brain in the outer membrane of mitochondria (Green and Youdim, 1975) and are encoded by different genes (Grimsby et al., 1991). Both genes are located on the X-chromosome (Grimsby et al., 1991).

In humans, *5-htt* gene is located on chromosome 17q11.2 and is composed of 14 exons (Ramamoorthy et al., 1993). The 5-HT system is involved in the pathology of depression and the action of antidepressants. The main regulator of 5-HTergic neurotransmission is 5-HTT. It transports 5-HT from synaptic cleft into presynaptic neurons, effectively regulating 5-HTergic tone (Gorman and Kent, 1999a). 5-HTT is situated both in perisynaptic membranes of nerve terminals and in dendritic arbors in close proximity to 5-HT-containing cell bodies in the midbrain and brain stem raphe nuclei. 5-HTT mediates rapid removal and recycling of released serotonin following neuronal stimulation (Murphy et al., 2004a). The 5-HTT is the primary molecular target for many antidepressants, especially the serotonin selective reuptake inhibitors (SSRIs). SSRIs are used as the first-line treatment for psychiatric conditions such as major depression, generalized anxiety disorder, panic disorder, and obsessive compulsive disorder (Gorman and Kent, 1999b). SSRIs increase 5-HTergic tone, and this biochemical effect is thought to be the basis of their therapeutic actions.

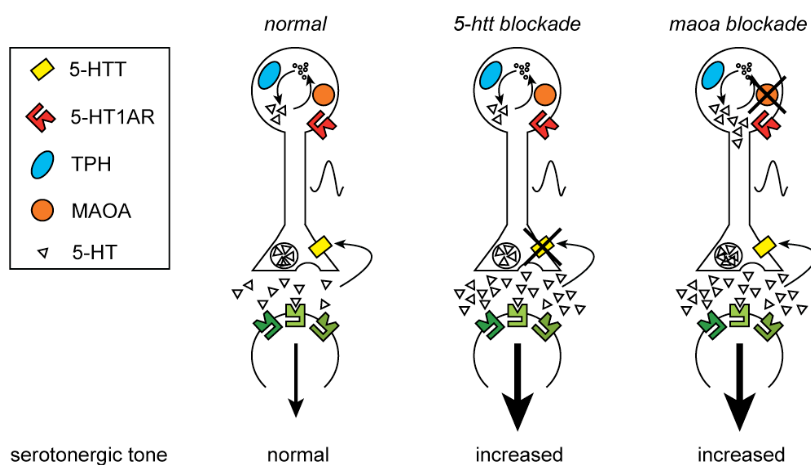


Figure 1-1. 5-HTT and MAOA. 5-HTT transports 5-HT from synaptic cleft into presynaptic neurons, effectively regulating 5-HTergic tone. MAOA is located in the outer membrane of mitochondria, and mainly metabolizes monoaminergic neurotransmitters, such as 5-HT, DA, NE, and E. [based on (Green and Youdim, 1975; Strolin Benedetti et al., 1992; Ansorge et al., 2007)]

I.IV.II. MAOA and MAOB expression

In humans and rodents, MAOA is present before MAOB in most tissues. MAOA is almost at adult levels at birth, whereas MAOB activity increases several-fold with aging (Saura et al., 1994a, b). Both *maoa* and *maob* are co-expressed in 5-HTergic neurons of the raphe from E12 to P7. During postnatal life, *maoa* expression declines, whereas *maob* expression remains stable. *maoa* is expressed in the noradrenergic and adrenergic neurons during embryonic and postnatal development. *maoa* is expressed in several dopaminergic cell groups including ventral tegmental area, substantia nigra, ventral thalamus, hypothylamas, paraventricular nucleus during embryonic or postnatal development. *maob* is expressed in the histaminergic and forebrain cholinergic cell groups. (Vitalis et al., 2002)

In adulthood, the distribution of MAOA and MAOB differs as well (Levitt et al., 1982; Saura et al., 1992; Jahng et al., 1997). *maoa* expression analysis in the rat using in situ hybridization shows that *maoa* has wide expression in the brain: *maoa* mRNA is localized in major monoaminergic cell groups such as locus ceruleus, the raphe nuclei, the substantia nigra and the ventral tegmental area; *maoa* mRNA is also found in forebrain structures such as the cortex, the hippocampus, the thalamus, and the hypothalamus (Jahng et al., 1997). In contrast to *maoa* expression, *maob* mRNA is expressed intensively in only three brain areas: the area postrema, the dorsal raphe, and the subfornical organ (Jahng et al., 1997). Immunohistochemical studies also showed that MAOB immunoreactivity is positive in two areas of the CNS: cells in the brain stem raphe and scattered cells in the hypothalamus (Levitt et al., 1982). Quantitative enzyme autoradiography analysis revealed that MAOA was most abundant in the locus coeruleus,

paraventricular thalamus, bed nucleus of the stria terminalis, median habenular nucleus, ventromedial hypothalamus, raphe nuclei, solitary tract nucleus, inferior olives, interpeduncular nucleus, claustrum. In contrast, MAOB is most abundant in the ependyma, circumventricular organs, olfactory nerve layer, periventricular hypothalamus, cingulum, hippocampal formation, raphe nuclei, paraventricular thalamus, mammillary nuclei, and cerebellar Bergmann glia cells (Saura et al., 1992). The different expression patterns of *maoa* and *maob* suggest that they exert different physiological functions.

I.IV.III. 5-HTT expression

5-htt becomes transcriptionally active at embryonic day 10 (Moiseiwitsch and Lauder, 1995). In situ hybridization and immunochemistry study showed that *5-htt* is expressed in 5-HTergic and non-5-HTergic cell groups from E13 in mice and E13.5 in rats (Lebrand et al., 1998). *5-htt* is expressed in the mouse forebrain by E15. *5-htt* is expressed in hippocampus, in dorsal subiculum, in endopiriform nuclei, and in cingulated, prelimbic, infralimbic, and retrospenial cortices (Lebrand et al., 1998). 5-HTT immunoreactivity is found in fiber tracts arising from the nuclei (Lebrand et al., 1998). The highest *5-htt* expression is present between P0-P7 in most regions such as cortex, hippocampus, and subiculum (Lebrand et al., 1998).

5-htt and *maoa* expression are present in brain regions implicated in anxiety/depression and aggressive behaviors and are evolving dynamically during the early postnatal period. Both 5-HTT and MAOA are important in keeping metabolic balance of monoamine systems. Thus manipulations that target 5-HTT or MAOA could

potentially change the dynamics of the monoamine system and lead to profound effects later in adulthood.

I.V Developmental hypothesis of neuropsychiatric diseases

I.V.I. The sensitive period during development

The concept of critical period has been clearly demonstrated in the development of visual system. Torsten Wiesel and David Hubel, who were awarded Nobel Prize for their significant contribution in visual information processing, made the first detailed observation. They showed that if you deprive one eye of a kitten during early life and let it mature to adulthood, there is a dramatic change in the Ocular Dominance (OD) distribution among the neurons in the visual cortex, while the same manipulation in adult cat dose not produce similar effects (Hubel and Wiesel, 1970). Further detailed characterization of the sensitive period demonstrated that four to six weeks after birth is the peak for this kind of OD distribution shift and it gradually declined until 6 months of age (Cynader et al., 1980). Similar phenomenon has also been shown in mice studies. The OD shift occurred during a critical period between P19 to P32 and the peak occurred between P28 to P32 (Gordon and Stryker, 1996). This OD plastic phenomenon is restricted to a critical period during development. Here the critical period refers to a strict time window during which experience provides information that is essential for normal development and permanently alters performance, which is a stricter definition than sensitive period (a limited time during development, during which the effect of experience on brain function is particularly strong) (Hensch, 2005a). Maturation of the inhibitory circuitry is responsible for the end of the critical period during development

(Hensch, 2005a, b). During this critical period in early life, neural circuitry or structure is sculpted by visual experience, which in turn results in changes in adult behavior. Multiple approaches have shown effective for recovery of function in adulthood, including environmental enrichment chronic administration of FLX, 10-day dark exposure (He et al., 2007; Sale et al., 2007; Maya Vetencourt et al., 2008). In particular, chronic FLX treatment could restore the visual plasticity caused by early occlusion of one eye. This is accompanied by reduced inhibition and increased Brain-Derived Neurotrophic Factor (BDNF) expression (Maya Vetencourt et al., 2008). This OD shift phenomenon provides a model for further understanding how early life experience shapes the brain development and result in different adult behaviors; in addition, it also carries further impact in understanding developmental disorders.

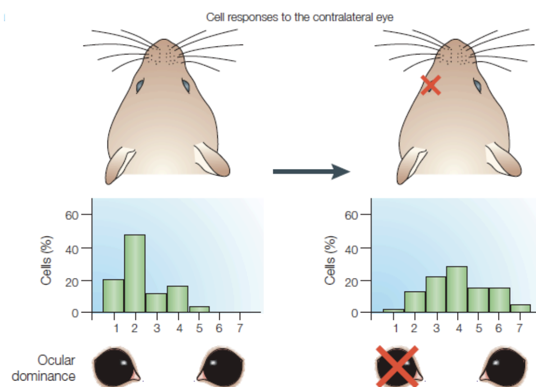


Figure 1-2. Occlusion of one eye during early development produces loss of response to the deprived eye. The OD of cells is rated on a seven-point scale of neuronal responsiveness. After 3 or more days of monocular deprivation, the distribution shifted to the open eye. [from (Hensch, 2005a)]

Neuropsychiatric disorders also have developmental origins and such developmental vulnerability is often restricted to sensitive periods. Early life blockade of 5-HTT from P4-P21 produced increased anxiety and depression like behaviors (Ansorge et al., 2004). Shifting the period of treatment to P90 failed to mimic the effect of earlier

exposure, demonstrating that 5-HT effects on adult behavior are developmentally specific (Ansorge et al., 2008). Postnatal pharmacological blockade of 5-HT_{1A} receptor from P13-P34 produced increased anxiety/depression like behaviors (Lo Iacono and Gross, 2008), and 5-HT_{1A} receptor expression in early development is required to establish the normal adult anxiety phenotype (Gross et al., 2002). The 5-HT_{1A} receptor have two distinct populations in the brain (autoreceptors and heteroreceptors) that could each impact the distinct circuitry underlying mood and anxiety in development or adulthood. Suppression of 5-HT_{1A} autoreceptors throughout life is sufficient to increase anxiety in the adult, while suppression of the heteroreceptors beginning either in early postnatal period or in adulthood is not sufficient to impact anxiety-like behavior, suggesting 5-HT_{1A} forebrain heteroreceptors are not the primary mediators on developing anxiety circuitry and autoreceptor mediated events might be responsible for this normal establishment of anxiety behaviors (Richardson-Jones et al., 2011). Risk factors that impact monoamine signaling during a sensitive period might alter brain maturation and circuitry formation, which influence vulnerability of neuropsychiatric disorders. Thus, it is very important to further elucidate the sensitive periods that determine the developmental trajectory of complex behaviors to improve preventive and therapeutic approaches towards neuropsychiatric disorders.

I.V.I. 5-HT system during development

The 5-HTergic system is among the earliest bioamine systems to appear during brain development. In the human, 5-HT neurons can be detected when the embryo is 5 weeks old (Sundstrom et al., 1993), with rapid growth and multiplication until at least the

10th week of gestation (Levallois et al., 1997). After 15 weeks of gestation, clustering of the 5-HT cell bodies in the raphe nuclei is observed (Takahashi et al., 1986). Levels of 5-HT increase during the first 2 years after birth and then decline to adult levels after the age of five (Sodhi and Sanders-Bush, 2004). In rodents, the first 5-HT neurons appear at the 11th day of rodent gestation. They continue to elaborate and innervate target structures throughout the early life of the organisms, finally reaching a mature status (Lauder, 1990). Although 5-HT axons reach target areas by birth, innervation and arborization is highly dynamic until postnatal day (P21) (Whitaker-Azmitia, 2005). During the first two postnatal weeks, rodent primary sensory cortex (notably layer IV of visual, auditory, and somatosensory areas) is transiently innervated by aggregates of 5-HT-containing neurons (D'Amato et al., 1987; Blue et al., 1991). In addition, several non-serotonergic neurons transiently express a serotonergic phenotype during early brain maturation in rodents (Cases et al., 1996; Lebrand et al., 1998; Salichon et al., 2001; Gaspar et al., 2003).

Electrophysiological studies have demonstrated differential effects of 5-HT on prefrontal cortex neurons during early and late development (Beique et al., 2004b; Beique et al., 2004a). For instance, in rat layer V pyramidal neurons serotonin induced excitatory response between P6 and P14 and inhibitory responses after P20. This effect has been attributed to a shift in 5-HT receptors, with $5HT_{2A}$ receptor mediating the depolarization and $5\text{-}HT_{1A}$ receptors mediating the hyperpolarization (Zhang, 2003; Beique et al., 2004b).

I.V.II. DA system during development

Midbrain DA neurons appear between embryonic days E12–15. These neurons begin to express tyrosine hydroxylase by E12.5, and then extensively migrate from the rhombic isthmus in a rostro-ventral direction to the ventral midbrain (Viggiano et al., 2003). Starting from E15 the first DA-positive fibers pass through the developing striatum to cortical regions. The development of the cortical DA innervation continues until P60. No difference in density and topography was observed between postnatal days 60 and 90 (Kalsbeek et al., 1988). In addition, DA transporter density in the striatum increases from postnatal day 25 through postnatal day 50, and then decreases continuously until P90 (Moll et al., 2000). During adolescence, the DA system has evident changes such as increased prefrontal cortex fiber density (Kalsbeek et al., 1988) and pruning of DA receptors (Teicher et al., 1995; Tarazi et al., 1998a, b).

I.V.III. Monoamine functions as trophic factors during development

During embryonic and postnatal development, monoamines act as trophic factors modulating neurodevelopmental processes, such as cell division, migration, and differentiation, axonal and dendritic elaboration and connectivity, myelination and apoptosis (Haydon et al., 1984, 1987; Lauder, 1990; Teicher et al., 1995; Tarazi et al., 1998b; Gaspar et al., 2003; Popolo et al., 2004; McCarthy et al., 2007). For example, studies on barrel field formation also provide insight into the role of developmental monoamine signaling on adult behavior. *maoa* and *5-htt* -/- mice exhibit disrupted barrel fields (Cases et al., 1996; Salichon et al., 2001). Barrels fields are the morphological substrate of the somatosensory cortical map in rodents. Each barrel receives sensory

afferents from one whisker through specific thalamic afferents, which normally form discrete clusters in cortical layer IV (Cases et al., 1996; Salichon et al., 2001). Increased 5-HT signaling through 5-HT_{1B} receptors on thalamocortical neurons during late-embryonic/early-postnatal development has been shown to be causal for this phenomenon (Cases et al., 1996; Vitalis et al., 1998; Salichon et al., 2001; Rebsam et al., 2002). These findings demonstrate that high levels of serotonin during a critical perinatal period cause permanent anatomical defects. The mechanism elucidated in these studies suggested that thalamocortical neurons transiently adapt a 5-HTergic phenotype during the critical perinatal period, and that enhanced 5-HTergic signaling disrupts axonal arborization and patterning of the thalamocortical neurons projecting to the somatosensory cortex, which in turn defines the overall barrel field pattern. 5-HT is also critical for growth cone elongation (Haydon et al., 1984, 1987) and plays a role in the formation of the dorsal and median raphé nuclei (Rumajogee et al., 2004). 5-HT influences the length of dendrites, the formation of the dendritic spines, and branches in the hippocampus and cortex (Mazer et al., 1997; Yan et al., 1997; Wilson et al., 1998; Norrholm and Ouimet, 2000). 5-HT influences synaptogenesis. 5-HT depletion significantly slows the progress of synaptogenesis (Wilson et al., 1998; Faber and Haring, 1999). Taken together, these data demonstrate the central role that 5-HT plays during development to modulate specific processes that impact brain maturation and structure long-lastingly.

DA regulates cell proliferation, differentiation and neuronal pruning (Tarazi et al., 1998b, a; Popolo et al., 2004; McCarthy et al., 2007). In addition, the developmental effects of monoamines may be linked to the transient expression of some molecules that are linked to monoamine neurotransmission. For instance, tyrosine hydroxylase, the first

enzyme in the catecholamine synthetic pathway, is transiently present in a subpopulation of cortical interneurons in the developing cortex of rodents (Berger et al., 1985). The 5-HTT and the VMAT2 are present in non-monoaminergic neurons of the sensory thalamus and in selected neurons of the cerebral cortex of rats and mice (D'Amato et al., 1987; Lebrand et al., 1996; Lebrand et al., 1998).

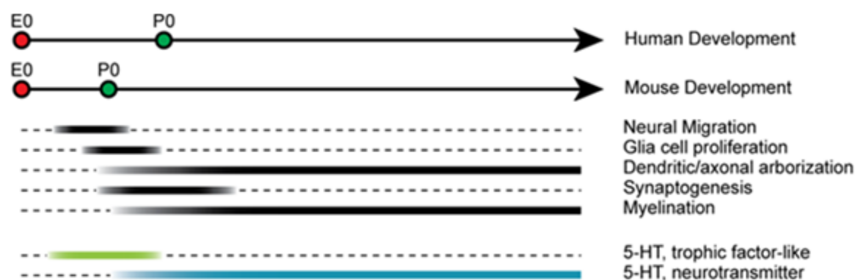


Figure 1-3. Serotonin acts as trophic factors during development impacting brain maturation. [based on (Gaspar et al., 2003; Innocenti and Price, 2005; Lenroot and Giedd, 2006)]

I.V.IV Brain structures and functions during development

Brain structures and circuitry modulating adult psychiatric behaviors are developing and maturing during early development. For example, cortical development and the establishment of cortical-cortical and cortical-subcortical networks take place in the first few postnatal weeks (Innocenti and Price, 2005; Dupont et al., 2006; Brockmann et al., 2011; Janiesch et al., 2011). Likewise, in rodent hippocampal development takes place during the early post-natal period, coinciding with the time that serotonergic neuronal innervation matures (Swann et al., 1989; Tansey et al., 2002; Gaspar et al., 2003). Glutamatergic neurons undergo rapid development postnatally, changing spine density and synapse characteristics (Hoftman and Lewis, 2011). Gamma-aminobutyric acid (GABA) neurotransmission is rapidly evolving during postnatal development, changing arborization patterns and synaptic connections (Lewis et al., 2004; Wonders

and Anderson, 2006). A critical developmental period for the neocortex in rodents occurs during the first 2 postnatal weeks, which is also a period of intense synaptogenesis, as synaptic density increases fivefold between P10 and P15 when it is almost at the level of adult brain (Micheva and Beaulieu, 1996). Thus, interference with monoamine signaling during early development conceivably impacts developmental trajectory of neuronal circuitry and brain maturation, which may ultimately lead to altered adult behavior.

I.V.V. Paradox of 5-HTT genetic life-long inhibition vs pharmacological blockade

Mice constitutively lacking the 5-HTT exhibit increased anxiety/depression like behaviors (Holmes et al., 2003; Lira et al., 2003). In humans, people with low-expression allele of *5-htt* gene are more likely to develop depressive episodes in face of stressful life events (Caspi et al., 2003; Kendler et al., 2005; Taylor et al., 2006; Cervilla et al., 2007; Mandelli et al., 2007; Aguilera et al., 2009; Karg et al., 2011). Hence, genetic inhibition of 5-HTT constitutes a risk factor for affective abnormalities in rodents, primates, and humans (Murphy et al., 2001; Hariri and Holmes, 2006; Canli and Lesch, 2007). However, blocking 5-HTT in adulthood using SSRIs produced decreased anxiety and depression-related behaviors in both humans and rodents (Dulawa and Hen, 2005; Berton and Nestler, 2006). Thus, lacking the 5-HTT throughout life produces opposite effects on behavior when compared to transiently blocking 5-HTT in adulthood. This leads to the hypothesis that genetically reduced *5-htt* function during critical periods of development alter brain function in a way that predisposes the organism to affective behaviors later in life. This hypothesis is supported by research showing that the postnatal (P4) to P21 period in mice constitutes an important developmental window during which FLX

treatment leads to increased anxiety and depression-like behavior in adulthood. The pharmacological effect was dependent on the presence of the 5-HTT, as no effect of FLX treatment was seen in *5-htt* -/- mice (Ansorge et al., 2004). Furthermore, while early life blockade of 5-HTT increases anxiety and depression-like behavior in adult mice, transient FLX treatment in 3-month-old mice had no demonstrable effects on the same emotional behaviors after cessation of treatment (Ansorge et al., 2008).

Thus, the developmental component of 5-HTT blockade explains the contrasting effect of pharmacologic 5-HTT blockade during adulthood and genetic life-long 5-HTT inhibition. The results furthermore indicate that there exists a critical developmental time window during which 5-HTT blockade exerts its "negative" influence on adult behavior.

I.V.VI. MAOA genetic life-long blockade vs pharmacological blockade

MAOA deficient mice display increased aggressive behavior and increased anxiety (Cases et al., 1995; Popova et al., 2001; Scott et al., 2008). Recently, hypomorphic MAOA mutant mice (MAO-A *Neo*) were generated, featuring the insertion of a floxed neomycin-resistance cassette in intron-12 of the maoa gene. MAO-A *Neo* mice show low levels of MAO-A enzymatic activity instead of complete ablation of MAOA. MAO-A *Neo* mice showed significant reductions in social interaction comparable to the KO mice even though the MAO-A *Neo* mice did not show increased aggressive behavior in resident-intruder aggression test (Bortolato et al., 2011).

Monoamine Oxidase inhibitor (MAOI) drugs play an important role in the treatment of anxiety and depression disorders and Parkinson's disease (Youdim and Weinstock, 2004). MAOIs are particularly effective in treatment of refractory depression

(Thase et al., 1992; Nolen et al., 1993; Krishnan, 2007) and there is evidence that MAOIs are of more benefit than TCAs in treating patients with anxiety disorders (Sheehan et al., 1980; Bakish, 1994; Krishnan, 2007).

The divergent effects of genetic lifelong inhibition versus pharmacologic inhibition during adulthood again leads to the thought that perturbed MAOA function during developmental might increase the likelihood of aggressive and anxiety traits later in life.

I.VI 5-HT_{2A} receptor

Serotonin signals through at least 14 receptors to exert its functions. Among these different receptors, 5-HT_2 receptors are generally excitatory, couple positively to phospholipase C and stimulate the accumulation of inositol phosphates and intracellular Ca^{2+} (Berg et al., 1998; Sodhi and Sanders-Bush, 2004). The 5-HT_{2A} receptor is a member of the super-family of 7-transmembrane-spanning (7-TMS) receptors, also known as G protein-coupled receptors. Traditionally it is thought that stimulation of the 5-HT_{2A} receptor will only activate phospholipase C in brain tissues via G-protein coupling (Sanders-Bush et al., 1988). Further studies have however shown that 5-HT_{2A} receptors interact with diverse signaling pathways: Endogenously expressed 5-HT_{2A} receptors activate phopholipase A2 (PLA2), phospholipase D (PLD), and the JaK/Stat pathways (Guillet-Deniau et al., 1997; Kurrasch-Orbaugh et al., 2003). In addition, they also inhibit or activate adenyl cyclase in certain cell systems (Garnovskaya et al., 1995; Guillet-Deniau et al., 1997)

I.VI.I 5-HT_{2A} receptor distribution

The presence and expression pattern of 5-HT_{2A} receptors and *htr2a*, respectively has been mapped extensively by receptor autoradiography, in situ hybridization, and more recently, immunocytochemistry. In adult tissues, high levels of 5-HT_{2A} binding sites are present in many forebrain regions, especially cortical areas (neocortex, entorhinal and pyriform cortex, claustrum), the caudate nucleus, the nucleus accumbens, the amygdala, the olfactory tubercle and the hippocampus, of all species studied (Pazos et al., 1985; Pazos and Palacios, 1985; Li et al., 2003). *htr2a* expression appears as early as embryonic day 9 and exists throughout lifetime (Lauder et al., 2000). *htr2a* expression increases with age and reaches a relatively stable level after P10-P11 in various brain stem nuclei (Liu and Wong-Riley, 2010). Immunochemistry analysis in rat also showed that 5-HT_{2A} receptor abundance was at a low level at postnatal day 3 (P3) and increased greatly during the first 3 postnatal weeks reaching adult level at P21 (Li et al., 2004). This dynamic change of *htr2a* expression and 5-HT_{2A} receptor abundance in brain regions implicated in emotional and cognitive processing indicates that early postnatal 5-HT_{2A} receptor signaling might be involved in mediating the trophic-like effects of developmetnal 5-HT signaling, which impact adult behavior.

I.VI.II 5-HT_{2A} receptor and anxiety/depression

The 5-HT_{2A} receptor is involved in a number of different physiological functions including sleep, endocrine modulation, thermoregulation, pain modulation, cognitive function and memory (Harvey, 2003; Popa et al., 2005; Pawlyk et al., 2006; Morairty et al., 2008; Nakajima et al., 2009). In addition, it is linked to a series of psychiatric

disorders including anxiety, depression and schizophrenia (Leysen, 2004; Berg et al., 2008; Kato et al., 2009; Ebdrup et al., 2011). In the context of my dissertation, I will focus on its role in anxiety and depression related behaviors. Human studies revealed that *htr2a* variants interact with childhood maternal nurturance to influence depressive symptoms in adulthood (Jokela et al., 2007). Depressive patients show up-regulation of frontal 5-HT_{2A} receptors and down-regulation 5-HT_{2A} receptor binding in the hippocampus (Massou et al., 1997; Mintun et al., 2004). Mice lacking 5-HT_{2A} receptors show decreased anxiety in multiple behavior tests; in addition, cortical restoration of *htr2a* expression normalizes the anxiety phenotype (Weisstaub et al., 2006). 5-HT_{2A} antagonists have anxiolytic effects in different behavior tests (Griebel et al., 1997a; Griebel et al., 1997b; Millan et al., 2003), even though some reports suggested an anxiogenic effects (Zangrossi et al., 2001; Graeff, 2002; Ripoll et al., 2006).

Chapter II Effect of early life 5-HTT and MAOA blockade on adult affective and aggressive behavior

Introduction

MAOA catalyzes the oxidative deamination of bioamines including 5-HT, NE, and DA. MAOA transient blockade during adulthood has anti-depressant/anxiolytic effect (Youdim and Weinstock, 2004; Krishnan, 2007). Unlike the salutary effects of pharmacologic MAOA inhibition, constitutive mutations of *maoa* function result in a syndrome characterized by antisocial/aggressive behavior in humans (Brunner et al., 1993a). Moreover, low expressing maoa variants are also associated with aggression and anxiety traits in rhesus macaques and humans (Tadic et al., 2003; Buckholtz and Meyer-Lindenberg, 2008; Karere et al., 2009). Consistent with higher primates, mice with genetic inactivation of maoa exhibit heightened levels of aggression and neophobia (Cases et al., 1995; Scott et al., 2008; Godar et al., 2010). The divergent effects of genetic (lifelong) mutations versus pharmacologic inhibition (during adulthood) leads us to hypothesize that perturbed MAOA function during sensitive periods of brain maturation increases the likelihood of aggressive and anxiety traits later in life.

The role of monoamine signaling in brain development has been firmly established. During the embryonic and early postnatal period, monoamines act as trophic factors, modulating neurodevelopmental processes, such as cell division, migration, and differentiation (Gaspar et al., 2003; Homberg et al., 2010; Souza and Tropepe, 2011). In mice, genetic maoa ablation increases brain tissue concentrations of 5-HT, NE, and DA during development and early adulthood (Cases et al., 1995). The most prominent

teratogenic consequence of murine maoa ablation, the disruption of barrel fields in the somatosensory cortex, has been linked to increased 5-HT signaling during perinatal development (Cases et al., 1996; Vitalis et al., 1998; Rebsam et al., 2002). Increased 5-HT signaling during early postnatal development on the other hand increases anxiety/depression-like behavior including neophobia in adult mice (Ansorge et al., 2004; Ansorge et al., 2008). Thus, we hypothesized that increased 5-HT signaling during early postnatal development causes neophobia in maoa deficient mice. Furthermore, we predicted that the origins of aggressive behaviors are also developmental and postnatal, because re-expressing maoa from postnatal day P1 onwards restores normal aggressive behavior in conditional maoa knock-out mice (Chen et al., 2007). To test both hypotheses, we compared the effects of developmental MAOA inhibition and monoamine transporter blockade on adult behavior and monoamine signaling in mice.

Materials and Methods

Subjects.

Mice were injected intraperitoneally (i.p.) daily (2:00 pm - 5:00 pm) with vehicle (VEH, 0.9% NaCl, 5 ml/kg), fluoxetine (FLX, 10 mg/kg, 5 ml/kg), clorgiline (CLO, 20 mg/kg), desipramine (DMI, 20 mg/kg) or GBR12909 (GBR, 20 mg/kg). FLX is a 5-HT transporter (5-HTT) blocker; CLO is a MAOA inhibitor; DMI is a norepinephrine transporter (NET) blocker; GBR12909 is a dopamine transporter (DAT) blocker. Groups of mice were injected during different postnatal periods: P2-P21 or P22-P41. Animals were maintained on a 12-hour light-dark cycle (lights on at 8 am) and provided with food and water *ad libitum*. Animal testing was conducted in accordance with the *Principles of Laboratory Animal Care* National Institute of Health (NIH) guidelines and the

institutional animal committee guidelines.

Drugs

For the studies involving postnatal drug administrations, fluoxetine (ANAWA Trading SA, Wangen, Switzerland), clorgiline (ANAWA Trading SA), desipramine (ANAWA Trading SA), GBR 12909 (ANAVA Trading SA) were dissolved in 0.9% NaCl to achieve the following concentrations: fluoxetine, 2 mg/ml; clorgiline, 4 mg/ml.; desipramine 4mg/ml; GBR12909 4mg/ml. Solutions were prepared fresh every day. FLX is a 5-HT transporter (5-HTT) blocker; CLO is a MAOA inhibitor; DMI is a norepinephrine transporter (NET) blocker; GBR12909 is a dopamine transporter (DAT) blocker. GBR12909 has a very high binding selectivity and long half-life (Andersen, 1989; Ingwersen et al., 1993).

Behavior Testing

Anxiety/depression like behavioral testing

All animals were exposed to the same series of behavioral paradigms starting at 3 months of age. The tests were administered in the following order: open field, novelty suppressed feeding, and shock avoidance with a minimum of 7 days between each test. All behavioral testing took place during the light cycle between 12:00pm and 7:00 pm. To eliminate odor cues, each apparatus was thoroughly cleaned after each animal.

Open Field

Exploration and reactivity to a novel open field was assessed in Plexiglas activity

chambers as previously described (Ansorge et al., 2008). Mice were tested in Plexiglas activity chambers equipped with infrared beams located 1.5 cm above the chamber floor and spaced 2.5 cm apart to detect horizontal activity. Vertical activity was detected using another set of infrared beams affixed 6 cm above the chamber floor and spaced 2.5 cm apart. It consists of a simple square enclosure that is equipped with infrared detectors to track animal movement in the horizontal and vertical planes. The conflicting innate tendencies to avoid bright light and open spaces but explore novel environments influence locomotor behavior. Measures of total distance covered during locomotion are used as an index of activity, while the proportion of time or distance spent in the center is taken as a measure of anxiety. Mice were placed into the center of the open field and activity was recorded for 30 minutes. Testing took place under bright ambient light conditions. Total distance, total ambulatory time, and vertical activity were measured.

Novelty-suppressed feeding

The novelty-suppressed feeding test is a behavior paradigm that is sensitive to chronic but not acute antidepressants as well as acute treatment with benzodiazepines (Bodnoff et al., 1989). The test was performed as described previously (Santarelli et al., 2003): the testing apparatus consisted of a plastic box (50 x 50 x 20 cm). The floor was covered with 2 cm of wooden bedding. Twenty-four hours before behavioral testing, animals were deprived of all food in the home cage. At the time of testing, two food pellets were placed on a piece of round filter paper (12 cm diameter) positioned in the center of the box. The test began immediately after the animal was placed in a corner of the box. The latency to approach the pellet and begin feeding was recorded (maximum

time, 10 min). Immediately afterward, the animal was transferred back to its home cage and the amount of food consumed in 5 min was measured. Each mouse was weighed before food deprivation and before testing to assess the percentage of body weight loss. Immediately after initiating a feeding episode, mice were removed from the arena and placed into their home cage containing a pre-weighed food pellet and allowed to feed *ad libitum* for 5 min. Food consumption was determined by the difference in weight of the pellet.

Shock Escape

Shock escape is the primary dependent measure affected by uncontrollable stress that occurs during a learned helplessness procedure (Seligman, 1972). Learned helplessness has been studied as a model of depression since shock avoidance performance is improved after antidepressant treatment (Cryan et al., 2002). Shock escape was performed in a 2-chambered Plexiglas shuttle box with the two chambers separated by an automated guillotine door as previously described(Lira et al., 2003). Each apparatus is located within a sound attenuated chamber. At the beginning of each trial, the door was raised and a mild scrambled foot shock (0.2 mA; 10 s duration) was delivered to the subject. The end of a trial was signaled by the closing of the guillotine door and was triggered either by a transition to the opposite chamber or after 10 s. Transition latencies were recorded. If the subject failed to make a transition during the 10s duration of the foot shock, a maximum latency of 10 s was recorded. A session consisted of 30 trials separated by a 30 s inter-trial interval. Locomotor activity was assessed during inter-shock intervals by counting total infrared beam interruptions during

each session.

Isolation induced aggression test

The aggression test used a rectangular cage that is divided in half by a perforated partition made of clear plastic. A pair of mice with the same treatment was placed into each compartment respectively. The mouse in one compartment is able to see, hear and smell the other mouse through the holes in the plastic divider, but physical interaction is blocked. Mice were housed for at least 10 days before experiment was performed. On test day, dividers were taken out and mice were allowed to freely interact for at least 10mins. All behaviors were videotaped. The latency to tail rattling, the latency to first attack, the total biting time, the total mounting time and the total tail rattling time were scored to assess aggressive behavior. The aggressive behavior is assessed by parameters adding up total biting time, and total mounting time and total tail rattling time.

Locomotor activity in response to amphetamine

Adult mice were injected with amphetamine (3 mg/kg, i.p.) or VEH and locomotor activity was assessed in the open field. Mice were placed into the center of the Open Field chamber and freely run for 30 minutes. Immediately following this, mice were injected amphetamine at 3mg/kg and returned to the testing environment for 60 minutes.

High Pressure Liquid Chromatography (HPLC)

HPLC was carried out on several brain regions(Underwood et al., 1999). Concentrations of biogenic amines and metabolites were measured using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The dissected brain samples in a 1.5-ml microfuge tube were homogenized in 0.5-1.0 ml of 0.4M perchloric acid with an Ultra Cell Disruptor (Microson, NY). The homogenate was centrifuged for 5 minutes at 14,000g in cold room and a 50 µl of typically four-fold diluted aliquot of the supernatant was injected over the HPLC system. The HPLC system, equipped with Waters Millennium software, consists of a Waters 515 HPLC pump, a Waters 717 Autosampler, a Varian Microsorb 100-5 C18 reverse-phase column (DYNAMAX 150x4.6 mm) attached with a Guard column (4.6 mm) and an ESA Coulochem electrochemical detector (Model 5100A) with a guard cell (Model 5020) and a dual analytical cell (Model 5011A). The electrochemical detector was set at potential of +0.05 for the first cell and +0.5V for the second cell. The mobile phase contained 0.75 mM sodium phosphate (pH 3.1), 1.4 mM 1-Octanesulfonic acid, 10 µM sodium EDTA and 8% acetonitrile. The mobile phase was filtered through a Millipore 0.22 µm filter (Type GV) and degassed in vaccuo. The flow rate was maintained at 0.8 ml/min. A chromatography software package (Waters Millennium) was used for data acquisition and analysis. Values are calculated based on peak area and compared to standard solutions. The inter- and intra-assay coefficients of variation of the assay were each less than 5%. The sensitivity of the assay was less than 0.5 pmol/injection. No effect of storage time was detected.

Statistic analysis.

Statistical analysis was performed using StatView 5.0 software (SAS Institute, Cary, NC). Data were analyzed using Student's t test, one-way, or two-way ANOVA with Student–Newman–Keuls posthoc testing; survival analysis, Kaplan-Meier, Logrank Mantel-Cox test; or non-parametric Mann-Whitney test as indicated. The criterion for significance for all analyses was $p < 0.05$. Results from data analyses are expressed as mean ± SEM.

Results

The effect of SERT and MAOA blockade during different developmental periods on adult anxiety/depression like behavior

Early postnatal 5-HTT blockade increases anxiety/depression-like behavior in mice (Ansorge et al., 2004; Ansorge et al., 2008). Because 5-HTT and MAOA blockade both increase 5-HT signaling, we hypothesized that early postnatal MAOA blockade would also increase adult anxiety/depression-like behavior. To test this hypothesis, we treated mice with the 5-HT transporter blocker fluoxetine (FLX, 10 mg/kg/day, i.p.), the MAOA inhibitor clorgiline (CLO, 20 mg/kg/day, i.p.), or vehicle (VEH, 0.9% NaCl, i.p.) in the early postnatal period (PN), from postnatal day 2 to 21 (P2-P21) and assessed emotional behavior in adulthood (P90). To investigate developmental specificity, we also tested mice treated with FLX, CLO, and VEH in the peri-adolescent period (ADO), from postnatal day 22 to 41 (P22-P41). To control for the effects of the injection procedure, we included naïve littermates in our behavioral tests.

Using the novel open field, we assessed neophobic behaviors by measuring exploratory activity. We observed that FLX and CLO treatment from P2-P21 decreased

total ambulatory time (treatment effect: $F_{(2,127)} = 6.225$, $p = 0.0026$) and total time spent rearing (treatment effect: $F_{(2,127)} = 5.42$, $p = 0.0055$; developmental timing effect: $F_{(1,127)} = 13.56$, $p = 0.0003$) when compared to VEH-treated mice (Figure 2-1). Neither FLX nor CLO treatment from P22-P41 altered exploratory behavior compared with VEH treated mice. Behavior between VEH-treated mice and naïve littermates did not differ in either measure.

To further probe neophobia phenotypes, we used the novelty suppressed feeding paradigm, which assesses approach-avoidance behavior when a food-deprived animal is presented with a familiar food pellet placed in the center of a brightly-lit novel arena. Mice exposed to FLX or CLO from P2-P21 exhibited longer latencies to approach and feed when compared to PN-VEH controls (treatment effect: $p = 0.0001$, survival analysis, Kaplan-Meier, Logrank Mantel-Cox) (Figure 2-2a). However, neither FLX treatment nor CLO treatment from P22-P41 produced such behavioral effect. No effect for treatment, developmental timing or treatment x developmental timing was detected for weight loss during food deprivation (Figure 2-2b). Behavior between VEH-treated mice and naïve littermates did not differ in either measure.

To assess behavioral response to stress, we examined escape latency in the shock escape paradigm. Consistent with our findings in tests of neophobia, mice exposed to FLX or CLO from P2-P21 but not P22-P41 exhibited significantly increased escape latencies when compared to PN-VEH controls (treatment effect: $F_{(2,133)} = 21.106$, $p < 0.0001$; developmental timing effect: $F_{(1,133)} = 46.749$, $p < 0.0001$; treatment x developmental timing interaction: $F_{(2,133)} = 24.613$, $p < 0.0001$) (Figure 2-3a). No effect of treatment, developmental timing or treatment x developmental timing was detected for

pre-shock activity in the dark chambers with the door open (Figure 2-3b), indicating that the effect on escape latencies was not caused by reduced overall activity. Behavior between VEH-treated mice and naïve littermates did not differ in either measure.

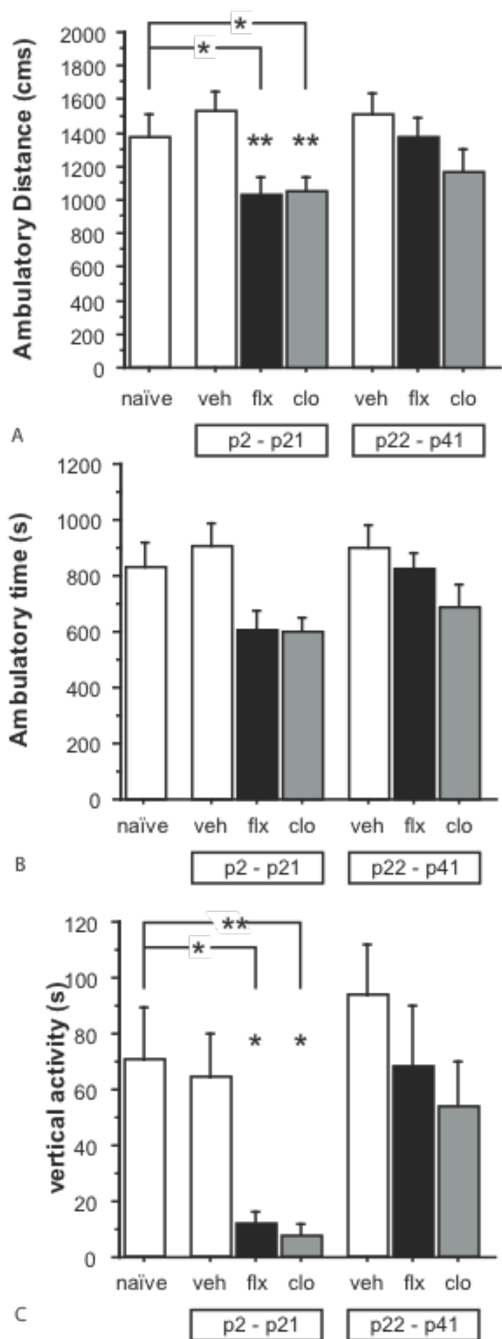


Figure 2-1 Exploratory behavior in the open field. In the open field, the following parameters were scored for 30minutes: total distance traveled (A), ambulatory time (B),

and vertical activity(C). FLX or CLO treatment from P2-P21 reduced the distance ambulating (A), the time ambulating (B) and the time rearing (C) when compared to control mice treated with VEH from P2-P21.*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with their respective controls. n = 19 - 32 mice per group. veh, vehicle; flx, Fluoxetine.

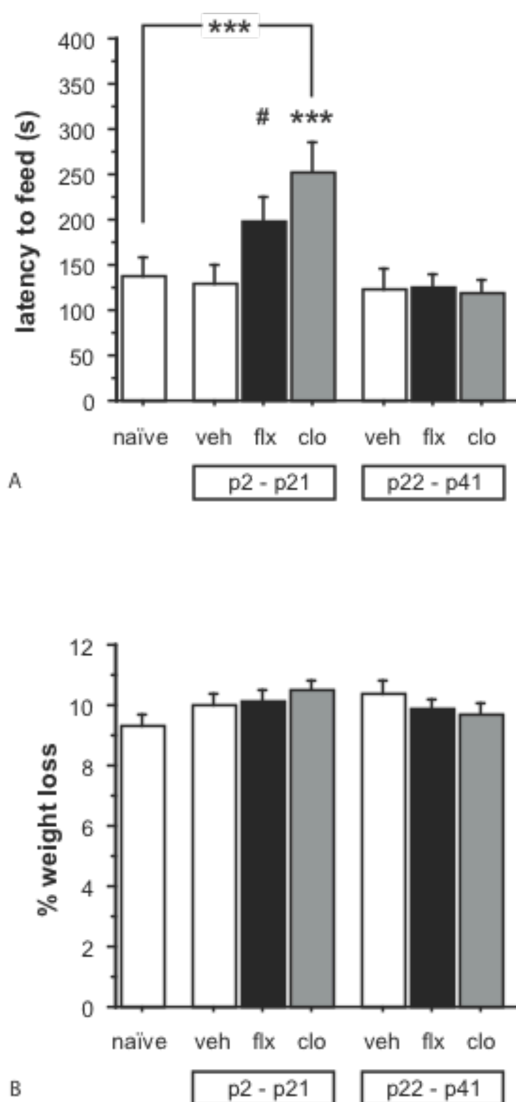


Figure 2-2. Novelty-suppressed feeding test. (A) The latency to begin feeding is shown in seconds. (B) Weight loss is expressed as a percentage of free-feeding body weight. n = 19 - 32 mice per group. FLX or CLO treatment from P2-P21 increased the latency to feed when compared to control mice treated with VEH from P2-P21 (A). No effect of treatment, period, or treatment x period interaction was detected for the weight loss after 24 hours of food deprivation (B). ***, $p<0.001$ compared with their respective controls. veh, vehicle; flx, Fluoxetine.

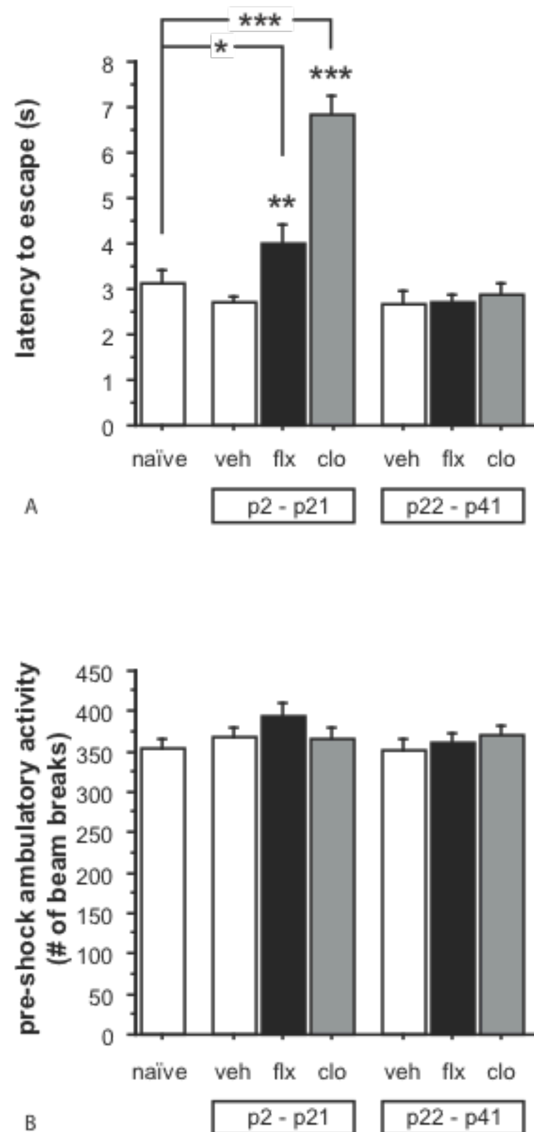


Figure 2-3. Shock escape paradigm. (A) Average latency to escape a foot shock shown in seconds. (B) Locomotor activity before shocks. n = 19 – 32 mice per group. FLX or CLO treatment from P2-P21 increased the latency to escape when compared to control mice treated with VEH from P2-P21 (A). No effect of treatment, period, or treatment x period interaction was detected for activity in the shuttle box before the onset of shock (B) *, $p<0.05$; **, $P<0.01$; ***, $p<0.001$ compared with their PN-VEH controls. veh, vehicle; flx, Fluoxetine.

The effect of 5-HTT and MAOA blockade during different developmental periods on adult aggressive behavior

Next we investigated aggressive behaviors in adult mice that had received FLX, CLO, or VEH during early postnatal or peri-adolescent development using the isolation-induced aggression paradigm. We measured the time mouse pairs spent engaged in aggressive behaviors consisting of mounting, tail rattling, or biting.

We found that CLO treatment from P22-P41 but not from P2-P21 increased aggressive behavior, when compared to VEH controls (effect of treatment: $F_{(2,69)} = 7.258$, $p = 0.0014$; effect of developmental timing: $F_{(1,69)} = 4.737$, $p = 0.033$; treatment x developmental timing interaction: $F_{(2,69)} = 3.828$, $p = 0.0265$) (Figure 2-4). FLX treatment reduced aggressive behavior when compared to VEH treatment, with no indication of developmental timing specificity. Aggressive behavior between VEH-treated mice and naïve littermates did not differ.

In summary, PN-FLX or PN-CLO treatment from P2 to P21 produced increased anxiety behavior; while adolescent FLX or CLO treatment from P22 to P41 did not produce increased anxiety behavior. Adolescent CLO treatment but not FLX treatment from P22 to P41 resulted in increased aggression behavior, while PN-FLX or PN-CLO from P2 to P21 did not generate increased aggressive behavior.

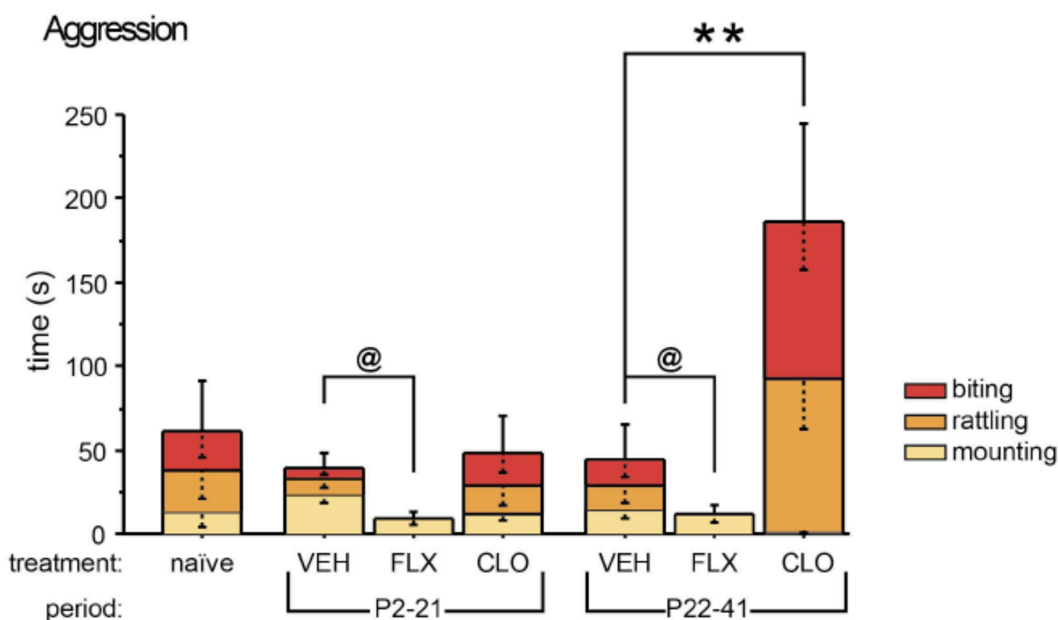


Figure 2-4. Altered aggression after developmental 5-HTT or MAOA blockade.

Isolation induced aggressive behavior was assessed in mice by scoring the time spent mounting, tail rattling, or biting during a 10-minute encounter. Aggressive behavior was increased in mice treated with CLO from P22-P41 when compared to control mice treated with VEH from P22-P41. Mice treated with FLX displayed reduced aggression when compared to VEH-treated control mice. Of note, FLX treated mice did not display any tail rattling or biting behavior. (n = 7–16 pairs per group). (*p < 0.05; **p < 0.01, ***p < 0.001).

<u>Peri-adolescent MAOA blockade reduces the metabolism of 5-HT, NE and DA.</u>

The differential consequences of P22-P41 5-HTT and MAOA blockade on adult aggressive behavior suggest that non-5-HTgic effects of peri-adolescent MAOA blockade increase adult aggression. In peri-adolescence, *maoa* is expressed in 5-HTergic, DAergic and NEergic neurons (Vitalis et al., 2002). To determine the consequences of P22-P41 CLO treatment on brain monoamine signaling, we assessed tissue monoamine and metabolite levels at P23 (24h after treatment initiation) and at P42 (24 h after treatment cessation). Specifically, we quantified 5-HT and its main metabolite 5-

Hydroxyindoleacetic acid (5-HIAA), DA and its two main metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and NE.

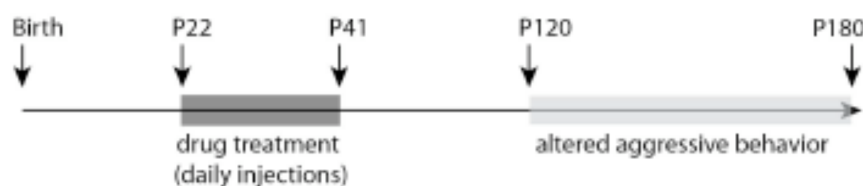
CLO treatment significantly increased 5-HT and reduced 5-HIAA levels in the brain stem at P23 ($F_{(1,12)} = 51.972$, $p < 0.0001$ and $F_{(1,12)} = 31.92$, $p = 0.0001$, respectively) and P42 ($F_{(1,11)} = 62.475$, $p < 0.0001$ and $F_{(1,11)} = 18.8$, $p = 0.0012$, respectively), when compared to VEH treated controls (Figure 2-5, Figure 2-S2 and Table S1). Norepinephrine (NE) levels in the brainstem were also increased by CLO treatment at P23 and P42 ($F_{(1,12)} = 68.717$, $p < 0.0001$ and $F_{(1,11)} = 40.45$, $p < 0.0001$, respectively) (Figure 2-5, Figure 2-S2 and Table S1). Striatal DA levels were not affected at P23 and significantly increased at P42 after chronic CLO treatment when compared to VEH treated controls ($F_{(1,12)} = 0.033$, $p = 0.8587$ and $F_{(1,11)} = 61.745$, $p < 0.0001$, respectively) (Figure 2-5, Figure 2-S3 and table S1). Striatal DOPAC levels were lowered at P23 and P42 ($F_{(1,12)} = 30.292$, $p = 0.0001$ and $F_{(1,11)} = 13.444$, $p = 0.0037$, respectively), while HVA levels were unchanged ($F_{(1,12)} = 0.003$, $p = 0.9586$ and $F_{(1,11)} = 2.41$, $p = 0.1489$, respectively) (Figure 2-5, Figure 2-S3 and Table S1). These data demonstrate that MAOA blockade using CLO treatment during peri-adolescence inhibits 5-HT, NE, and DA metabolism.

Peri-adolescent MAOA but not 5-HTT blockade reduces adult brainstem 5-HIAA levels and increases striatal DA and DOPAC levels.

To further investigate the differential effect of peri-adolescent MAOA and 5-HTT blockade on adult aggressive behavior, we next examined if either treatment produces long-lasting changes in brain monoamine or -metabolite levels. P22-P41 CLO-treatment

significantly reduced 5-HIAA levels in the brain stem at P180 when compared to either VEH- or FLX-treatment ($F_{(2,15)} = 8.505$, $p = 0.0034$) (Figure 2-5, Figure 2-S1 and Table S1). P22-P41 CLO treatment also significantly increased striatal DA ($F_{(2,15)} = 4.085$, $p = 0.0384$) and DOPAC levels ($F_{(2,15)} = 5.847$, $p < 0.0133$) at P180 when compared to VEH-treatment and VEH- or FLX-treatment, respectively (Figure 2-5, Figure 2-S1 and Table S1). Peri-adolescent FLX treatment did not produce significant changes in monoamines or their metabolites in the brainstem or striatum at P180, when compared to VEH treated controls.

a Experimental design



b Tissue levels

		P23	P42	P180	
		CLO	CLO	CLO	FLX
BRAIN STEM	5-HT				
	5-HIAA				
	NE				
STRIATUM	DA				
	DOPAC				
	HVA				

Legend:

	>200%
	200-150%
	150-110%
	90-75%
	75-50%
	<50%

Figure 2-5. Altered monoamine and –metabolite levels during and after peri-adolescent MAOA blockade. Tissue 5-HT, 5-HIAA, NE, DA, DOPAC, and HVA levels were measured by high performance liquid chromatography. (a) Mice were injected daily with CLO or FLX from P22-P41 and aggressive behaviors were investigated after P120. (b) Monoamine and –metabolite levels were assessed at P23, P42, and P180 in brain stem and striatum. Monoamine levels were increased and monoamine-metabolite levels were decreased during CLO treatment when compared to VEH treatment as indicated. At P180, brain stem 5-HIAA levels were decreased and striatal DA and DOPAC levels were increased after CLO treatment when compared to VEH treatment. FLX treatment did not alter monoamine or –metabolite levels at P180 when compared to VEH treatment. (n = 5–8 mice per group; colored fields indicate differences compared to VEH with p at least < 0.05).

<u>Peri-adolescent MAOA blockade increases while 5-HTT blockade reduces the behavioral response to amphetamine in adulthood.</u>

Dopaminergic hyper-activity has been implicated in aggression (de Almeida et al., 2005). We find that peri-adolescent MAOA blockade increases aggression and striatal DA and DOPAC levels in adulthood. Hence we hypothesized that peri-adolescent MAOA blockade produces long-lasting changes in the DA system. Conversely, we find that peri-adolescent 5-HTT blockade reduces adult aggression without altering striatal DA or DOPAC levels and might thus have no effect on the DA system. To test this hypothesized dissociation, we investigated the behavioral response to amphetamine challenge (3 mg/kg, i.p.) in adult mice (P180), which had received VEH, FLX, or CLO during P22-P41. We detected an effect of adult amphetamine treatment, an effect of peri-adolescent treatment and an interaction between both treatments ($F_{(16,480)} = 8.06$, $p < 0.0001$; $F_{(2,30)} = 8.4$, $p = 0.0013$ and $F_{(16,480)} = 4.141$, $p < 0.0001$, respectively) (Figure 2-6a). Adult VEH injection did not alter behavior in either of the P22-P41 treated groups (Figure 2-6b). Adult AMPH injection did alter behavior differentially in the P22-P41 treated groups: In comparison with P22-P41 VEH treated mice, P22-P41 CLO treated

mice showed a significantly increased response to amphetamine (peri-adolescent x adult treatment interaction: $F_{(16,416)} = 3.810$, $p < 0.0001$) (Figure 2-6a). Conversely, P22-P41 FLX treated mice showed a significantly reduced response to amphetamine when compared to P22-P41 VEH treated littermates (peri-adolescent x adult treatment interaction: $F_{(16,288)} = 1.896$, $p = 0.0375$) (Figure 2-6a). These data demonstrate that P22-P41 CLO and FLX treatment differentially impact the sensitivity of the adult DA system. Furthermore, the behavioral sensitivity to amphetamine correlates with levels of adult aggression.

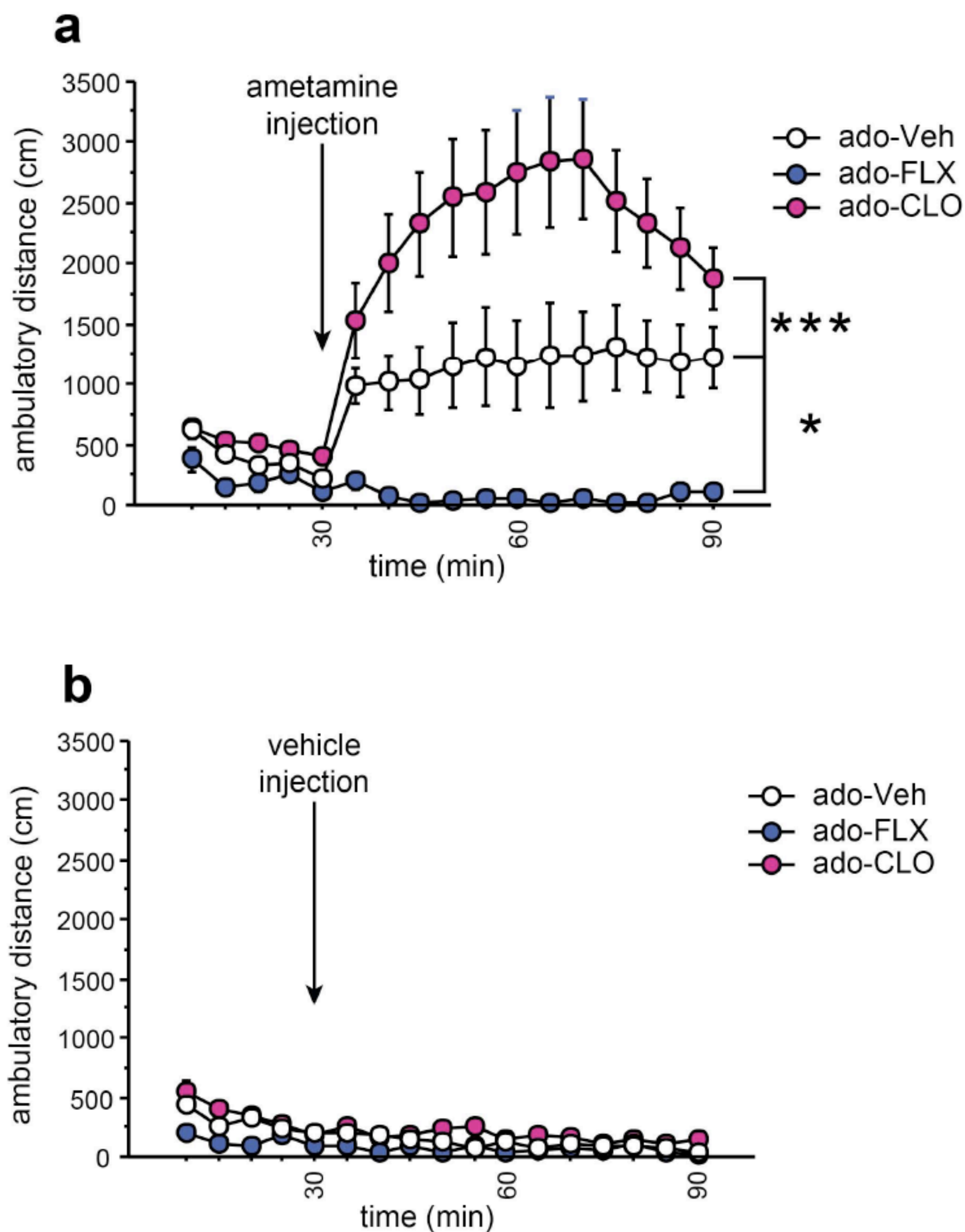


Figure 2-6 Altered behavioral response to amphetamine in adulthood after peri-adolescent MAOA or 5-HTT blockade. Behavioral amphetamine response was assessed in ADO-CLO, ADO-FLX and ADO-VEH treated mice using the open field. (**a**) Amphetamine injection (3 mg/kg) induced locomotor hyperactivity in ADO-VEH and ADO-CLO, but not in ADO-FLX treated mice. The behavioral response to amphetamine challenge in ADO-CLO treated mice was increased when compared to ADO-VEH treated controls. (**b**) VEH injection did not alter locomotor activity in any of the three treatment groups. (n = 5-15 per group) (posthoc comparisons indicate statistically significant interactions between time and peri-adolescent treatment. *p < 0.05, ***p < 0.001).

Peri-adolescent DAT but not NET blockade increases adult aggressive behavior.

Next we sought to identify the monoamine component in P22-P41 MAOA blockade, which leads to increased adult aggressive behavior. Because peri-adolescent MAOA blockade robustly increases central 5-HT, NE and DA levels, we examined whether increased peri-adolescent NE or DA signaling could increase adult aggressive behavior. To selectively and transiently increase NE or DA signaling we administered drugs blocking the NE transporter (NET) and the DA transporter (DAT). We used desipramine (DMI, 20 mg/kg/day) to block the NET and GBR12909 (GBR, 20 mg/kg/day) to block the DAT. DMI treatment from P22-P41, either alone or in combination with FLX treatment, did not result in increased aggressive behavior in adulthood (Figure 2-7a). GBR treatment from P22-P41 however lead to increased time spent in active aggressive behaviors (Mann Whitney, $p = 0.0104$) (Figure 2-7b).

Peri-adolescent DAT blockade increases behavioral response to amphetamine in adulthood.

Finally, we tested whether peri-adolescent GBR treatment produces long-lasting changes in the DA system, which mimic the effect of MAOA blockade. Strengthening the correlation between aggressive behavior and a sensitized/hyper-active DA system, we indeed found that P22-P41 GBR treated mice display an exaggerated response to adult amphetamine treatment when compared to adult VEH injection or P22-P41 VEH treated mice (effect of amphetamine challenge: $F_{(16,160)} = 2.682$, $p = 0.0009$; peri-adolescent

treatment x adult amphetamine challenge interaction: $F_{(16,160)} = 3.119$, $p = 0.0001$, respectively) (Figure 2-7c and d).

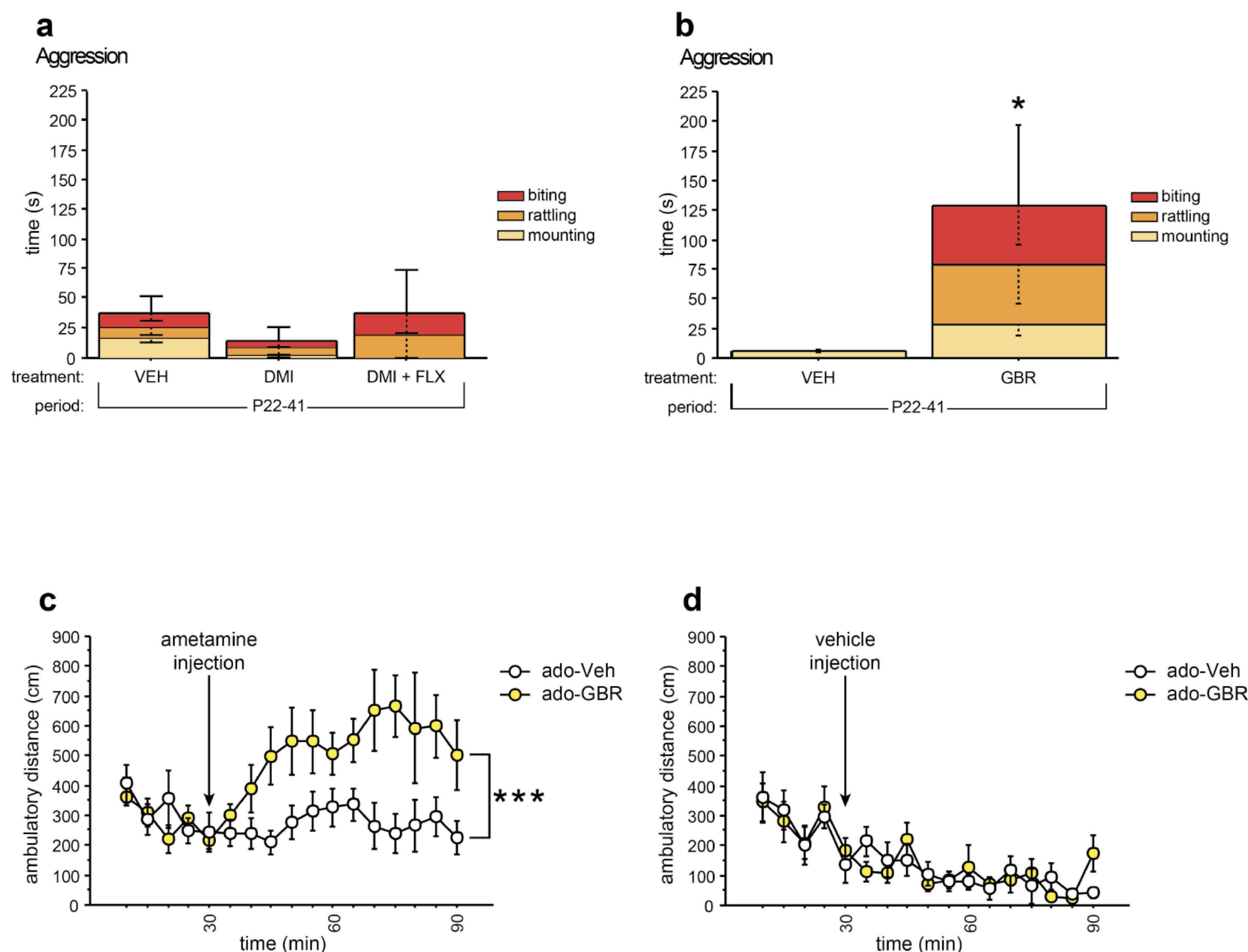


Figure 2-7 Increased adult aggressive behavior and response to amphetamine challenge after peri-adolescent DAT blockade**.** Isolation induced aggressive behavior was assessed in mice by scoring the time spent mounting, tail rattling, or biting during a 10 minute encounter (**a, b**). Behavioral amphetamine response was assessed in mice using the open field (**c, d**). Aggressive behavior was not altered in mice treated with DMI or DMI+FLX from P22-P41 when compared to VEH treated control mice (n = 5-15 per group) (**a**). Aggressive behavior was increased in mice treated with GBR from P22-P41 when compared to VEH treated control mice (n = 11 per group) (**b**). (**c**) Amphetamine injection (3 mg/kg) induced locomotor hyperactivity in ADO-VEH and ADO-GBR treated mice. The behavioral response to amphetamine challenge in ADO-GBR treated mice were increased when compared to ADO-VEH treated controls (n = 6 per group; posthoc comparisons indicate statistically significant interactions between time and peri-

adolescent treatment). (**d**) VEH injection did not alter locomotor activity in ADO-GBR or ADO-VEH treated mice (n = 6 per group). (*p < 0.05, ***p < 0.001).

		P23				P42				P180					
		VEH (N=6)		CLO (N=8)		VEH (N=6)		CLO (N=7)		VEH (N=5)		CLO (N=7)		FLX (N=6)	
		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Brainstem	5-HT (pmol/g)	6126.057	481.45	12318.215	646.418	6757.51	599.331	13242.893	559.544	8699.36	282.508	8249.643	354.639	9546.533	322.79
	5-HIAA (pmol/g)	6503.958	337.343	4532.359	168.841	5655.245	400.152	3725.939	229.598	9471.08	190.556	7552.6	413.79	8554.883	237.606
	NE (pmol/g)	5143.348	289.544	7591.22	138.393	7157.702	423.702	10216.157	259.698	17963.48	2527.924	14904.929	1588.878	16480.3	1470.395
Striatum	DA (pmol/g)	14904.563	1400.032	15556.771	2896.914	11091.06	1534.275	24050.601	787.488	16931.48	605.466	19216.857	460	17903.083	650.916
	DOPAC (pmol/g)	3117.928	415.694	939.151	150.203	2342.978	410.088	865.28	131.57	2492.66	189.909	3205.5	228.83	2298.567	172.134
	HVA (pmol/g)	1388.408	335.648	1368.491	208.545	1605.475	221.906	2022.536	160.946	2957.36	174.223	3604.857	320.824	3025.583	199.272

Table S1. Monoamine and –metabolite levels during and after peri-adolecent MAOA or 5-HTT blockade. Tissure 5-HT, 5-HIAA, NE, DA, DOPAC, and HVA levels were measured by high performance iquid chromatography. (n=5-8 mice per group; colored fields indicate differences compared to VEH with p at least <0.05)

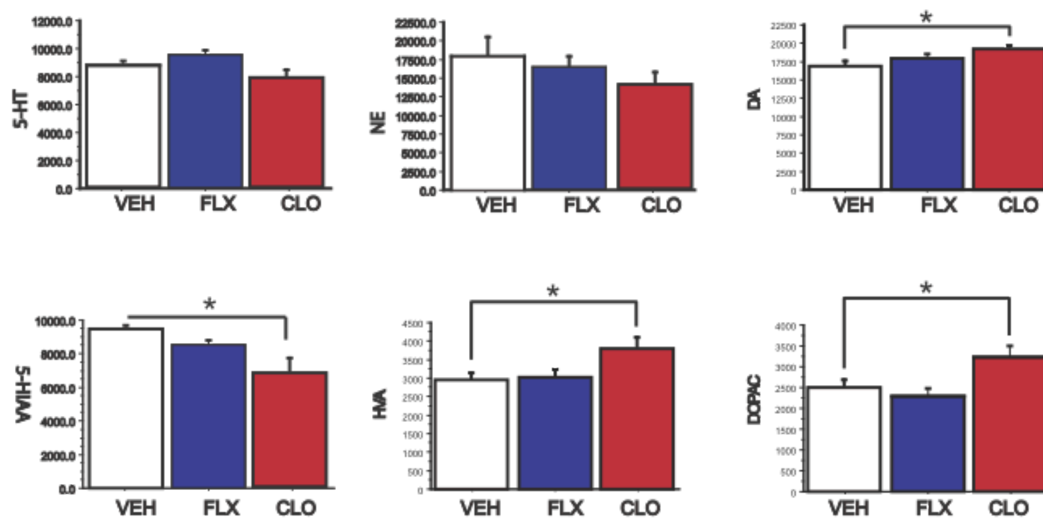


Figure 2-S1 Altered monoamine and –metabolite levels in adulthood after peri-adolescent MAOA blockade or 5-HTT blockade. Tissue 5-HT, 5-HIAA, NE, DA, DOPAC, and HVA levels were measured by high performance liquid chromatography. Mice were injected daily with CLO or FLX from P22-P41 and monoamine and –metabolite levels were assessed at P180 in brain stem and striatum. At P180, brain stem 5-HIAA levels were decreased and striatal DA and DOPAC levels were increased after CLO treatment when compared to VEH treatment. FLX treatment did not alter monoamine or –metabolite levels at P180 when compared to VEH treatment. (n = 5–8 mice per group).

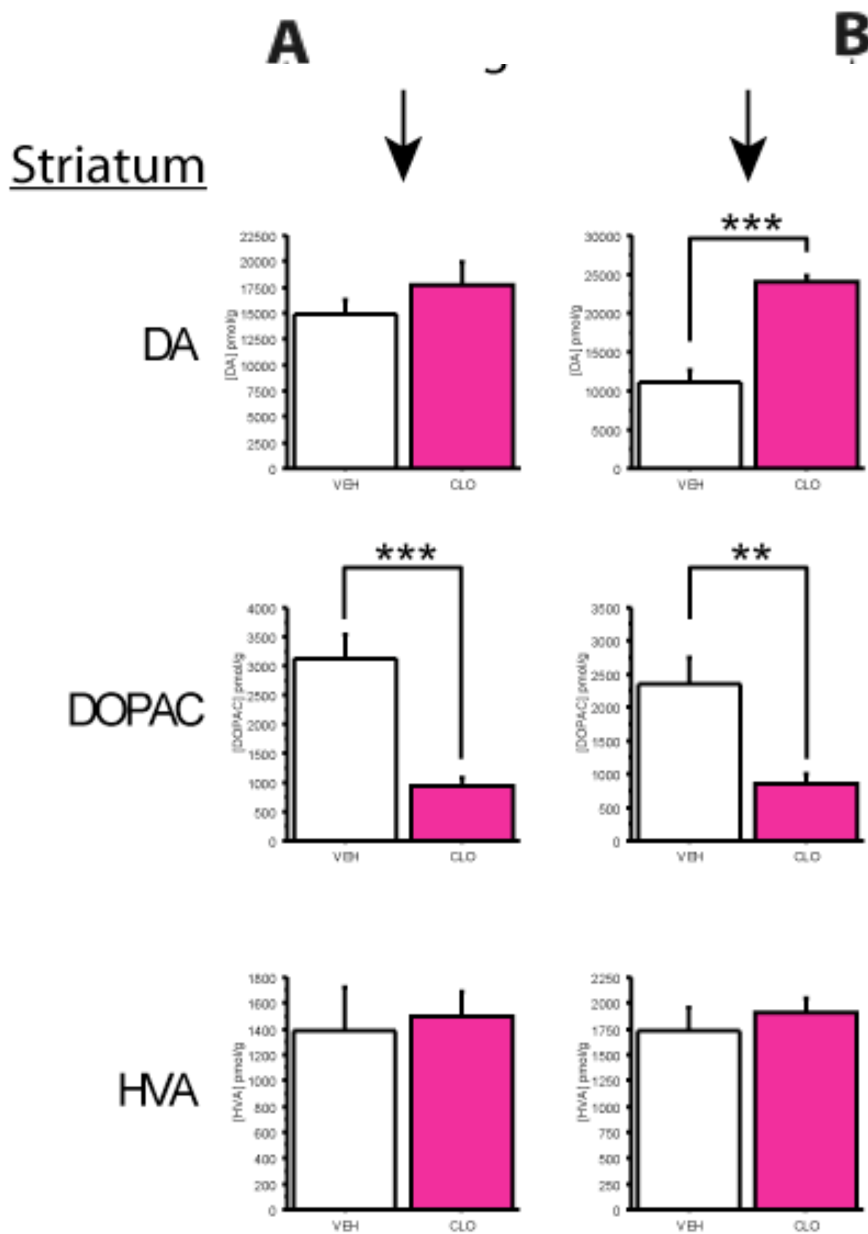


Figure 2-S2. Altered monoamine and –metabolite levels during and after peri-adolescent MAOA blockade or 5-HTT blockade. Tissue DA, DOPAC, and HVA levels were measured by high performance liquid chromatography. Mice were injected daily with CLO or FLX from P22-P41 and Monoamine and –metabolite levels were assessed at P23 (A), P42 (B) in brain stem and striatum. At P23, DOPAC levels were decreased after CLO treatment. At P42, DA levels were increased and DOPAC levels were decreased after CLO treatment when compared to VEH treatment as indicated. (n = 5–8 mice per group).

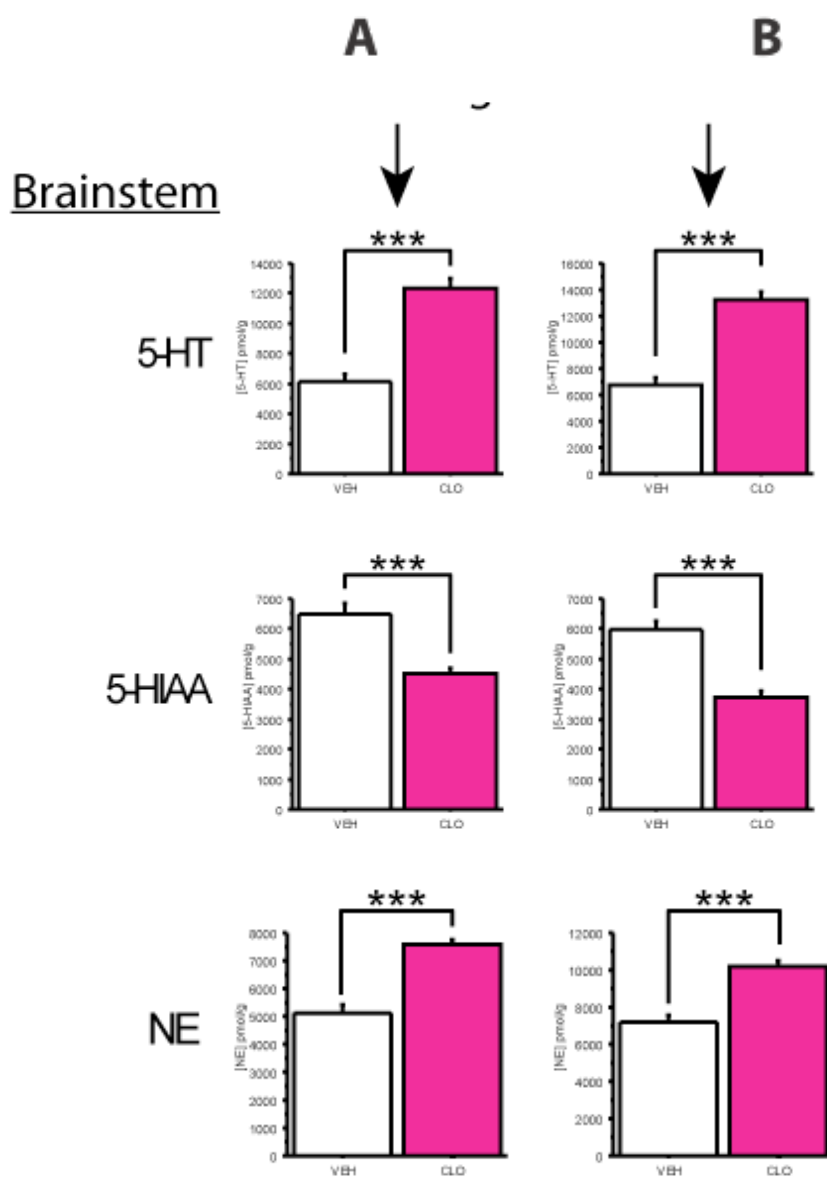


Figure 2-S3. Altered monoamine and –metabolite levels during and after peri-adolescent MAOA blockade or SERT blockade. Tissue 5-HT, 5-HIAA, and NE levels were measured by high performance liquid chromatography. Mice were injected daily with CLO or FLX from P22-P41 and Monoamine and –metabolite levels were assessed at P23 (A), P42 (B) in brain stem. At P23, NE levels and 5-HT levels were increased and 5-HIAA levels were decreased after CLO treatment; At P42, NE and 5-HT levels were increased and 5-HIAA levels were decreased after CLO treatment when compared to VEH treatment as indicated. (n = 5–8 mice per group).

Discussion

Our experiments demonstrate the existence of two developmental periods whereupon early-life perturbation of monoamine signaling alters adult behavior: an early postnatal (P2-P21) 5-HT-sensitive period that affects anxiety and depression-related behaviors and a later peri-adolescent (P22-P41) DA-sensitive period altering aggression. Moreover, serotonergic and dopaminergic signaling perturbations during peri-adolescence also exert profound and opposing effects on the behavioral response to psychostimulants.

Our findings can explain the paradoxical high neophobia/anxiety phenotypes of *5-htt-/-* mice (Holmes et al., 2003; Lira et al., 2003), *maoa-/-* mice (Cases et al., 1995; Scott et al., 2008; Godar et al., 2010), and pre-synaptic *htr1a* deficient mice (Richardson-Jones et al., 2011), because these mouse lines lack their respective gene products throughout life, including P2-P21. Likewise, the model can explain the increased aggression seen in constitutive *maoa*, *dat*, and *comt* loss-of-function mouse lines (Cases et al., 1995; Gogos et al., 1998; Rodriguiz et al., 2004; Scott et al., 2008). The moderating effect of developmental 5-HTT blockade on adult aggressive behavior does not follow the strict timing specificity (5-HTT blockade during P2-P21 or P22-P41 both decreased adult aggression), but nonetheless supports a developmental mechanism for the low-aggression phenotype of *5-htt-/-* mice (Holmes et al., 2002a).

Our findings also comport with human vulnerabilities to anxiety, depression, and aggression conferred by functional genetic polymorphisms. For example, endophenotypes of affective disorders have been associated with low expressing alleles

for the *5-htt* (Lesch et al., 1996; Canli and Lesch, 2007; Risch et al., 2009; Caspi et al., 2010; Karg et al., 2011), *maoa* (Schmidt et al., 2000; Tadic et al., 2003) and presynaptic *htr1a* (Le Francois et al., 2008). Likewise, aggressive behavior has been associated with loss-of-function and low-expressing *maoa* alleles (Brunner et al., 1993a; Caspi et al., 2002; Zalsman et al., 2005; Buckholtz and Meyer-Lindenberg, 2008), the *10R* variant of *dat1* (Guo et al., 2007; Bedard et al., 2010), and the low activity *met* allele of the *comt* (Volavka et al., 2004). Our model predicts that these risk alleles act primarily during sensitive developmental periods to alter brain maturation and circuit formation leading to altered behaviors. The temporal dynamics of transcription in the human brain and their genetic moderation support this hypothesis (Colantuoni et al., 2011).

Based on our data, pharmacologic interventions during sensitive periods of human development would be predicted to impact affective and aggressive behavior. The murine P2-P21 period roughly corresponds to the third trimester of human gestation and early childhood, while P22-P41 corresponds to peri-adolescent development. Thus SSRIs taken by pregnant mothers might impact fetal brain maturation and adult affective behavior while stimulant exposure during peri-adolescence could alter adult aggressive behavior. Indeed, chronic stimulant exposure increases aggressive behavior in rodents, non-human primates and humans (Martin et al., 1990; Sokolov et al., 2004; Dawe et al., 2009), even in abstinent stimulant users (Sekine et al., 2006). The long-term effects of gestational SSRI exposure on affect, aggression, and cognition are unknown (Oberlander et al., 2009; Homberg et al., 2010), but prenatal SSRI exposure has been associated with a 2-fold increased risk of autism spectrum disorder (Croen et al., 2011).

Our findings support the fundamental notion that circuitry and consequently behavior is most vulnerable to long lasting modulation during periods of maturation and high plasticity (Hensch, 2004; Crews et al., 2007). The 5-HT sensitive period described here coincides with the emergence of anxiety- and fear-related behaviors in rodents and the maturation of the underlying circuitry. For example, at around P10 the consequence of odor shock conditioning switches from preference to aversion, with amygdala engagement hypothalamic-pituitary-adrenal axis integration (Sullivan et al., 2000; Moriceau and Sullivan, 2006). Limbic structures including amygdala, prefrontal cortex and hippocampus are maturing during postnatal development and human *s* allele carriers as well as *5-htt-/-* mice exhibit disrupted mPFC-amygdala functional connectivity that likely arises during early circuit maturation (Heinz et al., 2005; Pezawas et al., 2005). The DA-sensitive, peri-adolescent period coincides with the developmental onset of play fighting in rodents (Pellis and Pellis, 1997; Pellis and Pasztor, 1999) and non-human primates (Suomi, 2006), and human adolescence is characterized by dynamic changes in aggressive behavior (Moffitt, 1993). Adolescent rodents spend more time in social interactions (Primus and Kellogg, 1989). The quality of the social interaction changes during adolescent period. For example, rats engage in more complex defensive strategies (Pellis and Pellis, 1997) and exhibit differential rates of attack and defense in play fighting during adolescent developmental period (Pellis and Pellis, 1990). Since the brain maturation continues until adolescent period, the development of brain circuits makes this age period critically vulnerable with respect to impulse control and development of social behavior. Neural chemical disturbances during this developmental period lead to behavior changes in adulthood.

Dopamine has been associated with a series of neuropsychiatric disorders. The dopamine transporter (DAT) gene variant has been implicated in ADHD (Todd et al., 2005), schizophrenia (Saiz et al., 2010), bipolar disorders (Pinsonneault et al., 2011), and cocaine abuse (Guindalini et al., 2006). Mice lacking DAT have shown hyperlocomotion (Giros et al., 1996) and exhibited increased rates of reactivity aggression following mild social contact (Rodriguiz et al., 2004). A broad spectrum of psychiatric disorders involves inappropriate social interactions that could lead to heightened state of aggressiveness. Cocaine, which inhibits dopamine uptake (Horn, 1990), administered during adolescent developmental period (P27-P57) results in highly escalated aggression behavior (Harrison et al., 2000). In addition, adolescent cocaine exposure leads to significant deficits in serotonin afferent innervations to many brain areas implicated in aggression (DeLeon et al., 2002). Our study has directly shown that DAT blockade during adolescent period leads to increased aggressive behavior in adult mice. Dopamine system during adolescence has undergone evident changes such as increased prefrontal cortex fiber density (Kalsbeek et al., 1988) and pruning of DA receptors (Teicher et al., 1995; Tarazi et al., 1998a, b). Increased dopaminergic tone by pharmacological treatment during this period might alter development of the structure and function of the brain and lead to changes of response towards social stimuli. This finding for the first time linked the developmental dopamine function manipulation to adult aggressive behavior. This finding also provides support for the notion that genetic polymorphisms of DAT gene may exert their effects during adolescent development of neuronal system by altering maturation of circuits that modulate responses to social stimuli.

We report that increased adult aggressive behavior elicited by peri-adolescent MAOA or DAT blockade correlates with increased locomotor-stimulating effects of amphetamine challenge in adulthood. Conversely, reduced adult aggressive behavior elicited by peri-adolescent 5-HTT blockade correlates with decreased locomotor-stimulating effects of amphetamine challenge in adulthood. Supporting phylogenetic conservation, human individuals with antisocial traits also show mesolimbic dopamine hypersensitivity to amphetamine, as impulsivity is positively correlated with the magnitude of amphetamine-induced DA release in the striatum (Buckholtz et al., 2010). Highlighting the notion that altered DAergic signaling impacts adult behavior as a function of developmental timing, transient *pre*-adolescent methylphenidate exposure *decreases* responsiveness to cocaine's locomotor-activating effects in adult rats (Andersen et al., 2002). Furthermore, transient adult methylphenidate exposure does not alter cocaine's locomotor-activating effects in later adulthood (Andersen et al., 2002). Taken together, our findings support the existence of sensitive periods that influence life-long vulnerability to anxiety, depression, aggression, and substance abuse. Such sensitive periods have been most extensively characterized for sensory systems (e.g. visual cortex), but conceptually similar principles may apply to the development and organization of brain circuitry that mediate the more complex behaviors described here. Furthering our knowledge of sensitive periods that determine the developmental trajectory of complex behaviors is a necessary step towards improving prevention and treatment approaches for neuropsychiatric disorders.

Considerations and remarks

Here we show that peri-adolescent MAOA blockade or DAT blockade (P22-P41) produced increased aggressive behavior, and adult MAOA blockade or DAT blockade does not increase aggressive behavior (unpublished data, Qinghui Yu). This points to a specific time window that is contained within P22-P41 period, during which increased dopamine signaling would impact adult aggressive behavior. Thus it is worthwhile further redefining this critical time window to probe the developmental events responsible for altered aggressive behavior.

Although the current study suggests that dopamine hyper activity might be the mechanisms underlying this aggressive behavior elicited by peri-adolescent MAOA blockade, the exact mechanisms are far from clear. For example, what brain areas might be relevant to this behavior readout? What receptors or signaling pathways or genes might mediate the increased dopamine on aggressive behavior? What physiological changes are the direct impacts of DAT blockade? Future characterization of the morphology, structure, and physiology would be necessary.

Another important and interesting consideration is whether we can reverse or rescue the changes exerted by peri-adolescent MAOA blockade. Optogenetic stimulation of the neurons in the ventromedial hypothalamus, ventrolateral subdivison (VMHvl) causes male mice to initiate attack and pharmacogenetic silencing of VMHvl reversibly inhibits inter-male aggression. Would silencing of the neurons in VMHvl or lesions of this region rescue the behavior phenotype in peri-adolescent MAOA inhibitor treated mice? Prefrontal cortex lesion has been associated with violence. Would optical

stimulation of the PFC neurons rescue the behavior changes? Answering these questions would hold very important translational promise.

Chapter III Early life blockade of 5-HTT on adult affective behavior and adult antidepressant-related behavioral response

Introduction

Genetic factors that impact serotonin levels alter adult emotional behavior both in humans and in animals (D'Souza and Craig, 2006; Kim et al., 2006). Low *5-htt* expression for example is associated with elevated trait anxiety and increased vulnerability to affective disorders (Lesch et al., 1996; Sen et al., 2004). However, pharmacologic inhibition of 5-HTT in adulthood generally produces therapeutic effects and ameliorates anxiety/depression symptoms (Dulawa and Hen, 2005; Berton and Nestler, 2006). This paradox led to the hypothesis that reduced 5-HTT function during critical periods of development may alter brain function in such a way as to predispose the organism to affective disorders later in life.

In support of this developmental hypothesis of anxiety and depression, it has been demonstrated that postnatal (P4) to P21 5-HTT blockade (using SSRIs) (Ansorge et al., 2004) or MAOA blockade (using the MAOA inhibitor clorgiline) (Qinghui Yu, Chapter II) increases depression/anxiety behavior in adult mice. The pharmacological effect of SSRIs is dependent on the presence of the 5-HTT, as no effect of treatment was seen in *5-htt* -/- mice (Ansorge et al., 2004). Furthermore, while early life blockade of 5-HTT increases anxiety and depression-like behavior in adult mice, transient FLX treatment in 3-month-old mice had no demonstrable effects on the same emotional behaviors after cessation of treatment (Ansorge et al., 2008). These results indicate that

increased 5-HT signaling during a critical time window (P4-P21) increases anxiety and depression-like behavior.

To better understand the manner in which increased 5-HT signaling during early life increases anxiety/depression-like behaviors, we further refined the developmentally sensitive period that confers the altered adult phenotype. We hypothesize that only a small period of exposure within P4-P21 is required to produce the same behavior effect as FLX treatment from P4-P21. We subdivided this period into P2-P11 or P12-P21, treated animals with FLX during these different postnatal periods and examined their anxiety- and depression-like behaviors in adulthood. In addition, we determined whether the increased anxiety/depression behavior could be normalized with adult SSRIs treatment.

Materials and methods

Animals

Pups were injected intraperitoneally daily (2:00 pm -5:00 pm) with either vehicle (0.9% NaCl, 5ml/kg) or FLX (10 mg/kg, 5ml/kg) during different postnatal periods P2-P11, P12-P21, P2-P21, P22-P41. Mice were treated once per day for all these experiments. For treatment, entire litters were removed from dams and placed into a petri dish (10 cm diameter) containing shavings from the respective home cage. The petri dish was resting on scales, to allow for weighing mice after picking them up. Mice were injected in random order, and an entire litter was injected within 2 min. Immediately after injection, mice were placed back into the home cage.

For adult mice undergoing chronic FLX treatment, they were injected

intraperitoneally daily (2:00 pm -5:00 pm) with either vehicle (0.9% NaCl, 5ml/kg) or FLX (10 mg/kg, 5ml/kg). Mice were treated individually once per day for four weeks before we started to test them in behavior tests. While testing for behavior, mice were also injected each day after behavior tests.

WT (129S6/SvEv) mice used for breeding were purchased from Taconic Farms. Animals were maintained on a 12-hour light-dark cycle (lights on at 8am) and provided with food and water. Animal testing was conducted in accordance with the Principles of Laboratory Animal Care National Institute of Health (NIH) guidelines and the institutional animal committee guidelines.

Drugs

For the studies involving postnatal drug administrations, fluoxetine (ANAWA Trading SA, Wangen, Switzerland) was dissolved in 0.9% NaCl to achieve the following concentrations: fluoxetine, 2 mg/ml. Fluoxetine is a 5-HT transporter (SERT) blocker. Fluoxetine is chosen due to its high specificity to SERT and its long half-life. PNFLX treatment schedule (10 mg/kg/day, i.p.) into mouse pups, produces blood levels that are in the expected therapeutic range for humans (Ansorge et al., 2004; Ansorge et al., 2008) and constitute the minimum dose behaviorally active in mice (Dulawa et al., 2004)

Behavior Testing

Refer to Chapter II

Data analysis

Data were analyzed using either Student's t-test, one-way or two-way analysis of variance (ANOVA). Adult treatments or postnatal treatments were assessed as the independent variable. The criterion for significance of all analyses is $p<0.05$. All experiment results are expressed as mean± SEM.

Results

The effect of PN-FLX treatment on exploratory activity in novel environment

Early postnatal SERT blockade from P4-P21 increases anxiety/depression-like behavior in mice (Ansorge et al., 2004; Ansorge et al., 2008). To further redefine the critical time window during which early life blockade of SERT results in increased anxiety/depression like behavior, we treated mice with SERT inhibitor FLX (10mg/kg/day, i.p.) or vehicle (VEH, 0.9% NaCl, i.p.) from P2-P21, P2-P11, P12-P21, or P22-P41, and tested the anxiety and depression like behaviors in adult mice. To control for the injection stress, we also tested the naïve mice in our experiment. We first assessed exploratory behavior phenotype in the open field. In comparison to vehicle treated mice, PN-FLX treatment from P2 to P21 significantly decreased exploratory behavior in adulthood. This was demonstrated by a reduction in the total distance travelled (Figure 3-1A; Posthoc analysis $p=0.0073$), time spent ambulating (Figure 3-1B; Posthoc analysis $p=0.0079$), and rearing in the open field (Figure 3-1C; Posthoc analysis $P=0.0463$). In addition, PN-FLX treated mice from P2 to P11 exhibit significantly decreased exploratory behavior in ambulatory distance (Figure 3-1A; $p=0.0002$), time spent ambulating (Figure 3-1B; $p=0.0005$), and rearing activity (Figure 3-1C; $p=0.0397$), which is comparable to PN-FLX treated group from P2 to P21. The decreased exploratory

behavior is not elicited in other groups who are treated with FLX from P22 to P41 or P12 to P21. This supports our hypothesis that a smaller time window (P2-P11) exists during which PN-FLX results in deficit in exploratory behaviors. The effects of PN-FLX was specific to exploratory behavior since we did not detect any differences in measures indicating anxiety level such as percentage of distance travelled in the center relative to the total distance traveled (Figure 3-1S).

The effect of PN-FLX treatment in test sensitive to chronic antidepressant treatment

To further assess the effect of PN-FLX treatment on adult emotional functioning, we examined their behavior in the novelty-suppressed feeding paradigm. This test is sensitive to chronic but not acute antidepressants as well as anxiolytics (Bodnoff et al., 1989). In the NSF, food-deprived mice are presented with a familiar food pellet in a novel, brightly lit environment and the latency to approach the food and begin a feeding bout is recorded. Consistent with previous findings, PN-FLX treatment from P2 to P21 prolonged the latency to feed (Figure 3-2A; p=0.0962). PN-FLX treatment from P2 to P11 significantly increased the latency to feed (Figure 3-2A; p<0.0001). PN-FLX treatment from P22 to P41 and from P12 to P21 does not produce the behavior deficit (Figure 2-2A). Weight losses during food deprivation and home cage food consumption were comparable across groups (Figure 3-2B), indicating the observed differences are not due to hunger levels.

The effect of PN-FLX treatment on behavioral stress response

We next examined the effects of PN-FLX in shock avoidance, a paradigm that assesses behavioral responses to stress. PN-FLX treatment from P2 to P21 produce behavior deficit in this paradigm as indicated by prolonged latency to escape shock (Figure 3-3A; p=0.0061). In addition, we found mice given FLX from P2 to P11 exhibited significant impairment in shock escape test (Figure 3-3A; p<0.0001). In contrast, PN-FLX treatments during other later postnatal periods do not produce this behavior deficit. No effect of treatment was detected for pre-shock activity in the dark chambers with the door open (Figure 3-3B), indicating that the effect on escape latencies was not caused by reduced overall activity.

The effect of adult FLX treatment in Postnatal treated mice

We next examined whether PN-FLX treated mice would response to adult antidepressant treatment. Since SSRIs are the most commonly prescribed anti-depressants, we gave mice chronic (>4 weeks) FLX treatment at 3-4 month old mice and tested the mice in novelty-suppressed feeding test. This test pharmacologically validated, sensitive to chronic but not acute antidepressants as well as anxiolytics. It has been previously demonstrated in 129 WT mice that adult chronic FLX treatment reduces latency to feed (Wang et al., 2008). Consistent with our previous findings, we detected an overall postnatal treatment effect. In terms of adult FLX response, in control mice, adult chronic FLX treatment significantly reduced the latency to feed (Figure 3-4; p=0.0156); in PNFLX mice, adult chronic FLX treatment increased the latency to feed. We also detected a significant interaction of adult chronic treatment and postnatal treatment (ANOVA p=0.0030).

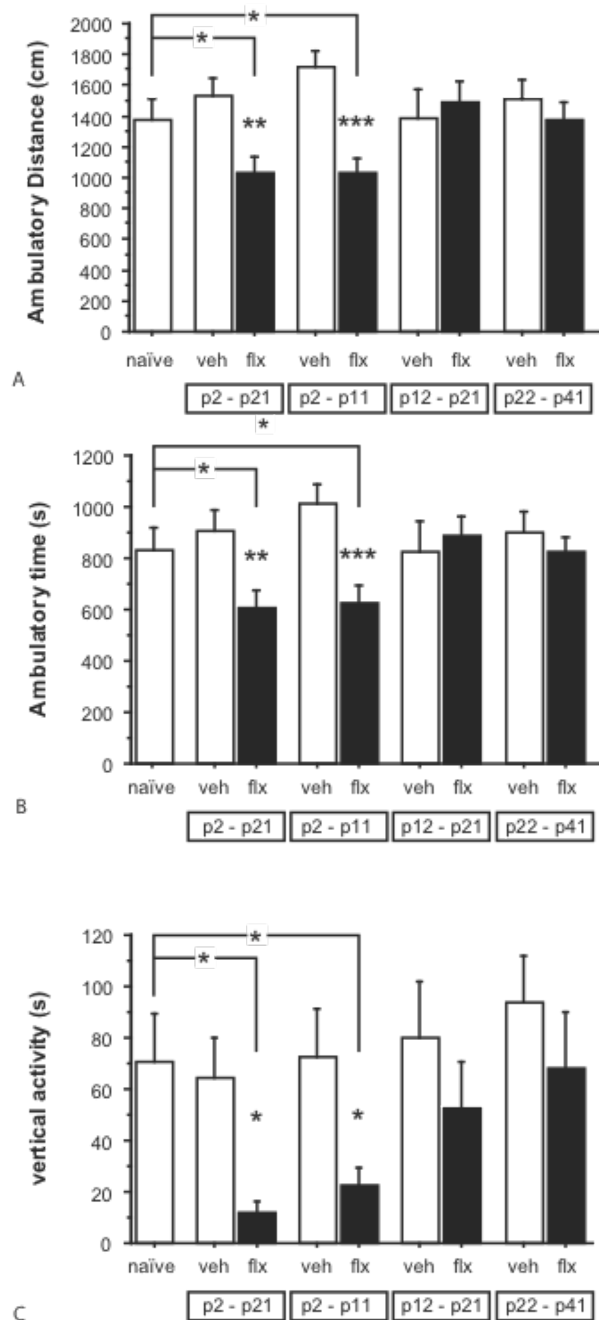
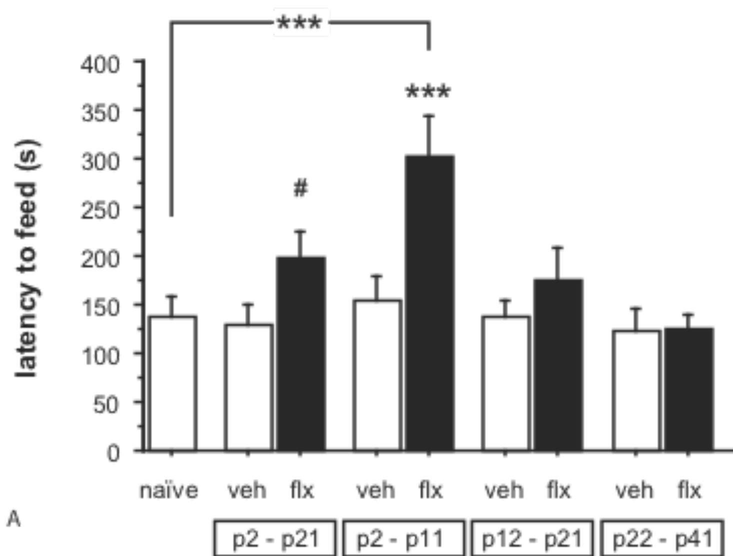


Figure 3-1. Exploratory behavior in the open field.

In the open field, the following parameters were scored for 30minutes: total distance traveled (A), ambulatory time (B), and vertical activity(C). PNFLX

treatment from P2-P11 reduced the ambulatory time, ambulatory distance, and vertical activity when compared with VEH from P2-P11. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with their respective controls. N = 19 to 32 mice per group. veh, vehicle; flx, Fluoxetine.



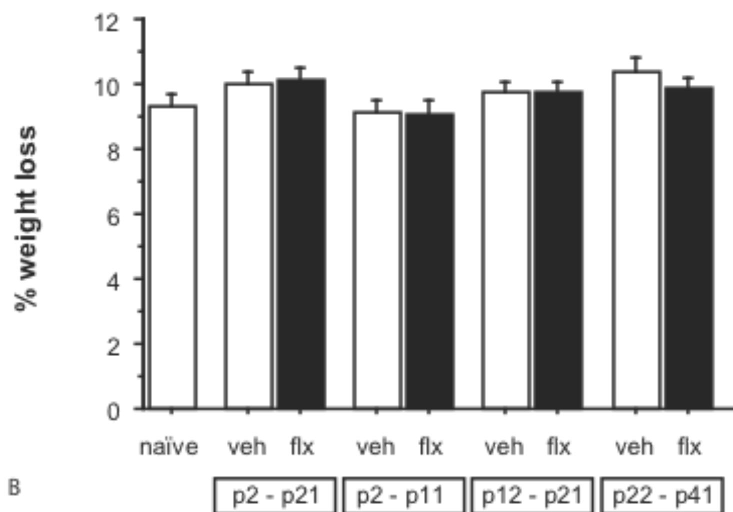


Figure 3-2. Novelty-suppressed feeding test. (A) The latency to begin feeding is shown in seconds. (B) Weight loss is expressed as a percentage of free-feeding body weight. PNFLX treatment from P2-P11 increased the latency to feed when compared with control mice treated with VEH. n =19 to 32 mice per group. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with their respective controls. veh, vehicle; flx, Fluoxetine.

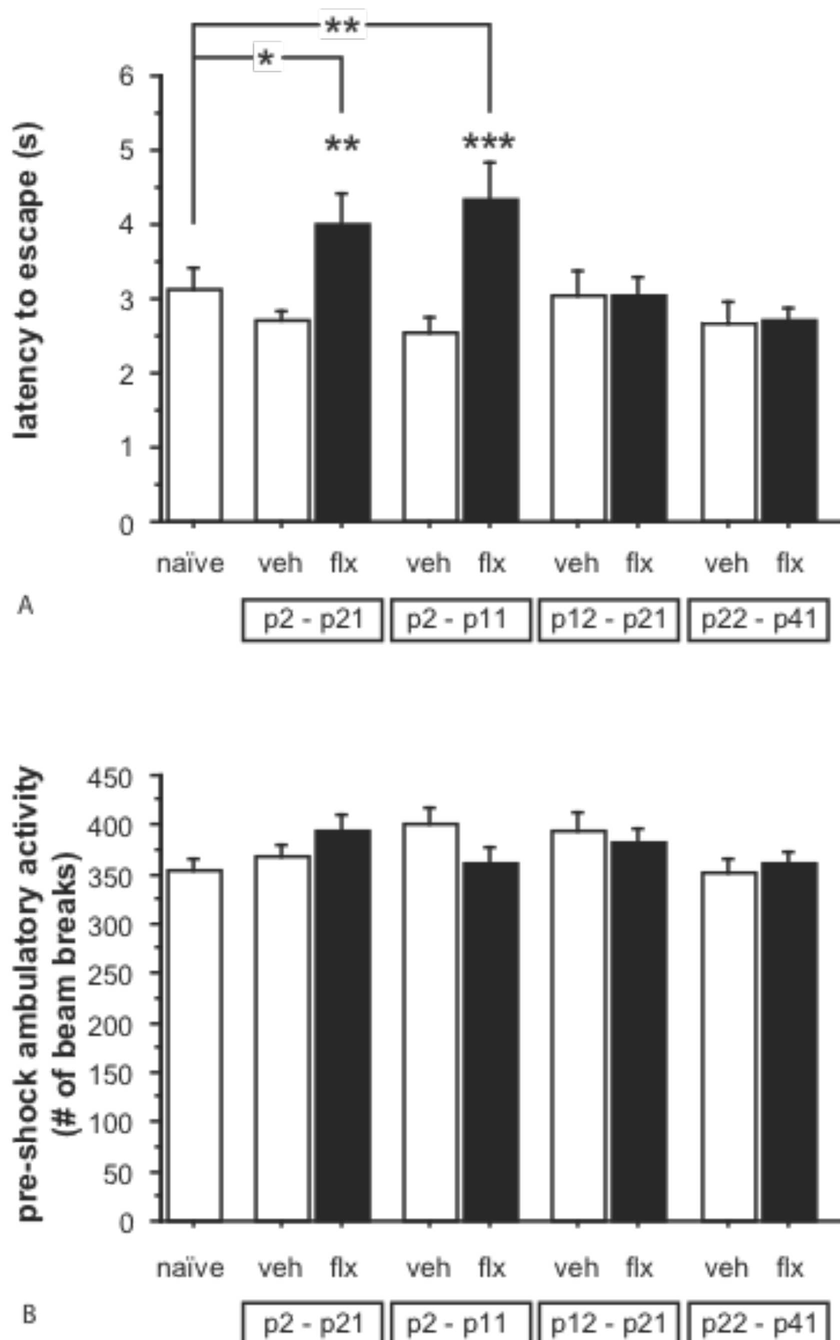


Figure 3-3. Shock escape paradigm. (A) Average latency to escape a foot shock shown in seconds. (B) Locomotor activity before shocks. n = 19 to 32 mice per group. PNFLX treatment from P2-P11 increased the latency to escape when compared with control mice treated with VEH. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with their respective controls. Veh, vehicle; flx, Fluoxetine.

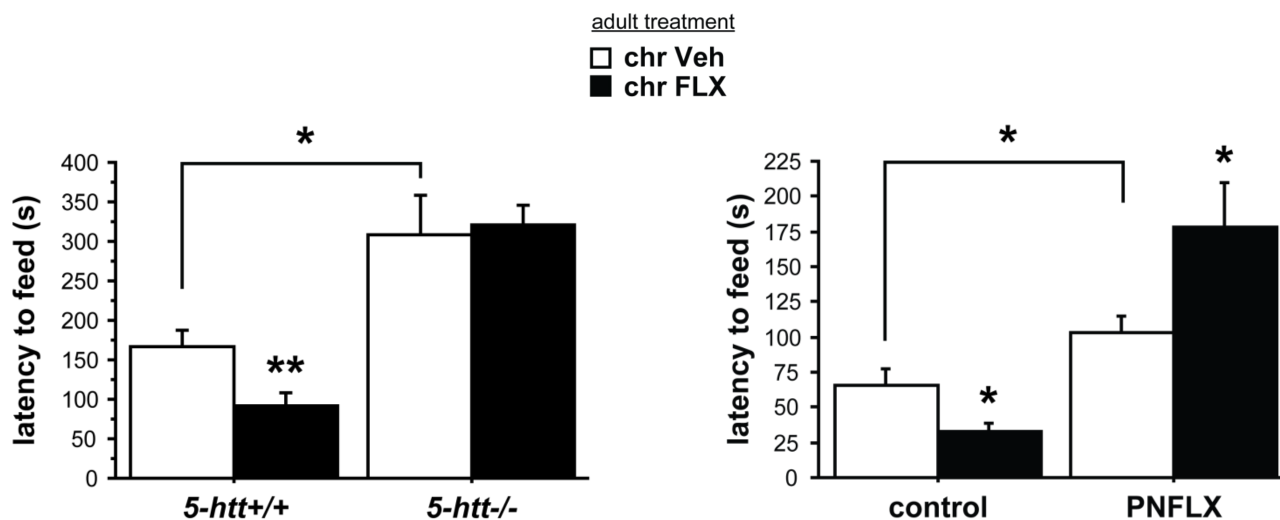


Figure 3-4. PN-FLX mice display anxiogenic response to chronic adult FLX treatment. 5-htt+/+ and 5-htt-/- mice (left), as well as PNVEH and PNFLX treated mice (right) were assessed in the novelty suppressed feeding test after chronic adult VEH or FLX treatment (10 mg/kg/day, 21 days, i.p.). 5-htt+/+ mice and control mice respond to chronic adult FLX treatment by reducing their latency to feed, and 5-htt-/- mice do not respond to chronic adult FLX treatment. PN-FLX display an anxiogenic response to adult chronic FLX treatment, which is indicative of fundamentally altered functional connectivity of 5-HTergic neurons to their postsynaptic targets. N = 17-30 per group, *: $p < 0.05$, **: $p < 0.01$.

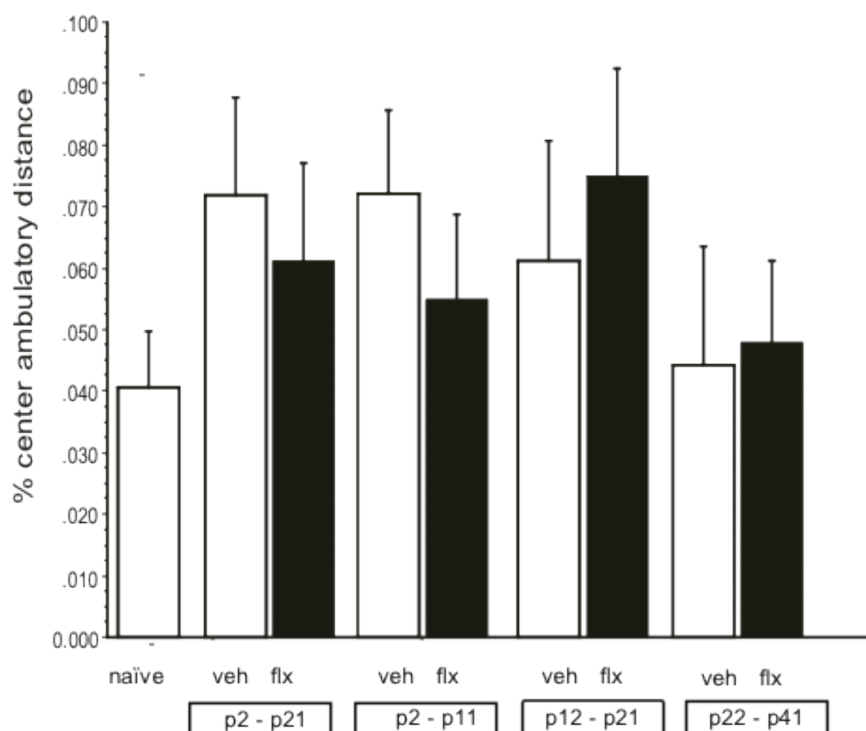


Figure 3-1S. PN-FLX treatment has no effect on anxiety measures in the open field. Behavior in the open field was recorded for 30 minutes and the percentage of distance traveled in the center portion of the open field relative to the total distance traveled was calculated. There is no difference in center activity among different treatment groups.

Discussions

Our findings demonstrated that P2 to P11 constitutes the critical period during which blockade of SERT resulted in increased depression and anxiety behavior. PNFLX treatment from P2 to P11 results in abnormal depressive and anxiety like behaviors in adulthood, while FLX treatment from P12 to P21 or from P22 to P41 does not produce this behavior deficit. The critical time window during which increased serotonin tone results in increased anxiety behavior is at least contained within P2-P11 period. In addition, adult FLX treatment failed to rescue the abnormal affective behavior in PN-FLX treated mice.

5-HTergic neurons are generated early in development on embryonic days (E) 10 to E12 in the mouse, and extend during early postnatal period (Levitt and Rakic, 1982; Luque et al., 1998). The full maturation of the axon terminal network requires more time and is achieved after birth in rodents (Lidov and Molliver, 1982). In addition to 5-HTergic neurons, a number of cells in the central and peripheral neuronal systems show a transient 5-HTergic phenotype during development (Lebrand et al., 1998). 5-HTT has transient expression in both serotonergic and non-sertonergic cell groups in early developmental period (Homberg et al., 2010). 5-HTT has broad expression during early developmental period not restricting to raphe regions. Previous experiment that analyzed expression of 5-HTT in P0-P21 mice showed that strong transient expression of 5-HTT was observed in the thalamic nuclei during the first two postnatal weeks. The highest 5-HTT expression was observed between P0-P7 in regions such as cortex, hippocampus, and subiculum (Lebrand et al., 1996; Lebrand et al., 1998). Recent genetic fate mapping studies more precisely showed that 5-HTT expression extends to non-serotonergic neurons, including thalamus, somatosensory cortex, the hippocampus (starting E14–E15), cingulated cortex (starting E14-E15) and the medial prefrontal cortex (starting P0). 5-HTT expression in non-serotonergic neurons ends rapidly during the second postnatal week, coinciding with the maturation of neural circuits (Narboux-Neme et al., 2008). The critical time window of P2 to P11 that we determined is congruent with the period of transient serotonergic phenotype and 5-HTT expression.

The 5-HT sensitive period described here coincides with the onset of anxiety and depression- related behaviors and the development of the underlying brain structure and circuitry. For example, at around P10 the consequence of odor shock conditioning

switches from preference to aversion, with amygdala engagement and hypothalamic-pituitary-adrenal axis integration. P2 to P11 is a critical period of synaptogenesis, axon growth and dendrite development (Pokorny and Yamamoto, 1981; Bahr and Wolff, 1985; Innocenti and Price, 2005). The cortical brain structure during this period is undergoing rapid changes in morphology and volume (Zhang et al., 2006). Hippocampus development takes place during post-natal period in rodents, with a critical maturation period coinciding with the time that serotonergic neuronal innervation occurs (Swann et al., 1989; Tansey et al., 2002; Gaspar et al., 2003). It is likely that the early-life dysregulation of serotonergic tone in PN-FLX mice would impact the proper development of many brain structures and cause permanent physiological, structural, and behavioral changes in adulthood.

Developmental disruption of serotonin system in mice has been shown to result in changes in serotonergic system as well as other critical structures. For instance, postnatal citalopram treatment from P8 to P21 can lead to a reduction in the density of 5-HTT immunoreactive (-ir) fibers in cortex (Maciag et al., 2006) and abnormal callosal connectivity and distorted activity profile of cortical neurons in primary auditory cortex when examined after treatment period (Simpson et al., 2011); postnatal treatment with SSRIs can lead to up-regulation of BDNF mRNA in the hippocampus (Karpova et al., 2009). PN-FLX treatment results in a decreased dendritic arborization of serotonergic fibers in the infralimbic (IL) sub-region of the mPFC, retraction of dendritic arbors in CA3 regions of the hippocampus, and reduced total spine number and density of CA3 neurons (unpublished data Tahilia Rebello). Administration of 5-HT_{1A} agonist (buspirone) to PCA-treated rat pups (PCA could deplete 5-HT) from P5 to P14 prevented

the loss of dendritic spines, and administration of 5-HT_{1A} antagonist (NAN-190) to naive pups from P3 to P14 produced a reduction in the number of dendritic spines and total dendritic length (Yan et al., 1997). Therefore, the brain structures and circuitry implicated in affective disorders might be permanently changed in a manner that contributes to the altered adult anxiety and depression behavior in adulthood.

Additionally, low-expression *5-htt* allele has been associated with neuroticism, anxiety and depression state in humans (Lesch et al., 1996; Sen et al., 2004). Our PNFLX animal model captured the behavioral aspects as shown in anxiety and depression related measures. Human *"s"* allele carriers exhibit diminished functional connectivity between the mPFC and the amygdale, in response to fearful stimuli in functional magnetic resonance imaging (fMRI) studies (Heinz et al., 2005; Pezawas et al., 2005). Moreover, there is reduced gray matter volume in short-allele carriers within the prefrontal cortical regions (Pezawas et al., 2005). This PNFLX animal model also exhibits morphological and physiological changes in these relevant brain regions in adulthood. Our PNFLX model serves a useful purpose for future study on the etiology and pathogenesis of depression and anxiety related disorders. This study also suggests that low-expressing allele may act during very early neonatal period to modify brain circuits that predisposes the carriers of these alleles to emotional disorders later in adult.

The SSRIs are used as a first-line treatment for psychiatric conditions such as major depression, generalized anxiety disorder, panic disorder, and obsessive-compulsive disorder. These agents are being used to treat these conditions in children and pregnant women, with 25% of depressed women continuing using anti-depressants throughout the preganancy (Ververs et al., 2006). Depression during pregnancy and the postpartum

period is a growing health concern that could affect up to 20% of women (Leung and Kaplan, 2009; Marcus, 2009). The current widespread use of SSRIs during pregnancy and childhood without knowing their long-term effects clearly constitutes an important public health concern. Our experiment in mice provides evidence on assessing the risk of antidepressants in pregnant woman. Since the P2-P11 period in rodents corresponds to human third trimester (Romijn et al., 1991), our experiment poses important health considerations for clinicians prescribing medicine to pregnant women with depression and anxiety disorders.

Currently up to 50% depression patients don’t respond to SSRIs. The serotonin transporter gene encodes a direct molecular target of SSRIs. *5-htt* genetic variant is associated with response to antidepressants. Human studies have shown that there exists a significant interaction of the low expression polymorphism and poor drug response (Lohoff; Smeraldi et al., 1998; Pollock et al., 2000; Murphy et al., 2004b; Lotrich and Pollock, 2005; Serretti et al., 2007; Wilkie et al., 2009). Animal studies in *5-htt* knockout mice have shown that deletion of *5-htt* gene renders the animals insensitive to the behavior effects of fluoxetine treatment (Holmes et al., 2002b). This thesis research shows early life 5-HTT blockade produces increased anxiety and depression like behaviors, which cannot be reversed by adult SSRI treatment. This finding provides a possible developmental explanation of the SSRI non-responders in depression patients. Inhibition of 5-HTT activity or excessive serotonin activity during development could be a risk factor for both etiology of affective disorders and poor treatment response to 5-HTT-sensitive antidepressants. The association of low expression “s” polymorphism and

poor drug response could be due to low 5-HTT activity the s allele carriers have during development.

Chapter IV Developmental mechanisms of early life 5-HTT blockade on adult affective behavior

Introduction

5-HT exerts its functions via 5-HT receptors and their downstream signaling pathways. These receptor subtypes have a central role in affective states and physiological functions. Among all the receptors, 5-HT_{2A} receptors have been implicated in the etiology and treatment of various psychiatric disorders. *hrt2a* -/- mice have shown increased anxiety phenotype (Weisstaub et al., 2006). 5-HT_{2A} receptor is also implicated in treatment of anxiety disorders and response to antidepressants (Carson and Kitagawa, 2004; McMahon et al., 2006; Millan, 2006; Aloyo et al., 2009).

Serotonin induces differential effects in rat layer V pyramidal neurons during different postnatal periods (Beique et al., 2004b; Beique et al., 2004a). Serotonin induced excitatory response in the first two postnatal weeks and inhibitory responses after P20 in rat layer V pyramidal neurons. This effect is due to a shift from 5-HT_{2A} receptor to 5-HT_{1A} receptor, with 5-HT_{2A} receptor mediating the depolarization effect and 5-HT_{1A} receptor mediating the hyper-polarization response (Zhang, 2003; Beique et al., 2004b).

Increased serotonin signaling from P2-P11 leads to increased anxiety- and depression- like behaviors in adulthood. This developmental period (P2-P11) is congruent with the time window that serotonin induces excitatory response via 5-HT_{2A} receptor. Moreover, pharmacological blockade of 5-HT_{1A} receptor from P13 to P34 is sufficient to produce the anxiety- and depression- like phenotype in adulthood (Lo Iacono and Gross, 2008). This P13-P34 period coincides with the time window of 5-HT_{1A}

mediated inhibitory response, which indicates that P13-P34 a critical time for 5-HT_{1A} mediated serotonin signaling. It is likely that developmental 5-HT signaling during P2-P11 period exerts its effect via 5-HT_{2A} receptor impacting adult behaviors. Taken together, this leads to the hypothesis that early postnatal 5-HT_{2A} receptor signaling mediates effects of developmetnal 5-HT signaling during P2-P11 period. Thus we tested the effect of postnatal FLX treatment on adult anxiety and depression- like behaviors by using *htr2a* -/- and *htr2a* +/+ mice.

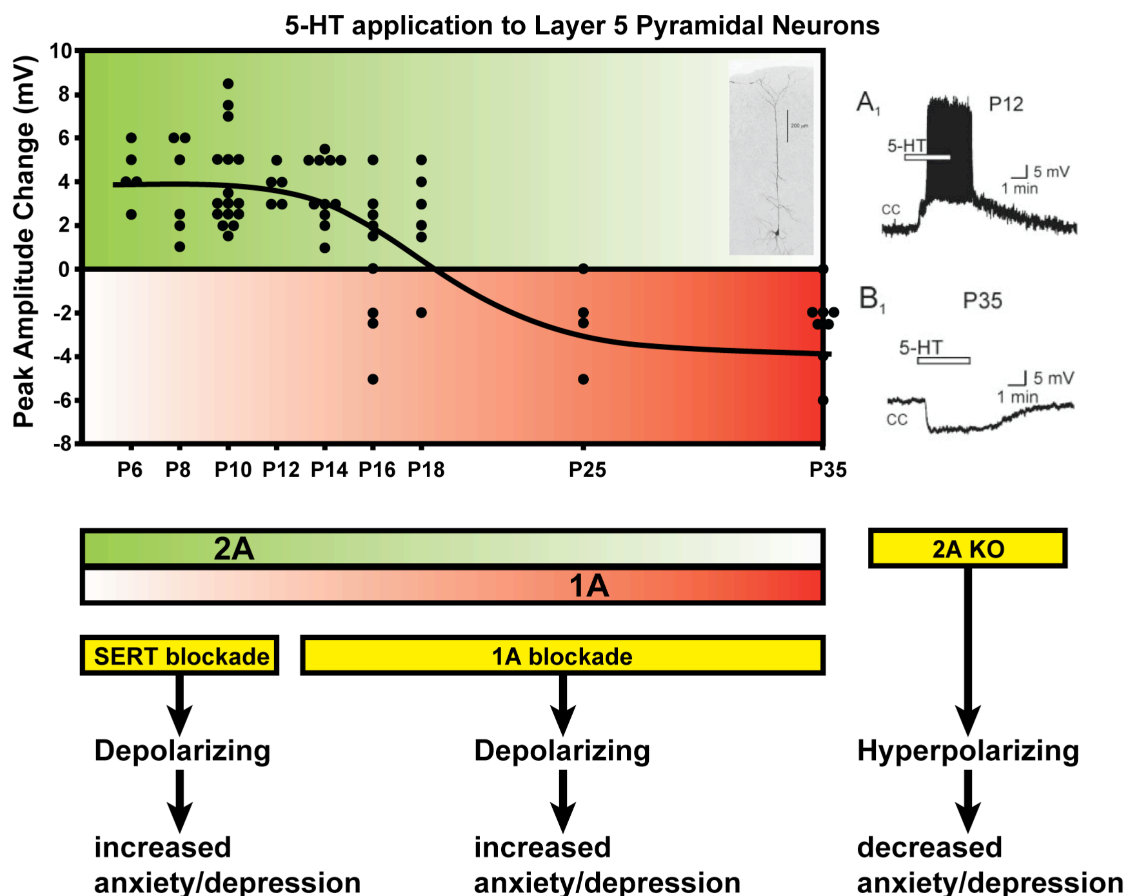


Figure 4-1 The critical period model. [Based on (Beique et al., 2004b; Weisstaub et al., 2006; Lo Iacono and Gross, 2008)]

Materials and methods

Subjects

Pups were injected intraperitoneally daily (2:00 pm -5:00 pm) with either vehicle (0.9% NaCl, 5ml/kg) or Fluoxetine (10 mg/kg, 5ml/kg) starting on postnatal day (P2) until P11. Mice were treated once per day for all these experiments. For treatment, entire litters were removed from dams and placed into a petri dish (10 cm diameter) containing shavings from the respective home cage. The petri dish was resting on scales, to allow for weighing mice after picking them up. Mice were injected in random order, and an entire litter was injected within 2 min. Immediately after injection, mice were placed back into the home cage. Mice were weaned after P22. Mice were genotyped using PCR of genomic DNA isolated from tissue samples following established protocols (Weisstaub et al., 2006). Animals were separated by sex and group housed with both WT and Knockout mice present in each cage.

The animals for behavior testing are bred via 5-ht$_{2A}$ +/- breeders. *htr2a* -/- mice were generated as described previously (Weisstaub et al., 2006). Animals carrying *htr2a+/-*mutation were intercrossed to obtain a mix of wild type (*htr2a+/+*), heterozygous (*htr2a+/-*), and knockout (*htr2a-/-*) offspring. The animals used for in-situ hybridization are WT animals. Animals were maintained on a 12-hour light-dark cycle (lights on at 8am) and provided with food and water. Animal testing was conducted in accordance with the Principles of Laboratory Animal Care National Institute of Health (NIH) guidelines and the institutional animal committee guidelines.

Drugs

For the studies involving postnatal drug administrations, fluoxetine (ANAWA Trading SA, Wangen, Switzerland) was dissolved in 0.9% NaCl to achieve the following

concentrations: fluoxetine, 2 mg/ml. Solutions were prepared fresh every day.

Behavior Testing

We used the same protocols as in chapter II.

In situ hybridization

In situ hybridization were performed on 15–20 µm cryostat sections as described (Demireva et al.). In brief, on the first day, prepare slides, add Hybridization Solution on each slide, incubate from 1hr to 6hrs at room temperature. On the second day replace pre-hyb solution with 100ul of hybrydization solution containing the probe (100ug/ul), cover slip slides carefully, place in sealed humidified black boxes and incubate in 72ºC incubator. Remove slides, wash slides in buffers, replace with anti-DIG Ab solutions (dilute AP-conjugated sheep anti-DIG Ab 1:5000, Roche # 1 093 274, in Buffer B2) and incubate slides with Ab at 4ºC in a humidified chamber. When signal is developed enough, stop reaction and mount slides.

Data analysis

Statistical analysis was performed using StatView 5.0 software (SAS Institute). Data were analyzed using Student's *t* test or one- or two-way ANOVA. The criterion for significance for all analyses was $p < 0.05$. Results from data analyses are expressed as mean ± SEM.

Results

Developmental expression of 5-ht2a receptor in prefrontal cortex

PNFLX treatment has caused physiological and anatomical changes in prefrontal cortex area such as reduced dendritic arborazation in the layer 2/3 pyramidal neurons in the IL of mPFC areas as well as changes in electrophysiological properties of the IL and PL pyramidal neurons (unpublished data Tahilia Rebello). In addition, the anxiety phenotype of *htr2a* -/- mice can be normalized by cortical 2A receptor re-expression. Thus we hypothesis cortical 5-HT_{2A} receptor might be critical for the increased 5-HT signaling during early developmental period. First, we performed in situ hybridization experiment at P8 and adulthood in prefrontal cortex area to examine 5-HT_{2A} receptor expression pattern in cortical areas. We observed that 5-HT_{2A} receptor has strong expression in frontal cortex (obital cortex, mPFC) at P8. In adulthood 5-HT_{2A} receptor has expression in frontal cortex (obital cortex, mPFC), which is congruent with other people's findings.

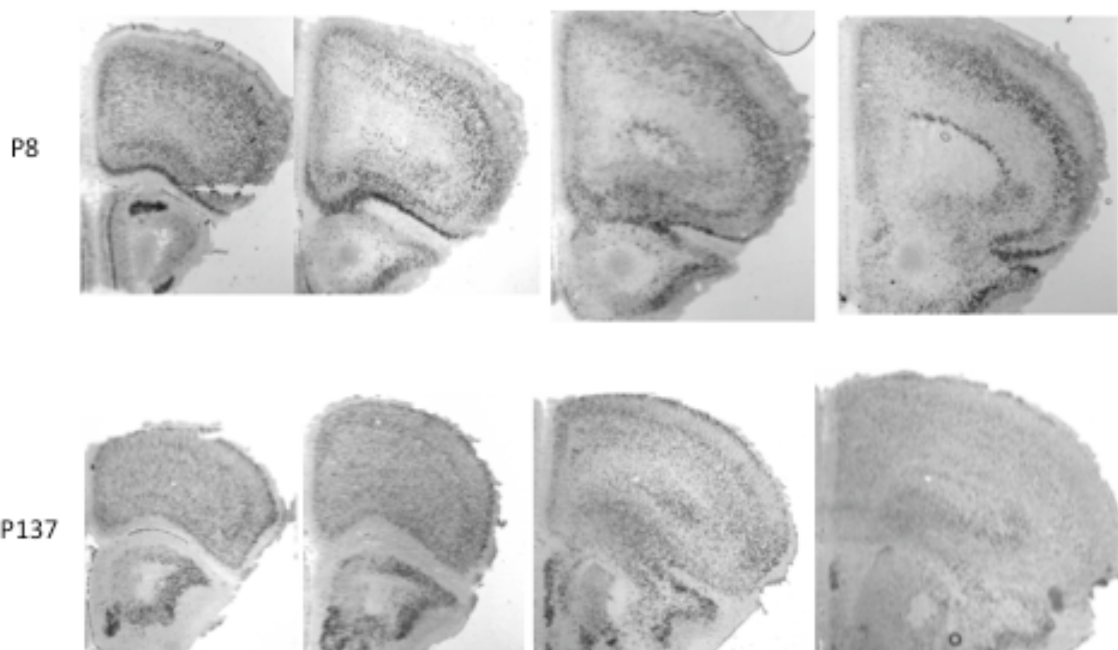


Figure 4-2 Representative pictures examining 5-HT_{2A} receptor expression in prefrontal cortical areas. The upper panel is coronal sections at P8; The lower panel is coronal sections at adulthood P137. Obtained from Elena Demireva

The effect of PN-FLX on exploratory activity in novel environment

To test whether 5-HT_{2A} mediated the PN-FLX effect on adult emotional behavior, we compared the effect of PN-FLX on *htr2a* +/+ and *htr2a* -/- mice. These mice were generated from crossing of htr2a+/- mice to obtain a mix of wild type (htr2a+/+), heterozygous (htr2a+/-), and knockout (htr2a-/-) offspring. The offspring is the mice that are undergoing PN-FLX treatment and behavior testing. We first tested mice in the open field. We detected an overall treatment effect in total distance traveled and vertical activity; we did not detect an interaction of genotype and treatment. In comparison to vehicle-treated groups, PN-FLX treatment decreased exploratory behavior significantly as demonstrated by total distance travelled (Figure 4-3A; ANOVA p=0.0145), total

ambulatory time (Figure 4-3B; ANOVA p=0.0078) and vertical activity (Figure 4-3C; ANOVA p=0.0163). The results indicate that the effects of PN-FLX in exploratory activity were not mediated by 5- HT_{2A} receptor.

The effect of PN-FLX treatment in test sensitive to chronic antidepressant treatment

To decide whether 5-HT_{2A} receptor mediates the postnatal FLX effects on conflict anxiety behavior, we examined the effect of postnatal FLX treatment in novelty-suppressed feeding paradigm. In the NSF, food-deprived mice are presented with a familiar food pellet in a novel, brightly lit environment and the latency to approach the food and begin a feeding bout is recorded. We detected the genotype and postnatal treatment interaction (ANOVA p=0.0076). The results showed that PN-FLX treatment from P2 to P11 increased the latency to feed in WT mice compared with VEH-treated mice (Figure 4-4A; p=0.0251); while PN-FLX treatment does not produce this behavior deficit in 5-HT_{2A} +/- or 5-HT_{2A} -/-mice. In terms of percentage of weight loss, no treatment effect was detected (Figure 4-4B).

The effect of PN-FLX treatment on behavioral stress response

We also examined the effects of PN-FLX in shock escape, a paradigm that assesses behavioral responses to stress. We detected the overall treatment effect (ANOVA p=0.0001). We did not detect any postnatal treatment and genotype interaction. The results showed that PN-FLX treatment significantly increased the latency to escape across all three genotype groups (Figure 4-5A) and PN-FLX treatment does not have

effect in pre-shock activity (Figure 4-5B). This indicates that the effect of FLX in shock escape paradigm is not mediated by 5-HT_{2A} receptor.

In summary these results indicate that 5-HT_{2A} receptor mediate PN-FLX response in conflict anxiety behavior paradigms. In exploratory behavior and shock escape paradigm, PN-FLX affects adult behavior in a 5-HT_{2A} receptor independent manner.

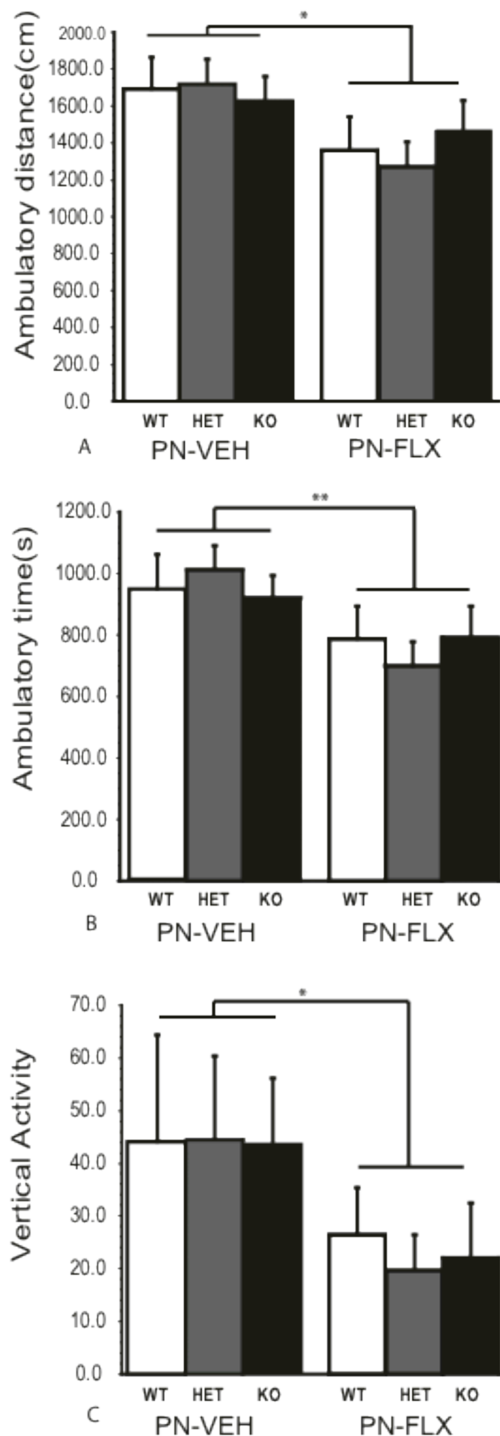


Figure 4-3. Exploratory behavior in the open field. In the open field, the following parameters were scored for 30minutes: total distance traveled (A), ambulatory time (B), and vertical activity(C). PNFLX treatment reduced ambulating time, distance, and rear activity in all three genotype groups. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with their respective controls. n = 15-20 mice per group. VEH, vehicle; FLX, Fluoxetine.

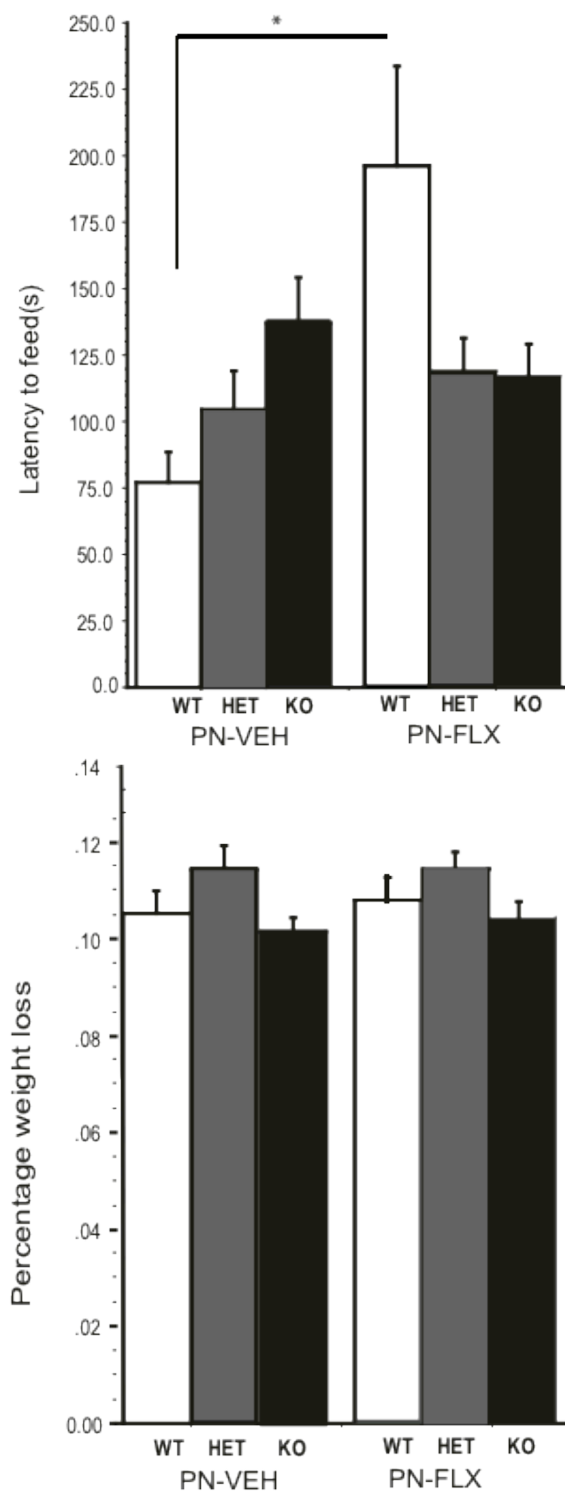


Figure 4-4. Novelty-suppressed feeding test. (A) The latency to begin feeding is shown in seconds. (B) Weight loss is expressed as a percentage of free-feeding body weight. FLX treatment during early developmental period increased latency to feed in WT mice, while

FLX treatment did not exhibit such effect in 5-HT_{2A} KO mice in comparison to VEH treated mice. n = 15-20 mice per group. *, p<0.05 compared with their respective controls. VEH, vehicle; FLX, Fluoxetine.

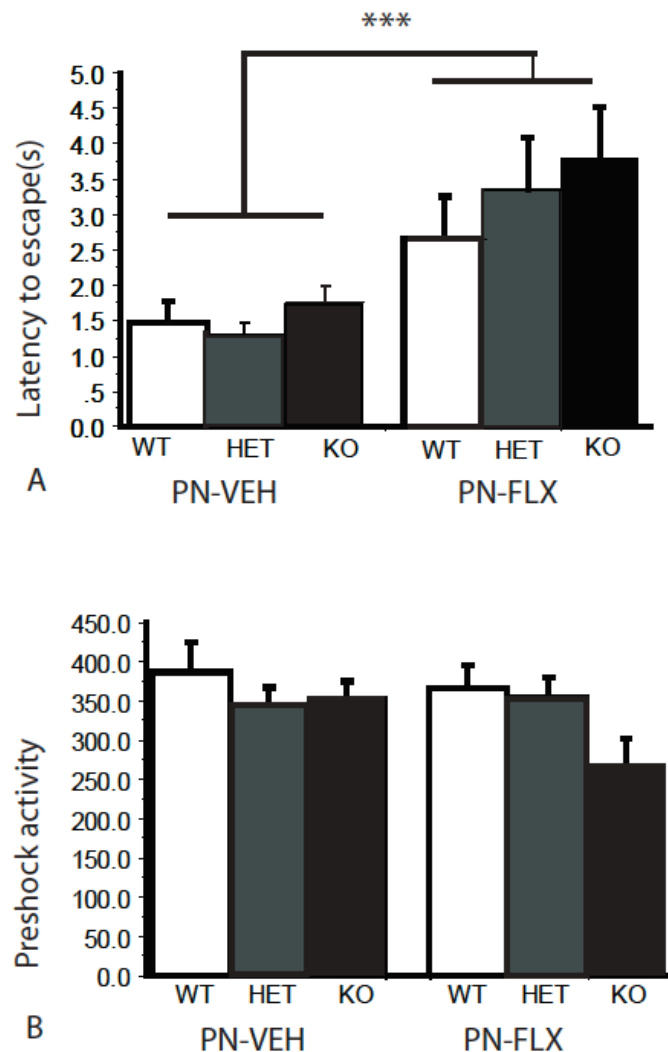


Figure 4-5. Shock escape paradigm. (A) average latency to escape a foot shock shown in seconds. (B) Locomotor activity before shocks. n = 15-20 mice per group. PNFLX increased latency to escape in all three genotype group in comparison to PNVEH controls. No treatment and genotype interaction was detected. Treatment dose not have effect on preshock locomotor activity. ***, p<0.001 compared with their PN-VEH controls. VEH, vehicle; FLX, Fluoxetine.

Discussion

This research shows that $5\text{-}HT_{2A}$ receptor is highly expressed in prefrontal areas during development as well as in adulthood. We find that blockade of $5\text{-}HT_{2A}$ receptor is sufficient to rescue some aspects (novelty suppressed feeding behavior in NSF test), but not all aspects of anxiety and depression-like behaviors (no change in helplessness behavior in SE test, no changes in exploratory behaviors in open filed). Our findings open new insights into the developmental mechanisms linking $5\text{-}HT_{2A}$ receptor with early life 5-HTT manipulations on adult emotional behavior.

The $5\text{-}HT_{2A}$ receptor is one of the most widely expressed 5-HT receptors in the brain. Several studies have indicated that $5\text{-}HT_{2A}$ receptors are important in neuronal differentiation and dendritic maturation (Lavdas et al., 1997; Azmitia, 2001). $5\text{-}HT_{2A}$ receptor is highly expressed during early postnatal period as demonstrated by previous studies (Roth et al., 1991; Volgin et al., 2003) and our own experiments, which implicates its involvement in the developmental etiology of anxiety/depression. Ablation of this receptor could potentially affect the whole serotonin system and this receptor mediated intracellular pathways, which could exert influence on physiology and behaviors.

Our experiment using *htr2a* -/- mice investigated the influence of $5\text{-}HT_{2A}$ receptor on adult affective behavior elicited by postnatal 5-HTT blockage. PN-FLX exerts no effect in $5\text{-}HT_{2A}$ -/- mice, while PN-FLX increased latency to feed in WT mice. This result suggests that PN-FLX exerts its effect through $5\text{-}HT_{2A}$ receptor in this anxiety aspect captured in novelty-suppressed feeding paradigm. Since depression is a multi-

factorial, multi-symptom heterogeneous disorder, it is unlikely that one behavior model of antidepressant action will encompass all aspects of this disease (Schmidt and Duman, 2007). Novelty-suppressed feeding (NSF) test is a conflict anxiety test that responds to chronic but not acute or subchronic, antidepressant treatments as well as to anxiolytic medications (Santarelli et al., 2003; Dulawa et al., 2004). Our own experiment in the lab has demonstrated that IL leisions in the mPFC could result in deficit in NSF test in comparison to controls, which implicates mPFC is a critical area for aspects of depression and anxiety disorders reflected in NSF test (unpublished data Tahilia Rebello). 5-HT_{2A} receptor has very strong expression in the frontal cortex. Electrophysiological studies also suggest that serotonin induced depolarization effect in the pyramidal neurons of the cortex are mediated by 5-HT_{2A} receptor during the first two postnatal weeks development (Beique et al., 2004b; Beique et al., 2004a). PN-FLX treatment will result in increased serotonergic tone; it is probable that concurrently deactivating the 5-HT_{2A} receptor in this developmental period could ameliorate this influence of increased serotonin tone on the maturation of brain structures such as mPFC or physiological functions. Additionally, it is noted that abolishment of hippocampus neurogenesis by X-ray of hippocampus suppresses behavioral responses to antidepressants in NSF test (Santarelli et al., 2003). Hippocampus lesions, particularly ventral hippocampus lesions, result in altered anxiety-like behaviors such as reduced hyponeophagia (Bannerman et al., 2002; Bannerman et al., 2003). 5-HT_{2A} receptor also has strong expression in hippocampus CA3 region (data not shown). Early-life exposure to FLX results in a retraction of the apical dendritic arbor in the CA3 hippocampus area and reduced spine number and spine density in the hippocampus region. Thus we cannot exclude the possibility that the hippocampus 5-

HT_{2A} receptor might also mediate the increased 5-HT signaling on anxiety measures. Taken together, it is probable that blockade of 5-HT_{2A} signaling counteracts the increased serotonin signaling during this early developmental (P2-P11) period, which leads to normalized behavior in this conflict anxiety behavior model.

In the open field and shock avoidance paradigm, PN-FLX has an overall effect on adult behaviors, and we did not detect genotype and treatment interaction. Open field test is a classical approach/avoidance paradigm in which the novel environment concurrently evokes both anxiety and exploration (Prut and Belzung, 2003). Shock avoidance paradigm reflects an innate helplessness. The results reflect that 5-HT_{2A} receptor deactivation is not mediating PN-FLX effect on exploration behavior and helpless behavior in adulthood. The fact that the ablation of 5-HT_{2A} receptor is not sufficient to normalize the exploratory behavior or helpless behavior deficit suggest that other receptors or neurotrophic factors or other developmental events, which are impacted by excessive 5-HT signaling during this early development, contribute to the altered emotional phenotype in open field and shock avoidance paradigm.

Considerations and remarks

Here in this study we use constitutive KO mice to investigate the developmental mechanisms by which early life 5-HTT blockade affects adult anxiety-and depression-related behaviors. Thus, it is unavoidable that some compensatory mechanisms might intervene the interpretation of the results. For future work, it is desirable to have a transgenic mouse line that could turn on or turn off 5-HT_{2A} expression in a tissue-specific and temporal manner.

Although we have shown 5-HT_{2A} receptor reverse some aspects of anxiety-and depression-like behaviors produced by early life 5-HTT blockade, the detailed mechanisms on how this affects specific intracellular signaling pathways and how this mediate the effects of serotonin on dendritic morphology and ultimately behavior, require further characterization.

In addition, other receptors, circuitry, transcription factors or a variety of molecules might be involved in the innate helpless behavior. The neurotrophin family including BDNF, NGF, and neurotrophin-3 et al plays very important roles in the development, differentiation, maintenance, and survival of neuronal populations in the nervous system (Levi-Montalcini, 1987; Barde, 1989). Chronic fluoxetine treatment can up-regulate BDNF mRNA in hippocampus (Nibuya et al., 1995; Nibuya et al., 1996). It is likely that the enhancement of neurotrophic factors will lead to normalized behaviors.
GABAergic inter neurons, or glutamate transmission might be involved in the aspects of anxiety- and depression- like behaviors. The net effect of the excitatory vs inhibitory input/output might regulate the effect of excessive 5-HT signaling on various maturation process including neuronal proliferation, migration, arborization, and integration contributing to altered behavior.

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Appendix A The role of 5-HT$_7$ receptor in adult antidepressant related behavioral response and adult neurogenesis

Introduction

SSRIs are the first line medicine for depression and anxiety disorders. SSRIs influence serotonin system to ameliorate symptoms of depression (Kent, 2000). However, SSRIs typically require chronic administration to produce beneficial effects in humans, suggesting that events downstream of serotonin signaling are ultimately altering behavior/emotion.

Structrual and functional changes associated with chronic SSRI treatments have been identified, however, there is lack of established causal relationship between these changes. The amounts of hippocampal camp response element binding protein (CREB) and adult hippocampal neurogenesis are both increased after chronic SSRI treatment. In addition, the causal relationship has been established. Blocking adult hippocampal neurogenesis (through focal irradiation or genetic ablation) abolishes the effects of SSRIs on anxiety and depression-like behaviors in rodents. Pharmacologically elevated cAMP signaling increases proliferation of progenitors and survival and maturation of adult born dentate gyrus neurons through CREB activation. Furthermore, dominant negative CREB expressed exclusively in newly generated cells reduces neuronal survival and impairs maturation (Merz et al).

Different receptors in serotonin system have been found to regulate anxiety and depression behavior. Activation of the adenylate cyclase pathway induces CREB (refs). Among 14+ 5-HT receptors which have been cloned and characterized thus far, only the 5-HT$_4$ and the 5-HT$_7$ receptors couple to Galphas. Htr7 is expressed in the entorhinal

cortex and the CA1, CA2, CA3 and dentate gyrus subfields of the hippocampus, and thus well positioned to impact neurogenesis through CREB signaling in a non cell-autonomous fashion (Bonaventure et al., 2002; Hedlund and Sutcliffe, 2004; Kvachnina et al., 2005). Furthermore, htr7 is expressed in newly born cells that proceed towards neuronal differentiation (personal communication Grigori Enikolopov), theoretically allowing for modulation of cell survival and maturation through CREB signaling. We hypothesized that 5-HT$_7$ receptor mediates the consequences of chronic SSRI treatment on CREB induction, neurogenesis activation and anxiety and depression behavior. Based on this information, we sought to investigate the involvement of the 5-HT$_7$ receptor in the cascade of events following chronic SSRI administration and the resulting reduced anxiety and depression-like behavior.

Materials and Methods

Animals

Mice were housed five per cage in a 12 h light/dark colony room at 22°C with available food and water. All experiments were performed in compliance with the institutional regulations and guidelines for animal experimentation. Mice were injected subcutaneously with fluoxetine (10mg/kg) daily for 4 weeks.

Immunohistochemistry and imaging.

Mice were anesthetized with ketamine/xylazine (100 and 7 mg/kg, respectively) and transcardially perfused (cold saline, followed by 4% cold paraformaldehyde in PBS). All brains were postfixed overnight in 4% paraformaldehyde at 4°C, then cryoprotected in

30% sucrose, and stored at 4°C. Serial sections were cut through the entire hippocampus using a cryostat and stored in PBS. Immunohistochemistry was performed in the following steps: For BrdU staining, euqilibrate with PBS containing 0.1% triton X/100 for 30 min; 30 min incubation in 2N HCl at room temperature, and 3×10 min rinse in PBS; 1 hr incubation in 0.1 M PBS with 0.1% Triton X-100, and 3% normal donkey serum. Sections were then incubated overnight at 4°C in primary antibodies for bromodeoxyuridine (BrdU; mouse; 1:100; Serotec, Oxford, UK). Biotinylated secondary antibodies were used. All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). DCX staining was done as follows: sections were rinsed in PBS, treated with 0.3% H_2O_2 in PBS for 30 min to quench endogenous peroxidase activity (and to enhance dendritic staining), incubated in 0.1M PBS with 5% normal donkey serum and 0.5% Triton X-100 for 1 hr, and then incubated overnight at 4°C in primary antibody for doublecortin (goat; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA). After secondary antibody incubation, sections were developed using Vector ABC kit and DAB kit. Stereological procedure was used to quantify labeled cells (Malberg et al., 2000).

Western blot

Brain tissues are prepared as followings: brains are dissected and put into Eppendorf tubes; tissues are added lysis buffer which is composed of 0.2M NaCl, 5nM EDTA, Glycerol 10%, NaPPi 2mM, HEPES 100mM, protease inhibitor cocktail, phosphotase inhibitor I &II cocktail, PMSF (0.5mM), NaF (2mM); homogenize tissue in ice cold lysis buffer until it is a uniform solution; centrifuge in cold room for 15 mins at

14000rpm to get supernatant A; transfer supernatant A to new set tubes on ice; repeat the last step; store sample at -80 degrees. Concentrations of final sample are measured using Pierce BCA Protein Assay Kit (Fisher Scientific). Western blot are done in the following steps: prepare the transfer buffer; add 2×loading buffer (Tris-Cl pH=6.8 100mM; SDS 4%; BPB 0.2%; Glycerol 20%; DTT 200nM); prepare a tube with molecular weight marker; boil samples in loading buffer for 5 mins at 100 degrees and spin them down; prepare running buffer (Trizma-base 25mM; Glycine 192mM; SDS 5.0g); run the gel first at a low voltage (~90V) until the dye is through the stacking gel and then it can be increased to 130-150V when samples are in the separating gel; set up the transfer equipment; run the transfer for 1 hr at constant 100V and 0.35 Amp; after 1 hr, dissemble the transfer sandwiches and place the membranes protein side up in tray and wash in water 2 x 30 sec; block membranes for 1 hr – 5% milk in TBS-T; incubate membranes in primary antibody overnight; pour off Primary Antibody solution; wash membrane 3 x 10 min in TBS-T at room temperature; add 1:5000 secondary antibody; wash membrane 3 x 10 min; develop the membrane using ECL kit (GE Healthscience).

<u>Novelty-induced hypophagia test</u>

Mice were trained to drink sweetened condensed milk for three consecutive days. Mice were presented with diluted (1:3; milk:water) sweetened condensed milk (Carnation) for 1 hour each day. Milk was presented in 10 ml serological pipettes with sippers attached with parafilm. Pipettes were closed with rubber stoppers and positioned through wire cage lids. Novel cage testing occurred when mice were placed into new clean cages of the same dimensions but without beddings with pipettes filled with

sweetened condensed milk after training days. The latency to start drinking and the amount consumed over a 30 min interval was recorded. On the following day, mice were presented a pipet with sweetened condensed milk in their homecage, and the latency to start drinking and the amount consumed over a 30 min interval was recorded. The latency to drink, and the volume consumed were recorded every 5 min for 30 min.

Novelty-suppressed feeding test

Refer to chapter II

Statistic analysis

Statistical analysis was performed using StatView 5.0 software (SAS Institute, Cary, NC). Data were analyzed using Student's t test, or two-way ANOVA with Student–Newman–Keuls posthoc testing; survival analysis, Kaplan-Meier, Logrank Mantel-Cox test as indicated. The criterion for significance for all analyses was $p < 0.05$. Results from data analyses are expressed as mean ± SEM.

Results

5-HT7 receptors are necessary for SSRIs to induce CREB in the hippocampus.

Chronic SSRI treatment has been shown to increase CREB expression (Nibuya et al., 1996). 5-HT_7 receptors couple to Gs and activation of the adenylate cyclase pathway induces CREB phosphorylation and creb expression (Meyer et al., 1993; Widnell et al., 1994). Thus, 5-HT_7 receptor activation has been hypothesized to mediate the effects of SSRI treatment on CREB induction (Duman et al., 1999).

To test this hypothesis we administered the SSRI fluoxetine (FLX, 10 mg/kg/day, drinking water) or vehicle (VEH) for 21 days to htr7+/+ and htr7-/- littermates and assessed CREB and GAPDH protein abundances in the hippocampus (HIP) using western blot analysis (Figure 2). We detected a significant interaction between genotype for CREB/GAPDH levels in the HIP ($F_{(1,27)}$=; p=0.0060). Post-hoc analysis revealed that FLX increases CREB/GAPDH levels in the HIP of htr7+/+ mice ($F_{(1,18)}$=8.884, p=0.0284). Conversely, FLX decreased CREB/GAPDH levels in the HIP of htr7-/- mice. In terms of pCREB levels, we detected a significant interaction between genotype and treatment for pCREB/GAPDH levels in the HIP ($F_{(1,27)}$=8.723, p=0.0064). Post-hoc analysis revealed that FLX dose not change pCREB/GAPDH levels in the HIP of htr7 +/+ mice, but decreases pCREB/GAPDH levels in the HIP of htr7-/- mice ($F_{(1,9)}$=15.227, p=0.0036). Taken together, these findings demonstrate that 5-HT7 receptor signaling is a necessary component for chronic FLX treatment to increase CREB levels in HIP and to keep stable levels of pCREB levels in HIP.

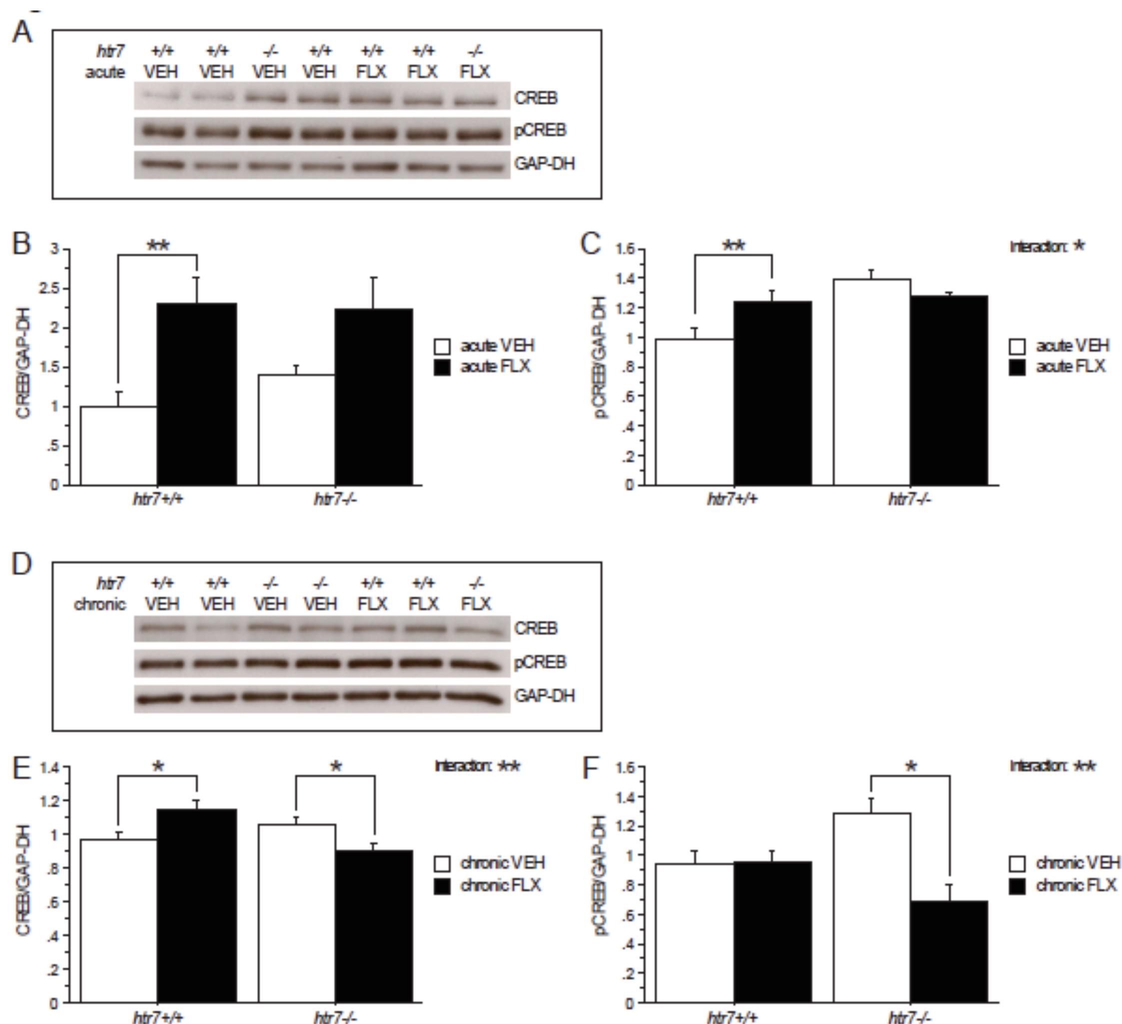

Figure A-1 5-HT_7 receptor is necessary for SSRIs to induce CREB in the hippocampus. A) Representative picture of western blot of acute fluoxetine (FLX) treatment on CREB and pCREB levels. B) Acute FLX significantly increased CREB levels in htr7+/+ mice while not in 5-htr7-/- mice in comparison to controls. C) Acute FLX significantly increased pCREB levels in htr7+/+ mice while not in 5-htr7-/- mice in comparison to controls. D) Representative picture of western blot of chronic fluoxetine (FLX) treatment on CREB and pCREB levels. E) Chronic FLX significantly increased CREB levels in htr7+/+ mice while reduced CREB levels in 5-htr7-/- mice compared with VEH controls. F) Chronic FLX did not change pCREB levels in htr7 +/+ mice while reduced pCREB levels in htr7 -/- mice in comparison to controls. n=4-7

<u>5-HT_7 receptors are not necessary for SSRIs to induce neurogenesis.</u>

We next investigated if the effects of chronic SSRI treatment on proliferation of DG progenitor cells and maturation of adult born DG neurons depend on 5-HT_7 receptor

signaling. Based on the established link between CREB signaling and adult hippocampal neurogenesis, we hypothesized that htr7-/- mice would have a blunted neurogenic response to chronic FLX treatment.

Htr7+/+ and htr7-/- mice were treated with chronic FLX (10 mg/kg/day, i.p.) or VEH for 21 days. On day 21, mice were injected with BrdU (150 mg/kg) and sacked 2h later. BrdU incorporation in the hippocampus was assessed using immunohistochemistry. We detected a main effect of FLX treatment ($F_{(1, 15)}$=15.380, p= 0.0014) and no effect of genotype or treatment X genotype ($F_{(1, 15)}$=0.295, p=0.5951; $F_{(1, 15)}$=0.128, p=0.7254 respectively) on BrdU-positive cells (Figure A-2A). Likewise, we detected a main effect of FLX treatment ($F_{(1,15)}$=13.108, p=0.0025) and no effect of genotype or treatment X genotype ($F_{(1,15)}$=0.074, p=0.7892; $F_{(1,15)}$=0.013, p=0.9096) on BrdU-positive cell-clusters (Figure A-2B). No differential effect of FLX in htr7+/+ versus htr7-/- mice was detected for either measure (Figure A-2B).

Doublecortin (DCX) is a marker of young neurons and is expressed in young neurons at 2-3 weeks of age (Couillard-Despres et al., 2005). Chronic SSRI treatment increases the amount of DCX-positive cells in the DG (Wang et al., 2008). To assess if SSRI treatment increases the amount of DCX positive cells in the DG through 5-HT7 receptor signaling, we treated htr7+/+ and htr7-/- mice with chronic FLX (10 mg/kg/day, drinking water) or VEH for 21 days. On day 21, mice were sacked and brains were stained for DCX. DCX positive cells in the hippocampus were quantified using stereology. We detected a main effect of FLX treatment ($F_{(1,14)}$=0.0075, p=0.0075) and no effect of genotype or treatment X genotype ($F_{(1,14)}$=0.752, p=0.4005; $F_{(1,14)}$=0.165,

p=0.6904 respectively) on DCX-positive cells (Figure A-2C). No differential effect of FLX in htr7+/+ versus htr7-/- mice was detected (Figure A-2C).

During the DCX-positive period, neurons undergo morphological maturation. One maturation indicator sensitive to chronic SSRI treatment is the dendritic tree complexity. Specifically, chronic SSRI treatment increases the number of DCX-positive neurons with tertiary dendrites. To assess if SSRI treatment increases the amount of DCX-positive cells with tertiary dendrites in the DG through 5-HT_7 receptor signaling, we quantified DCX positive cells with tertiary dendrites in the hippocampus of htr7+/+ and htr7-/- mice treated with chronic FLX (10 mg/kg/day, drinking water) or VEH for 21 days using stereology. We detected a main effect of FLX treatment ($F_{(1,14)}$=7.807, p=0.0109) and no effect of genotype or treatment X genotype ($F_{(1,14)}$=0.085, p=0.7731; $F_{(1,14)}$=0.411, p=0.5283) on DCX-positive cells with tertiary dendrites (Figure A-2D). No differential effect of FLX in htr7+/+ versus htr7-/- mice was detected (Figure A-2).

Taken together, our data demonstrate that 5-HT_7 receptor signaling is not necessary for SSRIs to increase proliferation of DG progenitor cells and maturation of adult born DG neurons, disproving our original hypothesis.

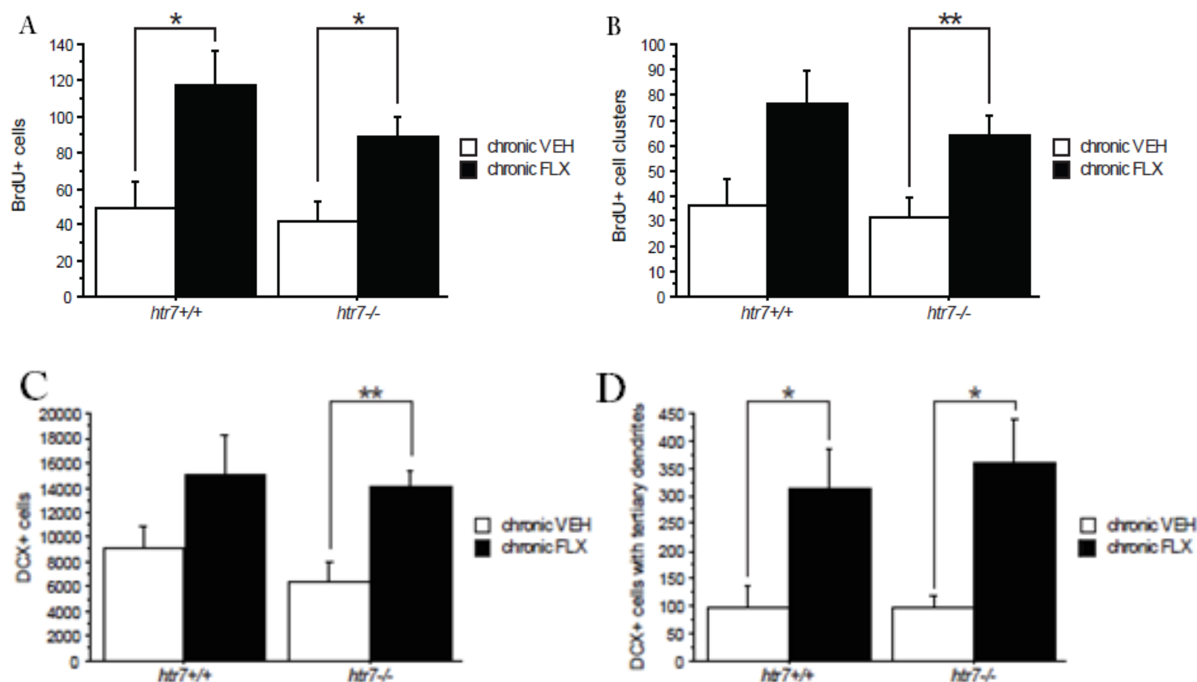

Figure A-2. 5-HT$_7$ receptors are not necessary for SSRIs to induce neurogenesis. A), B) Chronic FLX treatment increases the number of BrdU positive cells or cell clusters compared with VEH treated mice in 5-htr7 +/+ and 5-htr7 -/- mice. C) Chronic FLX treatment increases the number of DCX positive cells in 5-htr7-/- mice, and dose not change the number of DCX positive cells in 5-htr7 +/+ mice. D) Chronic FLX treatment increases the number of DCX positive cells with tertiary dendrites in 5-htr7 +/+ and 5-htr7 -/- mice. n=4-6 mice.

5-HT$_7$ receptor is not necessary for SSRIs to reduce anxiety and depression-like behavior.

We next investigated if the effects of chronic SSRI treatment on behavior depend on 5-HT7 receptor signaling. Behavior was assessed in three tests with predictive validity for chronic SSRI treatment: the NSF test and the novelty induced hypophagia test (NIH).

We treated htr7+/+ and htr7-/- mice with FLX (10 mg/kg/day, i.p.) for 21 days and then sequentially assessed behavior in the NSF and NIH. Behavioral tests were spaced by one week while FLX treatment continued. For the NSF, mice were food deprived for 24 hours and we assessed consequent body weight reduction, the latency to

start feeding in a novel arena, and homecage food consumption. In the NSF, the latency to start feeding is sensitive to chronic SSRI treatment (Wang et al., 2008). We detected main effects of treatment and genotype but no treatment X genotype interaction for the latency to start feeding ($F_{(1,88)} = 5.779$, P = 0.0183; $F_{(1,88)} = 11.332$, P = 0.0011 and $F_{(1,88)} = 0.020$, P = 0.8883 respectively) (Figure A-3). Posthoc analysis revealed that chronic FLX treatment reduced the latency to feed while htr7 ablation increased the latency to feed (Figure A-3). No effect of treatment or genotype and no treatment x genotype interaction was detected for body weight reduction ($F_{(1,88)} = 3.041$, P = 0.0847; $F_{(1,88)} = 2.959$, P = 0.0889 and $F_{(1,88)} = 0.097$, P = 0.7562 respectively) or homecage food consumption ($F_{(1,88)} = 1.479$, P = 2.271; $F_{(1,88)} = 1.077$, P = 0.3023 and $F_{(1,88)} = 3.158$, P = 0.0790 respectively) (Figure A-3).

For the NIH test, mice were first trained to drink sweetened condensed milk from a pipet. Subsequently, mice were placed into a novel environment having a pipet filled with sweetened condensed milk available. The latency to start drinking and the amount consumed over a 30 min interval was recorded. On the following day, mice were presented a pipet with sweetened condensed milk in their homecage, and the latency to start drinking and the amount consumed over a 30 min interval was recorded. In the NIH, the latency to start drinking in a novel environment and the % inhibition of sweetened condensed milk consumption are sensitive to chronic SSRI treatment (Dulawa and Hen, 2005). We detected main effects of treatment (p=0.0113) but no genotype or treatment X genotype interaction for the latency to start drinking (Figure A-4B, C). Posthoc analysis revealed that chronic FLX treatment reduced the latency to drink (Figure A-4B, C). We detected main effects of treatment and genotype but no treatment X genotype interaction

for the % inhibition ($F_{(1,61)}$ = 5.239, P = 0.0256; $F_{(1,61)}$ = 9.806, P = 0.0027; and $F_{(1,61)}$ = 0.027, P = 0.8689, respectively) (Figure A-4A). Posthoc analysis revealed that chronic FLX treatment and htr7 ablation both reduced novelty induced inhibition of sweetened condensed milk consumption (Figure A-4A).

Taken together, htr7-/- mice are fully sensitive to chronic SSRI treatment in multiple tests assessing anxiety and depression-like behavior.

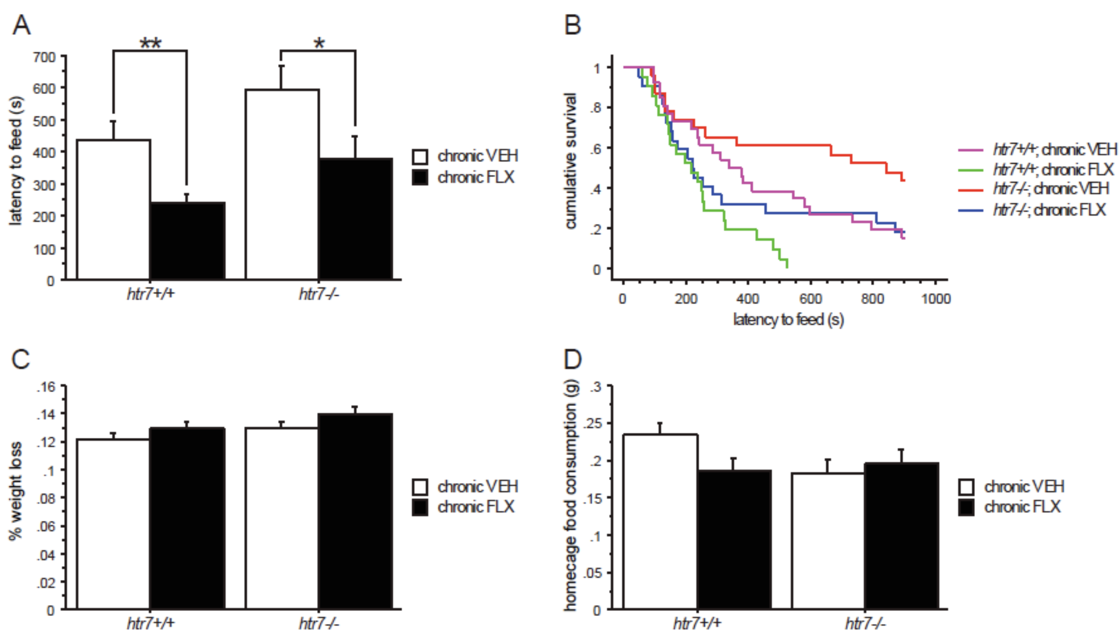

Figure A-3 Chronic SSRIs reduce latency to feed in novelty suppressed feeding test. (A), (B) Chronic FLX treatment reduced latency to feed in 5-htr7 +/+ and 5-htr7 -/- mice. No effect of treatment or genotype and no treatment x genotype interaction was detected for body weight reduction (C) and home cage food consumption (D). VEH, vehicle; FLX, fluoxetine. *p<0.05, **p<0.01

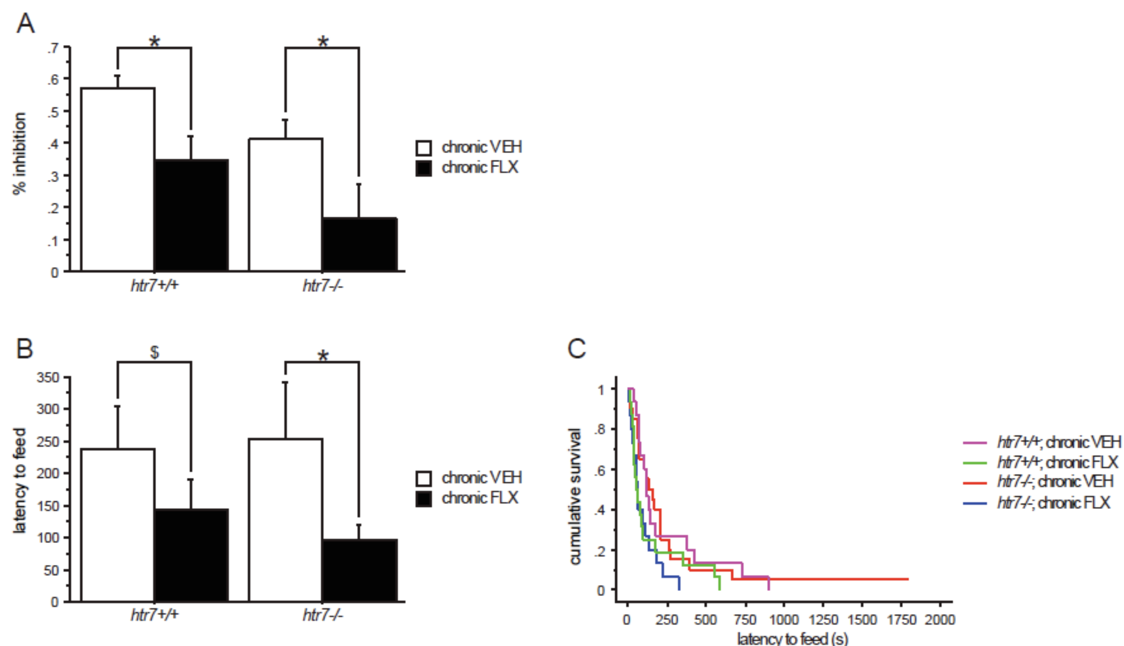

Figure A-4 Chronic SSRIs reduce anxiety-and depression-related behavior in novelty induced hypophasia test. (A) Chronic FLX treatment reduced the inhibition in 5-htr7 +/+ and 5-htr7 -/- mice. (B), (C) Chronic FLX treatment reduced the latency to feed in novel cage in 5-htr7 +/+ and 5-htr7 -/- mice. VEH, vehicle; FLX, fluoxetine. *$p<0.05$

Discussion

In the present study, we have demonstrated that chronic fluoxetine increases proliferation of immature neurons in the DG of hippocampus independent of 5-HT_7 receptor. We also demonstrate that 5-HT_7 receptor signaling is a necessary component for chronic FLX treatment to increase CREB levels in the hippocampus and to keep stable levels of pCREB levels in hippocampus, supporting the hypothesis that 5-HT_7 receptor mediates the effect of chronic antidepressants on transcription factor CREB. In assessing the behavioral consequences of the physiological actions of chronic fluoxetine, we investigated the effects of fluoxetine in the NSF test and NIH test and found that chronic fluoxetine produced anxiolytic effects in both 5-htr7+/+ and 5-htr7-/- mice.

Previous studies have demonstrated that activation of the cAMP cascade, including CREB, increases the proliferation and maturation of newborn neurons in adult mouse hippocampus (Fujioka et al., 2004). CREB is a transcription factor that is activated by its phosphorylation on Ser^{133} via cAMP-dependent protein kinase, as well as by Ca^{2+}- and neurotrophic factor-dependent signaling pathways. cAMP-CREB cascade exerts a positive influence on neuronal cell proliferation in the hippocampus (Nakagawa et al., 2002). In our study, increased neurogenesis is observed concurrently with reduced CREB or the active form of CREB (pCREB) expression, which implicates that CREB induction is not necessary for hippocampus neurogenesis. cAMP-CREB cascade is one of the key signal transduction pathways for regulation of cell proliferation and differentiation, however, other conditions such as Neurotrophic factors could directly regulate hippocampus neurogenesis.

Behavioral characterization of mice lacking 5-HT_7 receptor has been inconclusive. Previous studies using light-dark box paradigm show no significant differences between 5-HT7+/+ and 5-HT7−/− mice in C57BL/6J background in either the time spent in the light compartment or the number of transitions between the light and dark compartments (Roberts et al., 2004). Another strain of 5-HT7-/- mice in mixed129SvEV/C57BL/6J background was tested in elevated pluz mace and there was no difference in the time spent exploring the open arms or in the number of entries onto the open arms of the maze in both genotypes (Guscott et al., 2005). In our own experiments, we get mixed results. In novelty suppressed feeding test, we see increased latency to feed in 5-hrt7 -/- mice, which is indicative of increased anxiety level; in novelty induced hypophasia test, we observed reduced novelty-induced inhibition of sweetened condensed milk consumption in 5-htr7-

/- mice, which indicates reduced anxiety level. One thing that needs to be noted is that novelty suppressed feeding test requires food deprivation 24 hours before testing, thus we cannot exclude the possibility that the experiment results could be confounded by hunger level, motivational conditions et al. 5-ht7 receptor blockade by antagonist has shown consistent result: antagonist induce anxiolytic effect in rats and mice (Wesolowska et al., 2006a; Wesolowska et al., 2006b). Multiple conditions might explain such discrepancies: life-long genetic blockade might produce more dramatic and persistent changes, or develop some compensatory mechanisms to cope with the ablation in comparison to acute antagonist treatment; the genetic background might influence the behavior readout. Taken together, this shows the complex picture of 5-HT_7 receptor in mediating anxiety-related behaviors.

Our experiment results showed that 5-ht7 receptor is not required for chronic fluoxetine induced hippocampus neurogenesis and anxiolytic/antidepressant behavioral response. It is possible that other receptors might mediate this response. For example, 5-HT_{1A} receptors are generally thought to be involved in modulating both anxiety and depression-related behavior. It is shown that a 5-HT_{1A} autoreceptor levels in adulthood, prior to antidepressant treatment, has close relevance to the responsiveness to fluoxetine (Richardson-Jones et al.). It is possible that 5-HT_{1A} autoreceptor might mediate this chronic fluoxetine behavioral response. The fact that 5-HT_7 receptor is not necessary for the effect of adult chronic FLX treatment on anxiety-and depression-like behaviors might point to that 5-ht7 receptor might play more important roles in other aspects like learning and memory.