Serotonin Modulates the Maturation of the Medial Prefrontal Cortex and Hippocampus: Relevance to the Etiology of Emotional and Cognitive Behaviors

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ABSTRACT

Serotonin Modulates the Maturation of the Medial Prefrontal Cortex and Hippocampus: Relevance to the Etiology of Emotional and Cognitive Behaviors

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Increasing evidence suggests that anxiety and depression disorders have neurodevelopmental roots, with a strong case for early-life serotonergic dysfunction in their ontogeny. For instance, disrupting serotonergic tone during critical developmental periods results in dysregulated adult affective behaviors, in mice. However, insight into the mechanism by which early-life serotonin levels impact emotional behaviors in later-life, remains scarce. Based on its potent neurotrophic properties, we hypothesized that serotonin acts during development to guide the maturation of brain regions implicated in the modulation of both the emotional and cognitive features of anxiety and depression disorders, namely the medial prefrontal cortex (mPFC) and hippocampus (HC).

To address this hypothesis, we employed a pharmacological mouse model in which serotonin levels were increased using a selective serotonin reuptake inhibitor, fluoxetine (FLX), during a previously established critical post-natal time window (post-natal day 2-11; "PN-FLX" mouse model). The effect of this post-natal serotonergic disruption on the structural, physiological and functional properties of the adult mPFC and HC was assessed. In the mPFC, we found morphological and electrophysiological changes specific to cortical layer 2/3. Pyramidal neurons in layer 2/3 of the infralimbic (IL) sub-region of the mPFC, displayed decreased dendritic arborization, concomitant with reduced intrinsic excitability. Conversely,

prelimbic (PL) layer 2/3 neurons displayed normal dendritic arborization, but increased intrinsic excitability. Together, these changes produce a reduced IL/PL output ratio. In order to probe the functional consequences of these changes, we investigated the ability of PN-FLX mice to extinguish learned fear ("fear extinction"), as performance in this behavioral paradigm is modulated by IL/PL output. Congruent with their altered IL/PL balance, PN-FLX mice display deficits in fear extinction learning and recall. Mimicking the diminished IL/PL ratio by selectively lesioning the IL, in control mice, phenocopied some features of the PN-FLX anxiety and depression-behavioral profile, demonstrating a causal relationship between mPFC changes and the emotional phenotype of PN-FLX mice.

In the HC, PN-FLX treatment produced retraction of dendritic arbors but increased branching in the cornu ammonis 3 (CA3) sub-field. PN-FLX treatment also reduced total synapse number and synaptic density of CA3 neurons. In order to probe the functional consequences of these morphological changes, we investigated spatial learning in the Morris water maze and contextual fear conditioning in PN-FLX mice, as these behaviors are sensitive to CA3 manipulations. In line with reduced CA3 function, PN-FLX mice displayed deficits in both these learning paradigms.

Taken together, our findings demonstrate that serotonin acts during a key developmental epoch to set the structural and physiological properties, and ultimately the functional output, of two key brain regions that underlie affective and cognitive behaviors, the mPFC and HC. Our findings provide a mechanism by which reported genetic (polymorphisms, mutations) and environmental factors (exposure to pharmacological agents or stress) that alter serotonergic tone during development, have long-term, often indelible, consequences on adult affective and cognitive function.

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Dedication

This thesis has been written in the fondest memory of my cousin Anthony Colaco, who left us prematurely but whose lovely smile, innocence and spirit will remain with us always.
You were, and are, loved…

I also dedicate this work to my dear aunt and cousin, Evette and Anabelle Colaco, who have had to endure an unfair share of adversity. May the coming days (over)compensate for all the storms of the past and shower you instead with contentment, peace and, with time, sincere joy.

Acknowledgments

I have immense gratitude for all those who have supported and encouraged me, or simply walked beside me, through the last six years of this tumultuous yet enriching journey they call "graduate school". A huge thanks to Mark, Jay and my entire lab (namely Annette, Jeff, Qing and Elena) for providing me with mentorship, insight, inspiration, laughter and friendship. To my dearest friends and extended family scattered throughout the world, and also those here in NY (especially my grandma Juliet, my aunt Anne, Julia, Kathy, Priscilla, Minji, Da-In, Christine and Elena)—I value your friendship tremendously and hope I can give back even 30% (+/- 5) of all that you have given me.

An immeasurable thank you to my wonderful family—my father, Augustine, who always makes me feel safe and incredibly cherished, my mother Tessie who has taught me to be strong and positive and whose love I feel even from 3000 miles away, and to my brother Ayesaan who

is one of the best and kindest human beings I have ever met (…just not always to his little sister….j/k =). I have everything because I have the three of you.

Last but not least, I would like to acknowledge the incalculable support provided by the following: The Columbia Wine company, Advil®, The Peter Griffin family, my Asics running shoes, U.S. Immigration services for not deporting me back to Canada, my bike, the Columbia hiking club, Bard Hall Players, the Rom-Com genre, and of course the wonderfully maddening city of New York.

CHAPTER 1: Introduction

1.) Anxiety and Depression Disorders

a.) Clinical presentation and epidemiology

Anxiety, in its non-pathological form, is a natural response to threatening or fearful circumstances. In this sense, anxiety can be perceived as an evolutionarily beneficial phenomenon as it mobilizes the physiological systems that enhance arousal, vigilance and preparedness, all of which aid in dealing with, or escaping from, potential threats ("fight or flight response") (Nutt, 2005). An increase in the intensity and duration of anxiety yield its pathological form. In this form, anxiety becomes maladaptive and is considered a disorder (Nutt and Ballenger, 2003; Nutt, 2005). Anxiety disorders are the most pervasive of the psychiatric maladies with a lifetime prevalence of 31.2% in the United States (Kessler et al., 2005), and are classified into five general categories: obsessive-compulsive disorder (OCD), panic, (social and simple) phobias, post-traumatic stress disorder (PTSD) and generalized anxiety disorder (GAD) as specified in the *Diagnostic and Statistical Manual of Mental Disorders, 4th edition, Text Revision* (DSM-IV-TR, 2000). The unifying symptom of all categories of anxiety disorders is excessive uncontrollable worry and fear, often in response to non-threatening, nebulous, or no specific stimuli (Nutt, 2005). Patients, such as those with PTSD, often have a disproportionately high fear response to environmental cues that serve as reminders of their initial traumatic experience (Heim and Nemeroff, 2009). Without intervention, these triggered fear responses tend to stay consistently high throughout a patient's lifetime (Heim and Nemeroff, 2009). Additional somatic and psychological symptoms

characteristic of pathological anxiety include increased heart rate, sweating, nausea, sleep disturbances and avoidance and/or helpless behaviors in response to fearful and anxiety-provoking situations (DSM-IV-TR, 2000; Nutt and Ballenger, 2003). Excess stress is another key characteristic and/or consequence of anxiety disorders which may also participate in its etiology, as will be described in subsequent chapters (Kendler et al., 2003; Nutt and Ballenger, 2003; Lupien et al., 2009). Cognitive symptoms such as memory and attention deficits and diminished cognitive flexibility are also frequently described in anxiety disorder patients (Castaneda et al., 2008; Gualtieri and Morgan, 2008).

As with anxiety, mood disturbances associated with depression lie on a spectrum. Short-lived, mildly intense feelings of sadness may be normal responses to adversity, but when recurrent, enduring or intensified, indicate a psychiatric disorder (DSM-IV-TR, 2000). Depression has been categorized into several classes including major depressive disorder (MDD), dysthymic disorder and minor depression (DSM-IV-TR, 2000). Further sub-typing within these classes exists based on symptom and origin specificity such as hormonal changes, aging, seasonal variations, psychotic, manic or catatonic features, injury and co-morbid neurobiological disorders (DSM-IV-TR, 2000, Ninan and Berger, 2001). MDD is one of the severest forms of unipolar depression and is associated with negative affect, pessimism, hopelessness and a loss of interest in formally pleasurable activities (anhedonia), concomitant with changes in appetite, sleep patterns and energy levels (DSM-IV-TR, 2000, Ninan and Berger, 2001). The lifetime prevalence of a mood disorder in the US is about 20% with the probability of having a depressive episode being around 5% (Kessler et al., 2005).

Although anxiety and depression can be considered distinct phenomena, they are highly co-morbid (Kendler et al., 1992; Kaufman and Charney, 2000; Ninan and Berger, 2001; Hettema et al., 2006). The *National Co-morbidity Survey Replication* (NCS-R, 2001-2003) found that in the United States, almost 50% of patients with MDD also suffered from an anxiety disorder, and the lifetime prevalence of a co-morbid anxiety/depression disorder was around 28% (Kessler et al., 2005; Khan et al., 2005). Factor analyses showed a similar high correlation between GAD and MDD (Kendler et al., 1992; Hettema et al., 2006). Underscoring the co-morbidity between these two disorders is the finding that pharmacological agents used to treat one disorder often alleviate the symptoms of the other (Kaufman and Charney, 2000; Blier, 2003; Nutt et al., 2005; Ravindran and Stein, 2010). For instance, the frontline class of anti-depressant drugs, known as the selective serotonin reuptake inhibitors (SSRIs), have been used efficaciously to treat various subtypes of anxiety disorders including OCD, PTSD and GAD (Ninan and Berger, 2001; Ravindran and Stein, 2010). There is also significant overlap in both the genetic risk and symptomatology of the two disorders (Kendler et al., 1992; Roy et al., 1995; Ninan and Berger, 2001). For instance, GAD and PTSD patients often display depressive symptoms such as negative affect, helplessness and anhedonia as well as many of the cognitive deficits characteristic of mood disorders (DSM-IV-TR, 2000; Kaufman and Charney, 2010). Taken together, these findings suggest that anxiety and depression-related behaviors may be mediated by similar neurocircuits and may have commonalities in their etiologies and pathophysiology.

b.) Pathophysiology

Although studies in humans have provided some insight into the neuropathology of anxiety and depression, studies in animals, such as rodents, whose neurobiological systems are highly homologous to those of humans (Tarantino and Bucan, 2000; Cooper et al., 2003; Strand et al., 2007), have greatly advanced our understanding. Additionally, the presence of pharmacologically validated tests to gauge anxiety and depression behaviors in rodents (Fig A.4 – A.6) and the high amenability of these species to genetic and other experimental interventions, makes them useful models to study the pathology of emotional dysfunction (Kalueff and Tuohimaa, 2004). As such, this review will present data from both human and animal studies.

c.) Dysregulated neurocircuits

As anxiety and depression are multifaceted disorders with diverse symptomatology, it is not surprising that they are associated with dysfunction in multiple brain regions. This review will focus primarily on the alterations in corticolimbic regions/circuits as they are the most consistently altered in depression and/or anxious states (Nestler, 2002; Drevets et al., 2008; Price and Drevets, 2010), most comprehensively studied, and the focus of the current body of work.

Putative anxiety/depression circuits were described as early as the late nineteenth century but began to really coalesce in the mid-1900s with the work of such scientists as Papez and Maclean, who proposed the existence of an interconnected network of forebrain and subcortical structures (collectively described as the "*limbic system*" or

"*Papez circuit*" in its antiquated form) which regulated emotional responses (Andersen et al., 2006; Baars and Gage, 2007; Price and Drevets, 2010). Notable structures include the prefrontal cortex (PFC), hippocampus (HC) and amygdala. Extant data in humans and rodents supports the involvement of these regions in emotional regulation, as depression and anxiety-like states are often associated with structural, physiological and functional abnormalities in these areas (Drevets et al., 2008; Price and Drevets, 2010). For instance, in the medial prefrontal cortex (mPFC) changes in gray-matter volume, dendritic morphology, receptor expression, theta power and functional connectivity to lower limbic structures are found in human patients and rodent models of affective disorders (Pezawas et al., 2005; Gordon et al., 2005; Altamura et al., 2007; Bhansali et al., 2007; Wellman et al., 2007; Adhikari et al., 2010). Similar associations between anxiety/depression and abnormal structure and physiology of the HC have been observed. For instance, volume reductions, decreased neuronal metabolism, viability, proliferation, theta power and receptor expression in the HC of depressed patients/rodent models have been well documented (Sheline et al., 1996; Reagan and McEwen, 1997; Bhansali et al., 2005; Czeh and Lucassen, 2007; Sahay and Hen, 2007; Taylor Tavares et al., 2007; Price and Drevets, 2010). Interestingly, pro-depressive and anxiogenic factors such as the maternal separation, learned helplessness and chronic stress paradigms in rodents, yield similar mPFC and HC phenotypes (Sunanda et al., 1995; Sousa et al., 2000; Cook and Wellman, 2004; Kole et al., 2004; Radley et al., 2005; Izquierdo et al., 2006; Hajszan et al., 2009; Farrell et al., 2010; Magariños et al., 2011). Conversely, diverse therapeutic regimens, such as treatment with SSRIs, deep brain stimulation or electroconvulsive therapy reverse some of these abnormal structural and functional features in the mPFC and HC (Mayberg

et al., 2003; Ansorge et al., 2007; Sahay and Hen, 2007; Price and Drevets, 2010). These data suggest that the structural/physiological changes in the mPFC and HC may be causal to at least some aspects of the depressive/anxious phenotype.

The mPFC and HC also have well characterized impacts on cognitive functions such as mnemonic processes, various types of learning, information processing and attention (Moser and Moser, 1998; Bannerman et al., 2004; Nachev et al., 2005; Bars and Gage, 2007). As such, dysregulation in these regions may also account for the cognitive deficits commonly observed in patients. Moreover, these regions have profuse reciprocal connections to the amygdala, a structure that has been shown to be a major processor of fearful stimuli and a mediator of fear-induced behaviors (LeDoux et al., 1988; Milad and Quirk, 2002). Cortico-amydala connectivity and its role in responding to anxiety-provoking and fearful stimuli has been comprehensively characterized and alterations in functional connectivity between these regions are found in human subjects with, and rodent models of, anxiety and depression disorders (Milad and Quirk, 2002; Pezawas et al., 2005; Quirk et al., 2006; Hariri et al., 2006; Price and Drevets, 2010). HC-amygdala connectivity also plays an important role in the integration of cues (contextual, auditory, and olfactory) in the process of learned fear, which might be particularly relevant to some subtypes of anxiety such as PTSD (Phillips and LeDoux, 1992; Fanselow, 2000; Maren, 2008). These data reinforce the strong correlation between mPFC and HC dysfunction and emotional dysregulation.

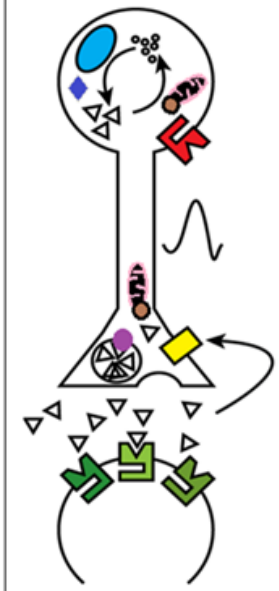
d.) Dysregulated neurosystems

Changes in various brain systems have been implicated in the pathophysiology of anxiety and depression disorders (Nestler et al., 2002; Mann and Currier, 2006). Alterations in the hypothalamic-pituitary-adrenal (HPA) axis, variations in growth factors and changes in glutamate, γ-amino butyric acid (GABA), cannabinoid, opioid, dopaminergic and noradrenergic transmission have all been implicated in some aspect of the neuropathology of affective disorders (Leonard, 1997; Sibille et al., 2000; Park et al., 2005; Nestler, 2002; Paul and Skolnik, 2003; Ansorge et al., 2007; Hill et al., 2007; Dobson and Dozois, 2008; Peters et al., 2011). Perhaps the most comprehensive studies, however, have been done on the involvement of the neurotransmitter serotonin in regulation of anxiety, fear and depressive states (Hirschfeld, 2000; Nutt, 2002). Serotonin, also known as 5-hydroxytryptamine (5-HT), is part of the monoamine family of neurotransmitters and, as its chemical name and classification suggests, is biochemically derived from the essential amino acid tryptophan via sequential metabolic reactions (Fig. A.1). Evidence for the involvement of serotonin in mood disorders has been especially robust with both genetic and pharmacological data underscoring its important role in regulation of affective states (Hirschfeld, 2000; Nutt, 2002; Gaspar et al., 2003; Mann and Currier, 2006; Ansorge et al., 2007). Genetically, there is evidence that polymorphisms/mutations in genes encoding various elements of the serotonin system, such as transcriptional factors that specify serotonergic fate, enzymes in the 5-HT synthetic or degradatory pathway, 5-HT receptors and 5-HT reuptake transporters impact emotional behaviors (summarized in Fig. A.1; Gaspar et al., 2003; Sodhi and Sanders-Bush, 2004). For instance, variations in expression, or complete loss of, the serotonin

reuptake transporter (5-HTT) or the inhibitory 5-HT1A receptor have been associated with enhanced vulnerability to anxiety and depression-like behaviors, and treatment response, in both humans and mice (Lesch et al., 1996; Gutierrez et al., 1998; Caspi et al., 2003; Gross et al., 2002; Lira et al., 2003; Richardson-Jones, 2011). Additionally, many of the early-life genetic and environmental states that promote the development of depressive and anxious symptoms in adulthood typically involve changes in the serotonin system. For instance, animals that are exposed to the learned helplessness and maternal separation paradigms display enhanced anxiety/depression-like behaviors with concomitant changes in 5-HT release, receptor expression and 5-HT signaling in brain regions associated with the mediation of affective behaviors, such as the mPFC and HC (Meaney et al., 1994; Smythe et al., 1994; Petty et al., 1994; Inoue et al., 1994; Kaufman et al., 2000; Bland et al., 2003; Vicentic et al., 2006; Bhansali et al., 2007; Goodfellow et al., 2009; Benekareddy et al., 2010). Pharmacologically, drugs that target the serotonin system, such as 5-HTT inhibitors (the SSRIs) have anti-depressant and anxiolytic effects, while tryptophan and 5-HT depletion tend to be pro-depressive (Neumeister, 2002; Sodhi and Sanders-Bush, 2004; Fernandez and Gaspar, 2012). Firing rates of serotonergic neurons may also modulate emotional states, as reduced firing of these neurons is often observed in animal models of depression and anxiety (Kinney et al, 1997; Lira et al., 2003), while enhancement of firing partly mirrors onset of therapeutic efficacy of anti-depressant drugs (Blier, 2003). Cumulatively, these findings underscore the role of serotonergic transmission in regulating anxiety/depression behaviors.

Apart from its function in the *adult* brain as a neurotransmitter regulating various psychiatric states, serotonin may influence adult emotional behaviors in an alternate way,

by acting as a potent neurotrophic factor during development and guiding the formation of the previously described cortico-limbic circuits underlying anxiety and depression behaviors. This unique role for serotonin is the focus of the current work and will be discussed in greater detail in the following sections.



Component of the 5-HT system	Effect of depletion, variations or loss	References
tryptophan	depressogenic	in some, but not all studies. Reviewed in Neumeister, 2003
TPH-2	increased suicidality, depression	Zill et al, 2004; Zhou et all, 2005
AADC		
5-HT	depressogenic	in some, but not all studies. Reviewed ir Neumeister, 2003
MAO-A	increased fear, reduced exploration	Tadic et al, 2003;Guitierrez et al, 2004
mitochondria		
5-HT1A-R	increased anxiety and depression dysregulated stress and fear responses	Klemenhagen et al, 2006, Gross et al, 2001; Richardson-Jones et al, 2011; Reviewed in Olivier et al, 2001
vesicle containing 5-HT		
VMAT2	increased depression and anxiety (in heterzygotes)	Fukui et al, 2007; Taylor et al, 2009
5-HTT	increased anxiety and depression dysregulated stress and fear responses	(Lira et al, 2003; Holmes et al, 2007; Wellman et al, 2007; Muller et al, 2011)
postsynaptic 5-HT-R		

Fig. A.1. The serotonergic system. Serotonin (5-HT) is produced from the amino acid tryptophan via two sequential steps catalyzed by tryptophan hydroxylase 2 (TPH-2) and amino-acid decarboxylase (AADC). 5-HT is packaged into synaptic vesicles by the vesicular monoamine transporter-2 (VMAT2), and released 5-HT is bound by both post-synaptic 5-HT receptors (in green), but also by auto-inhibitory 5-HT1A receptors (5-HT1A-R). Re-uptake of synaptic 5-HT is mediated by the serotonin transporter (5-HTT). After reuptake, 5-HT may be recycled, or metabolized by the mitochondrial enzyme monoamine oxidase-A (MAO-A). (Figure modified from Ansorge et al., 2007; Gaspar et al., 2003; Sodhi and Sanders-Bush, 2004).

e.) Etiology

Although there are several psychosocial and biological models that attempt to elucidate the origins of emotional disorders, all of them concede that there is both a genetic and environment component in the vulnerability to psychiatric conditions (Torgensen 1986; McGuffin et al. 1991; Kendler et al. 1993; Bierut et al. 1999; Sullivan et al. 2000; Levinson et al., 2006; Kendler et al., 2011; Hettema et al., 2001). Genetic factors contribute vulnerability towards the acquisition of affective disorders, but those with susceptibility genes do not necessarily develop frank anxiety or depression (Caspi et al., 2003; Levinson et al., 2006). Since an organism's behavioral repertoire is guided by both the underlying genes that sculpt the circuits/systems modulating behavior, but also by the unique molecular and psychological terrain they encounter during their lifetime, it is unsurprising that models taking into account both these factors are the most comprehensive. One such paradigm is the *stress-diathesis model* which highlights the gene by environment (G x E) interaction in the manifestation of affective disorders (Monroe & Simons, 1991; Gross and Hen, 2004). A specific example of this interaction is found in epidemiological data of human carriers of the short or "*s*" allele of the *5-htt* (*slc6A4*) gene, which results as a consequence of an insertion/deletion polymorphism in the promoter region of the *5-htt* gene. Human "*s*" allele carriers display an underlying vulnerability to adult emotional dysfunction. However, this dysfunction manifests itself primarily with exposure to traumatic experiences, such as childhood maltreatment, with a demonstrable dose-response effect both in terms of number of "*s*" alleles (homozygotes have enhanced reactivity to stressful life experiences compared to heterozygotes) and

also in terms of dosage of stress exposure (positive correlation between probability of depression/anxiety and number of stressful life events).

An important question is *how* genetic and environmental factors influence vulnerability. One model suggests that they influence the formation and/or function of brain regions or neurosystems that modulate emotional behaviors (Ansorge et al., 2007; Leonardo and Hen, 2009; Daubert and Condron, 2010). Since circuitry is fundamentally set early in development (although some plasticity may be retained throughout life) it is conceivable that these factors, acting in development, could influence the maturation of circuits thereby permanently altering their function. Thus, a germane next question is *when* are these factors acting to influence vulnerability? This question is especially pertinent when considering the existence of sensitive or critical periods in brain development (Knudsen, 2004; Hensch, 2004). If, for instance, the impact of the genetic changes or environmental perturbations occur during these highly plastic critical time windows, it is possible for the developmental trajectory of the brain to be altered in a long-lasting and permanent fashion (Knudsen, 2004). These critical time windows have been thoroughly characterized in the development of sensory circuits such as those mediating auditory cue learning, filial recognition, and vision. Whether or not similar critical periods exist in the development of emotional circuits is unclear, however as the following section will depict, there is evidence that they do. This G x E x *time* interaction, as it relates to affective disorders, is best captured by the neurodevelopmental hypothesis on the origins of pathological anxiety and depression.

2.) Neurodevelopmental Origins

Increasing evidence suggests that adult psychiatric conditions have their roots in developmental perturbations, with a particularly strong case for the presence of critical time windows in the development of circuits underlying depression and pathological anxiety (Gross et al., 2002; Gingrich et al., 2003; Ansorge et al., 2007; Leonardo and Hen, 2009). Initial work in the field revealed that, in humans, early-life anxiety levels are strong predictors of vulnerability to both anxiety and depression later in life (Breslau et al., 1995; Parker et al., 1999; Weissman et al., 2005). Exposure to either enriched or adverse environmental factors in early life can, respectively, decrease or enhance an organism's vulnerability to affective and fear-related disorders in adulthood (Kendler et al., 1992; Kessler et al., 1997; Bulik et al., 2001; Caspi et al., 2003; Moffitt et al., 2007). Moreover, many psychiatric disorders often have an early onset. For instance, according to the National Institute of Mental Health, almost 50% of lifetime cases of mental illness begin on average at age 14, with anxiety disorders having a median age of onset of 11 (Kessler et al., 2005). Taken together, these findings suggest that the circuits regulating affective and fear behaviors are established early on and are highly sensitive to perturbations occurring during their formation.

a.) Involvement of Serotonin

Work investigating the neurotransmitter pathways regulating the formation of anxiety, depression and fear circuits has implicated the serotonin (5-HT) system. Insight into this idea has come from findings in humans with the previously mentioned lower-expressing "*s*" allele of the *5-htt* gene, who have enhanced vulnerability to emotional

dysfunction in adulthood, especially with exposure to adverse life-events (Lesch et al., 1996; Caspi et al., 2003). This behavioral phenotype was also found in *5-htt -/-* mice, an extreme rodent model of the human "*s*" polymorphism, that underwent testing in pharmacologically validated behavioral paradigms used to gauge anxiety/depression behaviors in rodents (Lira et al., 2003; Holmes et al., 2003; Olivier et al., 2008; Muller et al., 2011). The behavioral profile of human "*s*" allele carriers and *5-htt -/-* mice was contrary to what was expected based on the fact that pharmacological inhibition of 5-HTT (via treatment with SSRIs such as fluoxetine; FLX) has anti-depressant/anxiolytic effects (Gingrich et al., 2003; Ansorge et al., 2007; Leonardo and Hen, 2009). One key difference between drug-induced 5-HTT inhibition during adulthood versus having reduced (or lacking) the 5-HTT protein throughout life, is that in the genetic conditions, 5-HTT hypofunction is present during key developmental periods, whereas the pharmacological treatment circumvents the developmental period, and is instead applied to a mature substrate –the developed adult brain. A hypothesis that emerged to reconcile these paradoxical findings was that the dysregulation of the serotonin system that arises from genetic 5-HTT hypofunction likely precludes the proper development of brain circuits that underlie these behaviors (Gingrich et al., 2003; Ansorge et al., 2004; Ansorge et al., 2007; Homberg et al., 2010). This hypothesis is contingent on the potent neurotrophic properties of serotonin and its demonstrable impact on brain maturation (Fig. A.2).

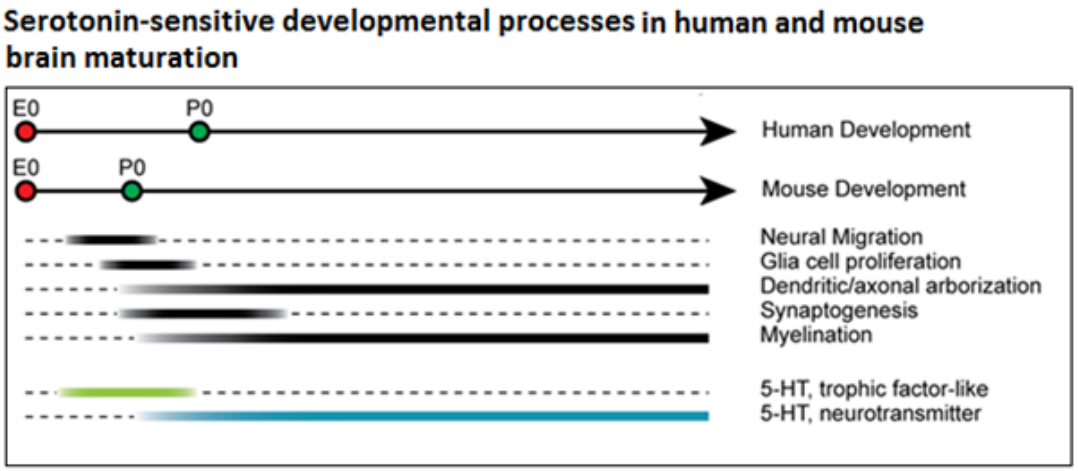


Fig. A.2 In early life, 5-HT acts as a potent neurotrophic factor influencing various maturation processes that comprise brain development, in both humans and mice (based on Giedd et al., 1999; Gaspar et al., 2003; Tau and Peterson, 2010).

The putative mechanism by which 5-HT influences brain development is presented in Fig. A.3. Briefly, serotonin interacts with, and can affect the expression of, various elements important in the highly orchestrated series of events comprising brain development (Gaspar et al., 2003). These elements include transcription factors in mitogenic pathways, signaling molecules, growth factors (such as brain-derived neurotrophic factor and S100β), axon-guidance molecules such as netrin-1, cytoskeletal proteins, synaptic proteins and various receptor types (Whitaker-Azmitia et al., 1996; Gaspar et al., 2003; Sodhi and Sanders-Bush, 2004; Homberg et al., 2010). Through these diverse relationships, 5-HT influences various maturation processes including neuronal proliferation, migration, axonal/dendritic arborization and synaptogenesis (Gaspar et al., 2003). As a consequence, proper serotonergic tone needs to be conserved while these

developmental processes are underway in order to avoid aberrant maturation of brain circuits, such as those mediating affective behaviors. Complementarily, perturbations of serotonergic tone during these critical periods could permanently alter the formation of these circuits such that they impact adult emotional behaviors. As such, the paradoxical anxiety and depression-like behavioral phenotype of *"s"* allele carrier and *5-htt* -/- mice may be due to the abnormal formation of affective circuitry, due to the altered serotonergic tone they experienced during key developmental windows. In line with this hypothesis, disrupting serotonergic tone strictly during a critical developmental time window would be sufficient to phenocopy the emotional phenotype of *5-htt* -/- mice and human *"s"* allele carriers.

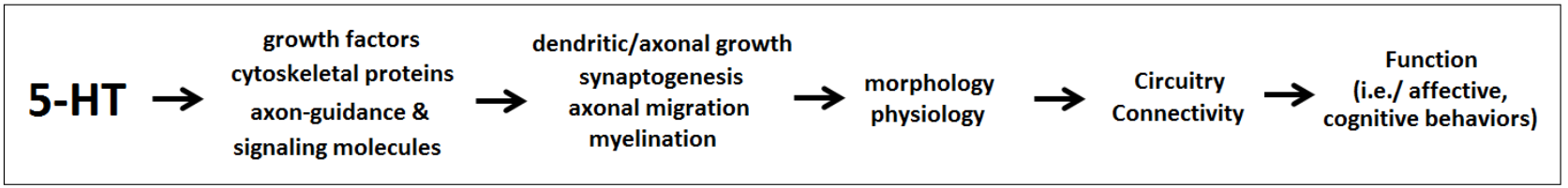


Fig A.3. Mechanism by which early-life 5-HT levels influence the maturation of the brain (Azmitia and Whitaker-Azmitia, 1997; Gaspar et al., 2003; Sodhi and Sanders-Bush, 2004)

b.) Testing the neurodevelopmental hypothesis: Post-natal FLX mouse model

Part 1

The preceding hypothesis was tested directly in our lab in two parts. First, we determined whether altering serotonergic tone during a critical developmental time window would be sufficient to yield abnormal adult emotional behaviors and reproduce the phenotype of *5-htt -/-* mice and *"s"* allele carriers. To this end, we employed a pharmacological mouse model in which 5-HTT was inhibited via FLX treatment, during various temporally restricted periods in post-natal development ("PN-FLX mouse model), and the outcome of this blockade on adult behaviors was examined. The post-natal period was selected as this epoch represents a time of major brain development in rodents and is thought to be analogous to the third trimester of gestation in humans (Gaspar et al., 2003; Ansorge et al., 2007; Leonardo and Hen, 2009; Homberg et al., 2010). During this time, many of the systems and brain regions implicated in the pathophysiology of anxiety and depression disorders are rapidly evolving (Sodhi and Sanders-Bush, 2004; Homberg et al., 2010). For instance, in the first few weeks of life, the HC and mPFC undergo rapid and substantial changes in anatomy and volume (Whitaker-Azmitia et al., 1996; Zhang et al., 2006; Andersen et al., 2007), with data suggesting that between P0 and P7, the rate of cortical growth exceeds that of any other brain region (Zhang et al., 2006). Synaptic connections, axon tracts and dendritic arbors are also being sculpted at this time, fortifying the importance of this epoch in circuit formation (Gaspar et al., 2003). This post-natal period also corresponds to the time in which the 5-HT system itself is rapidly maturing. 5-HT levels gradually increase in this period, peaking in the fourth post-natal week, and subsequently decreasing to adult levels

(Sodhi and Sanders-Bush, 2004; Sodhi; Whitaker-Azmitia, 2005). In rodents, only about 30% of adult levels of serotonin receptors are present at birth and are thought to increase in expression during the first weeks of post-natal life (Azmitia and Whitaker-Azmitia, 1997; Beique et al., 2004; Gaspar et al., 2003; Whitaker-Azmitia, 2005). For instance, the 5-HT1A receptor is present at P0, but is dramatically upregulated within the first three weeks of life, with near adults levels of this receptor achieved around P21 (Gross et al., 2002). Serotonergic innervation to behaviorally relevant areas is also developing during this time. In the mPFC, serotonergic innervation is present at birth but adult patterns are achieved only around the sixth week of life, implying that axonal arbors are being dramatically sculpted in the early post-natal period (Azmitia and Whitaker-Azmitia, 1997). Interestingly, during this time, the mPFC and HC are also expressing 5-HTT and can thus take up, store and potentially release 5-HT, a feature which may be important in their functional development (Narboux-Neme et al., 2011).

Based on the above data, we hypothesized that the critical period in which changes in serotonin impact adult anxiety and depression behaviors is captured in the early post-natal period. To experimentally test this hypothesis, wildtype mice were given daily intraperitoneal injections of either FLX (10mg/ml) or Vehicle (0.9% NaCl) during the following time windows: P4-21 (Ansorge et al., 2004), P2-21, P2-11, P12-P21, P22-41 (Qinghui Yu, unpublished data). After post-natal treatment, animals were allowed to mature in standard facility conditions and behavioral testing was performed at P90 and above. We have previously shown that this treatment scheme results in therapeutic and physiologically relevant brain levels of both FLX and its active metabolite nor-fluoxetine

without any overall effects on viability and growth (Ansorge et al., 2004 and 2008). A treatment naïve cohort was also included to control for injection and handling stress.

c.) PN-FLX mouse phenotype

The behavioral phenotype of PN-FLX treated mice was characterized using specific, pharmacologically validated tests for anxiety and depression-like behaviors (Figs. A.4 to A.6, methodology described in detail in Chapter 2). PN-FLX mice treated between P4-21, P2-21, or the narrower P2-11 window, displayed anxiety and depression-like behaviors in adulthood. In particular, PN-FLX mice show decreased exploratory behavior (Fig A.4) and suppression of feeding in a novel, brightly lit and thereby anxiogenic, environment (Fig A.5).

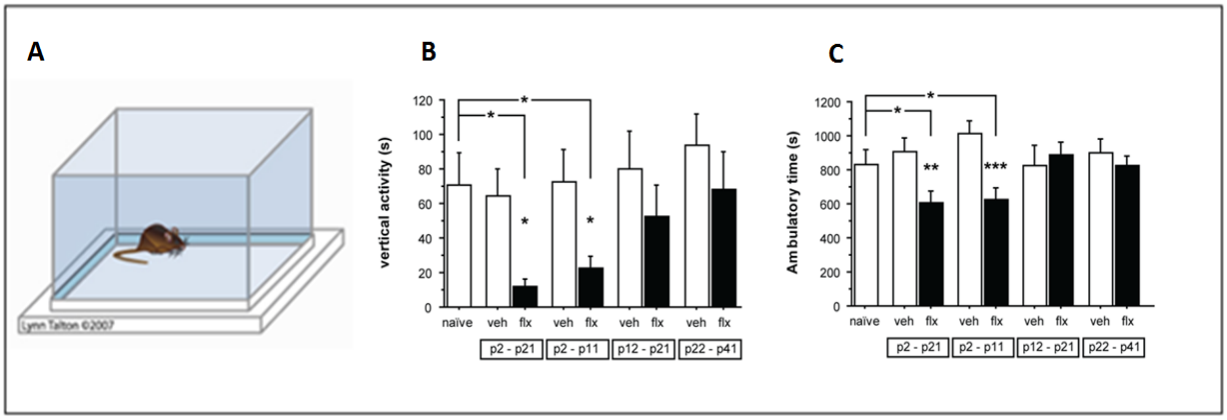


Fig. A.4. Mice treated with FLX from either P2-21 or the narrower P2-11 display anxiety and depression-like behavior in the Open Field Test (A), as evidenced by significantly reduced vertical activity or "rearing" (B) and decreased time engaging in exploratory activity (C). FLX treatment during any of the other windows had no effects relative to either controls, or naïve mice. Rearing activity is decreased in other mouse models of emotional dysfunction and can be reversed by anti-depressant and anxiolytic drug treatment (Hughes, 1993; Angrini et al., 1998;

Choleris et al., 2001; Holmes, 2003). Moreover, diminished ambulatory activity in a novel, brightly-lit environment is indicative of reduced exploratory activity and is observed in other mouse models of anxiety/depression (Gross et al., 2002; Richardson-Jones et al., 2011).

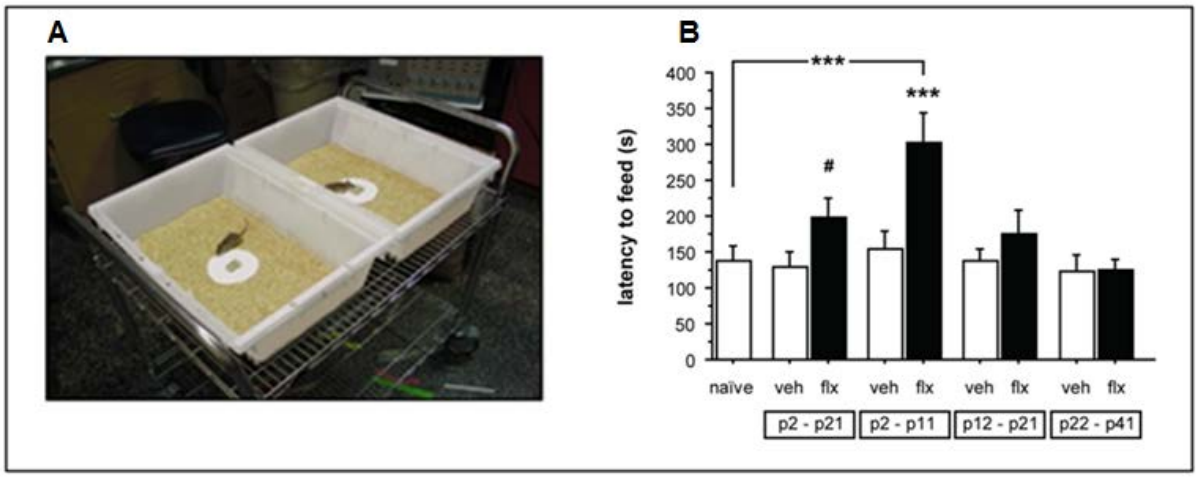


Fig. A.5. Only mice treated with FLX from either P2-21 or the narrower P2-11 display anxiety and depression-like behavior in the Novelty Suppressed Feeding (NSF) test (A), as evidenced by significantly longer latencies to feed (B). Feeding latency times are sensitive to chronic anti-depressant and acute anxiolytic drug treatment and are elevated in other mouse models of anxiety and depression (Bodnoff et al., 1989; Gross et al., 2002; Santarelli et al., 2003; Lira et al., 2003).

PN-FLX mice also displayed an innate helpless-like phenotype and ineffectual responses to acute stressors (Fig. A.6).

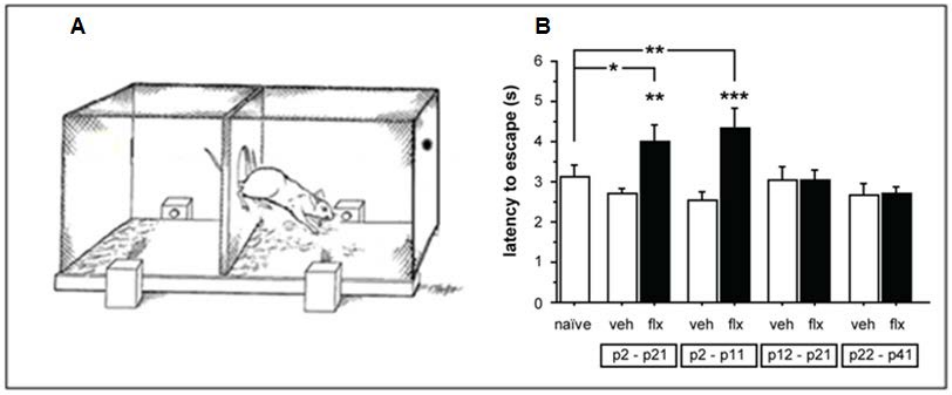


Fig. A.6. Only mice treated with FLX from either P2-21 or the narrower P2-11 display anxiety and depression-like behavior in the Shock Escape (SE) test (A), as evidenced by significantly increased latencies to escape (B), relative to controls. Increased escape latencies are found in other mouse models of anxiety and depression and are decreased with anti-depressant and anxiolytic agents (Seligman, 1972, Cryan et al., 2002; Lira et al., 2003; Muller et al., 2011).

The critical time window was contained within the narrower P2-11 epoch as treatment schedules that did not include this window, did not produce abnormal emotional behaviors in adulthood (summarized in Fig A.7). Thus, we were able to show that 5-HTT inhibition solely during a key developmental period was sufficient to phenocopy the behavioral profile of constitutive *5-htt -/-* mice. Moreover, these long-term behavioral effects were not specific to the use of FLX, as PN-treatment with other SSRIs and monoamine oxidase A (MAOA) inhibitors, also alter adult emotional behaviors (Vogel et al., 1990; Maciag et al., 2006a and 2006b; Ansorge et al., 2008, Qinghui Yu, unpublished results). Nor are these behavioral effects specific to manipulations of the 5-HTT, as variations or loss of the inhibitory 5-HT1A receptor or MAOA also result in enhancements of anxiety and depressive behaviors in both humans and mice (Zhuang et

al., 1999; Gross et al., 2000; Tadic et al., 2003; Guitierrez et al., 2004; Savitz et al., 2010). In line with our finding from the PN-FLX model, restoration of proper 5-HT1A R signaling in forebrain in the second to third post-natal week was sufficient to rescue the behavioral phenotype of constitutive *5-ht1a* -/- mice (Gross et al., 2002). These data provide evidence for a strong correlative relationship between altered developmental serotonergic tone (irrespective of mechanism of disruption) and adult affective dysfunction.

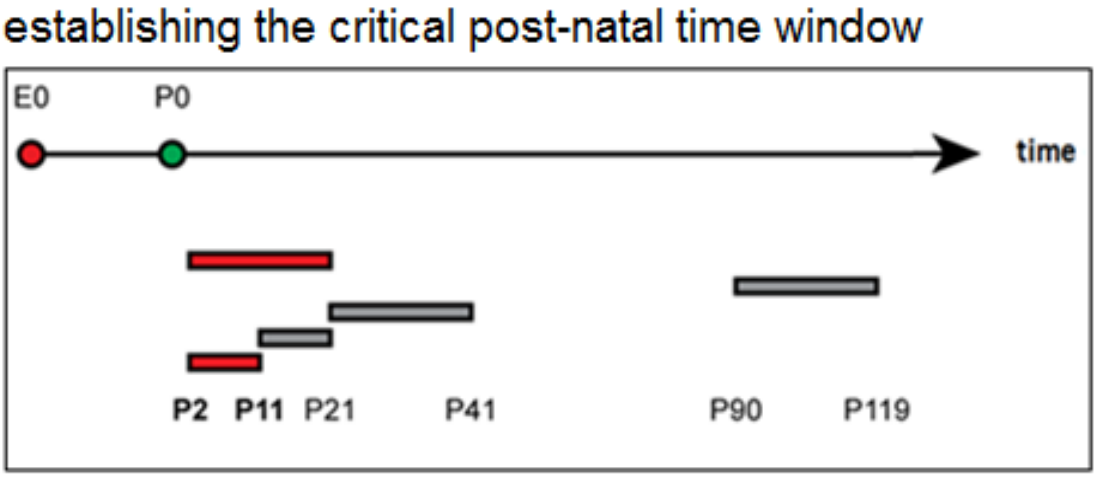


Fig. A.7. Summary of PN-FLX treatment phenotypes. P2-11 and P2-21 treatment with fluoxetine altered adult emotional behavior in a similar fashion. P12-21 and P22-41 treatments had no effect. Behavior was assessed in the open field test, the novelty suppressed feeding test and the shock escape test. The summary depicts treatment windows with (red boxes) and without (grey boxes) long lasting "negative" effects on affective behavior. E0 = conception; P0 = birth.

Part 2

The compelling next question relates to the mechanism by which early-life perturbations of 5-HT result in altered adult behaviors. Since behaviors are mediated by specific brain regions, and the connectivity between them, the serotonin-sensitive critical time windows for emotional behaviors likely represent epochs in which the circuits underlying these affective behaviors are developing (Gingrich et al., 2003; Gross and Hen, 2004b; Ansorge et al., 2007; Leonardo and Hen, 2009). Indeed, the crucial role of 5-HT in sculpting connectivity between brain regions has already been shown for sensory circuits. Specifically, serotonin signaling via the 5-HT1B and 5-HT1D receptors is essential in thalamocortical circuit maturation, which transmits sensory information from the whiskers to the somatosensory cortex (Rebsam et al., 2002; Gaspar et al., 2003; Bonnin et al., 2007). The axonal arbors of cortically projecting thalamic neurons are disrupted in *5-htt -/-* mice, which results in significantly impaired tactile sensation (Pang et al., 2011). These findings provide a convincing proof-of-principle that illustrates how abnormal serotonergic tone alters the maturation of circuits/brain areas resulting in altered behavior. Accordingly, serotonin may play a similar role in sculpting brain regions that underlie emotional behaviors, such as the mPFC and HC. Addressing this question has been the focus of my dissertation work.

HYPOTHESIS

The overarching hypothesis of this body of work:

During development serotonin influences the maturation of brain regions that underlie affective behaviors such as the mPFC and hippocampus. Thus perturbations of serotonergic tone in early development alter the developmental trajectory of these regions thereby enhancing the vulnerability to anxiety and depression-like disorders in later life.

The specific aims pertinent to the above hypothesis will be presented in following chapters.

CHAPTER 2: Serotonin modulates the structural, physiological and functional development of the medial prefrontal cortex

Introduction

In this chapter, we address the first part of our overarching hypothesis, by determining if early-life perturbations of 5-HT affect the maturation of the mPFC. Having identified changes in mPFC development, we next asked whether these alterations were causal to at least some component of the emotional phenotype of PN-FLX mice.

The mPFC is a key regulator of affective state, whose output modulates fear-related behaviors, anxiety levels, stress responses and depressive states (Vertes, 2006; Milad et al., 2007). As such, variations in its proper development may impact its functional output, thereby resulting in a dysregulation of emotional behaviors. As *5-htt -/-* and PN-FLX mice display an enhanced anxiety and depression-like phenotype, we hypothesize that they have abnormal mPFC function. Indeed, there is ample, although circumstantial, evidence indicating that 5-HT influences the developmental course of the mPFC. For example, humans with the lower-expressing "*s*" allele of the *5-htt* gene, display a marked decrease in gray matter volume in the prefrontal cortex (Pezawas et al., 2005). These changes are found in regions that are homologous, in terms of their connectivity and function, to sub-regions of the rodent mPFC, namely the infralimbic (IL) and prelimbic (PL) cortices (Uylings et al., 2003). Similar structural changes in the mPFC have also been characterized in *5-htt -/-* mice, an extreme model of the "s" polymorphism, who display volume changes in the mPFC (Altamura et al., 2007), likely

due to alterations in dendritic branching and complexity in this region that we, and others, have characterized (Appendix B, Fig. B.1-B.2; Wellman et al., 2007). Changes in mPFC volume have also been characterized in humans with anxiety and depression (Sheline et al., 1996; Vasic et al., 2008; Lorenzetti et al., 2009), both of which are pathologies associated with serotonergic dysfunction (Gingrich et al., 2003). Together, these data suggest a strong association between serotonergic tone, mPFC structure and affective dysfunction.

5-HT also influences the physiological properties of the mPFC. Dysregulation in 5-HT signaling affects spine density (Nietzer et al., 2011), receptor expression (Mossner et al., 2003; Yuen et al., 2005; Englander et al., 2005; David et al., 2005; Guidotti et al., 2011), receptor coupling (Manouri la Cour et al., 2001; Bhanshali et al., 2007; Stockmeier et al., 2009), afferent input (Maciag et al., 2006; Neil Gray, unpublished results) and electrophysiological responses (Iyer et al., 1996; Manouri la Cour et al., 2001; Goodfellow et al., 2009; Adhikari et al., 2010;) in the mPFC. Congruently, many of the above physiological parameters are abnormal in mice displaying both altered serotonergic tone, and dysregulated affective behaviors, such as *5-htt -/-* and those lacking the 5-HT1A receptor (*5-ht1a -/-* mice), suggesting a correlation between these phenotypes (Gobbi et al., 2001; Mannoury la Cour et al., 2001, Murphy et al., 2001; Li et al., 2003). Additionally, theta oscillations within the mPFC, which are generated by the synchronized rhythmic activity of neuronal electrical conductances in the 4-12 Hz range, are also influenced by 5-HT signaling (Gordon et al., 2005). This suggests that 5-HT might affect network activity and synchronization in the mPFC. Given the established

influence of 5-HT on mPFC neuronal physiology, we hypothesize that mPFC neurons of PN-FLX mice display altered physiological properties.

Furthermore, since mPFC output is essentially a composite of the firing and physiology of the individual neurons of which it is composed, changes in mPFC neuronal cytoarchitecture and physiology may impact its functional output. Functional probing of this structure using mPFC-dependent behavioral paradigms suggests mPFC output is indeed altered in *5-htt -/-* mice (Wellman et al., 2007). Specifically, the mPFC, through its connections to other limbic structures such as the amygdala, modulates an organism's ability to extinguish learned fear, termed *fear extinction* (Burgos-Robles et al., 2007; Milad et al., 2007; Quirk and Mueller, 2008). Lesion and microstimulation studies have shown that specific manipulations of mPFC output result in either enhanced or dampened fear extinction learning and memory (Lebrón et al., 2004; Sierra-Mercado et al., 2006; Santini et al., 2008; Farrell et al., 2010; Sierra-Mercado et al., 2011) . In line with their structural/physiological mPFC phenotype, *5-htt -/-* mice display deficits in fear extinction learning and its subsequent recall (Wellman et al., 2007) signifying altered mPFC function. Congruently, this decreased corticolimbic connectivity, suggestive of altered mPFC output, has also been demonstrated in human "*s*" allele carriers. Functional magnetic resonance imaging (fMRI) studies revealed diminished functional connectivity between the mPFC and the amygdala in "*s*" allele carriers, in response to fearful stimuli known to recruit corticolimbic circuits (Heinz et al., 2004; Pezawas et al., 2005). Inclusively, these data indicate a strong correlation between abnormal serotonergic tone, altered maturation of the mPFC and affective dysfunction. To directly address whether early-life serotonergic tone affects the functional maturation of the mPFC, we

investigated whether PN-FLX mice displayed structural, physiological and functional changes in the mPFC, by addressing the following aims:

Aim 1a: *Does development-specific disruption of serotonergic tone alter the maturation of the medial pre-frontal cortex (mPFC)?*

i. Are there structural alterations within the mPFC of PN-FLX mice?

ii. Are there electrophysiological changes in mPFC neurons from PN-FLX mice?

iii. Is the functional output of the mPFC altered in PN-FLX mice?

Next, we sought to determine whether there was a causal relationship between the 5-HT-mediated changes in the mPFC and the emotional behaviors generated as a result of early-life modulation of 5-HT. As such, the following aim was addressed:

Aim 1b: *Are the structural/functional changes in the mPFC causal to the affective behavioral phenotype of PN-FLX mice?*

Methods

Drug preparation and Subjects

Fluoxetine (FLX; ANAWA Trading SA, Wangen, Switzerland) was dissolved in 0.9% NaCl to a final concentration of 2mg/ml. The solution was filtered using a 0.45um filter tip and filtrate was collected in a fresh tube and stored at 4º until ready to use. Prior to use, FLX solution was warmed to room temperature. Male and female WT 129SvEv mice pups were intraperitoneally injected with the 2mg/kg FLX (in 0.9% sodium chloride, NaCl) solution. Control mice received injections of vehicle (0.9% NaCl solution). For treatment, entire litters were removed from dams and placed into a bucket (10 cm diameter) containing shavings from the respective home cage. The bucket was resting on scales, to allow for weighing mice after picking them up using a subtractive technique. Mice were injected in random order, and an entire litter was injected within 2 min. After injections, mice were placed back into the home cage and were observed for 5mins to ensure mothers attended to them. For the morphological analyses mice were treated from P4-21 as this period yields an identical behavioral profile as mice treated form P2-11, as it contains the narrower critical time window (Ansorge et al., 2000). For all other experiments mice were treated from P2-P11. After weaning, which occurred on P28, mice were separated by sex, randomly mixed for treatment and group housed with 5 mice per cage.

Analysis of dendritic complexity

Brains from adult (>P90), male PN-Veh and PN-FLX mice processed with the Golgi stain as previously described (Rosoklija et al., 2003). Stained intact brains were cut

using a vibratome to generate 150μm sections which were mounted on 3% gelatin-subbed slides. Pyramidal neurons (n = 6-9 cells per mouse; 3 mice per treatment group) in the infralimbic (IL) and prelimbic (PL) regions (layer 2/3 and 5) were visualized at 100x magnification and identified based on their location (verified using the Comparative Cytoarchitectonic Atlas of the 129/Sv mouse brain by Hof et al., 2000) and characteristic morphology such as the pyramidal shape of their somas and the presence of a prominent apical dendrite. Cells that were in the middle of the z-plane and possessed intact dendritic arbors were marked as potential candidates for analysis. From this candidate pool, 7 to 10 cells per brain were randomly selected and traced. Neurons were manually traced using Neurolucida software (MBF Bioscience, Williston, VT). Cells from both hemispheres were included in a balanced manner. Extent of dendritic arborization was determined via Sholl analysis (Sholl, 1953) using NeuroExplorer software (MBF Bioscience) with 10μm set as the radius parameter (Fig. 2.1). Dependent measures from the Sholl analysis output included number of nodes (branch points, both bifurcating and trifurcating nodes were included in analysis, however >95% of total nodes were bifurcating), intersections and total dendritic material. In addition to the Sholl parameters, the length of dendritic tree (distance between the soma and farthest point of dendritic tree) was also recorded using the maximum Sholl radius as the index of how far out the trees protruded from the soma. Brain sections were coded such that the experimenter was blind to treatment during the tracing and analyses.

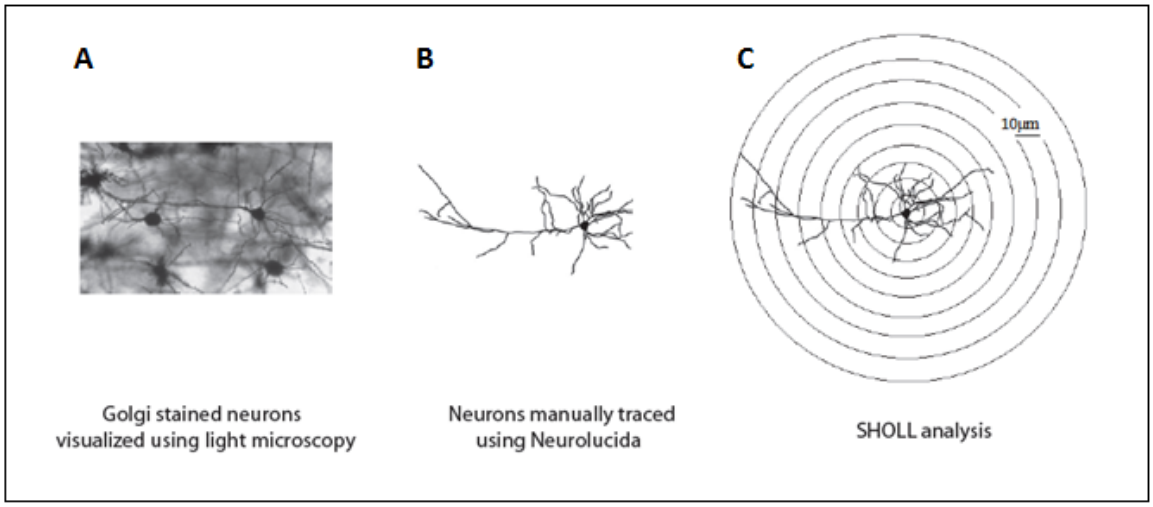


Fig 2.1. Schematic displaying the steps in analyzing dendritic complexity of mPFC neurons. Cells were visualized at 100x magnification (A), manually traced (B) and finally analyzed using Sholl analysis (C). Sholl analysis works by setting concentric spheres of a fixed radius (10µm) starting from the epicenter of the soma. In each sphere or "shell" the number of nodes (branch-points), intersections (number of branches crossing through a specified shell) and dendritic material is reported.

Electrophysiology

Experiments were performed at the University of Toronto (data acquisition and analysis was performed by Nathalie Goodfellow- laboratory of Dr. Eveyln Lambe) and approved by the University of Toronto Animal Care and Use Committee and conformed to the international guidelines on the ethical use of animals. Male PN-FLX and PN-Veh mice aged P100-P120 were shipped to the University of Toronto Animal facility and were kept in quarantine and allowed to habituate to the new environment for a period of >6 weeks. The following methods have been modified from, and based on: Goodfellow et al., 2009. Briefly, animals were rapidly decapitated using a guillotine and the brain was

excised and immediately cooled by placement in 4°C oxygenated sucrose artificial CSF (sACSF; 254 mM sucrose, 10 mM D-glucose, 24 mM NaHCO3, 2 mM CaCl2, 2 mM MgSO4, 3 mM KCl,1.25 mM NaH2PO4; pH 7.4). The sACSF was specifically formulated such that it contained no NaCl in order to dampen any sodium ion-mediated firing activity that might occur during the processing of the tissue in preparation for recordings, thereby protecting the tissue from excitotoxicity. Brains were mounted on a vibratome and cut to generate 400μm thick slices of the medial prefrontal cortical region. Prefrontal slices were cut from anterior to posterior using the appearance of white matter and the corpus callosum as anterior and posterior guides (from approximately 1.4 to 2.3 mm anterior to Bregma). Slices were cut on a Dosaka Linear Slicer (Sci-Media) and were transferred to 32°C oxygenated "regular" (including NaCl) ACSF (128 mM NaCl, 10 mM D-glucose, 24 mM NaHCO3, 2 mM CaCl2, 2 mM MgSO4, 3 mM KCl,1.25 mM NaH2PO4; pH 7.4) in a pre-chamber and allowed to recover for at least 2 h before the beginning recordings. For whole cell recordings, slices were placed in a superfusion chamber (Warner Instruments) and mounted on the stage of an OlympusBX50WI microscope. Regular ACSF at 31 to 33 °C was bubbled with 95% oxygen and 5% carbon dioxide and flowed over the slice at a rate of 3–4 ml/min. The temperatures used during brain removal and recordings were found to be critical for maintaining healthy brain slices which was especially important given the advanced age of our mice.

Whole cell recordings of neurons were performed as previously described (Goodfellow et al., 2009). Briefly, whole-cell recording electrodes (2–3 MΩ) contained 120mM potassium gluconate, 5 mM KCl, 2 mM MgCl, 4 mM K2-ATP, 0.4 mM Na2-GTP, 10 mM Na2-phosphocreatine, and 10 mM HEPES buffer (adjusted to pH 7.33 with

KOH). Prefrontal cortex layer 2/3 pyramidal neurons in the IL and PL were visualized using infrared differential interference contrast microscopy. Neurons were identified based on their location, the pyramidal shape of their somas and presence of a prominent apical dendrite. Care was taken to select neurons in the central part of each region (IL and PL) so as to avoid any confusion of whether a cell belonged to either IL or PL. Under these conditions, and currents were recorded using continuous single electrode voltage-clamp mode with a Multiclamp700b (Molecular Devices), acquired and low-pass filtered at 3 kHz using pClamp10.2/Digidata1440 software (Molecular Devices). For input-output curve generation, neurons were held at their resting membrane potentials, or at -75 mV, as indicated, and 50 pA current steps up to 450 pA were used and spike responses to current injections were measured (Fig 2.2). Statistical comparisons between treatment groups were analyzed using unpaired t-tests. Data is presented as means +/- standard error of the mean (S.E.M.).

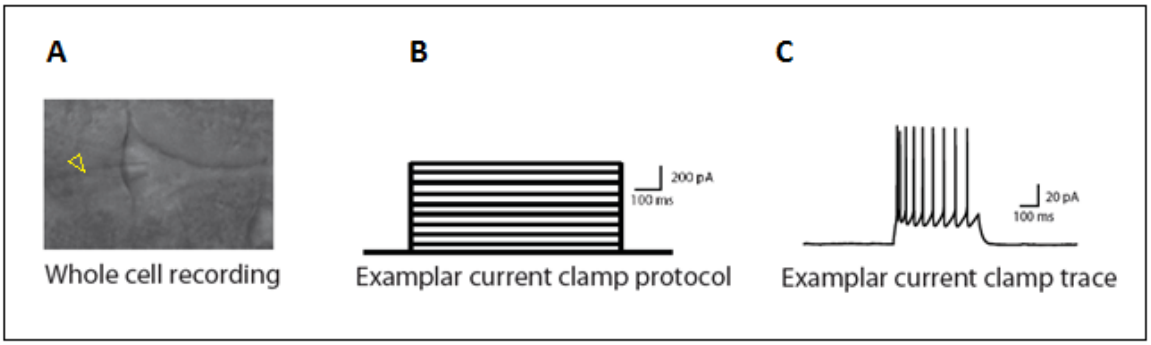


Fig. 2.2 Neurons in the mPFC were patched (A, yellow arrow indicates pipette) to make whole-cell recordings in responses to incremental 50 pA current steps (B). Spike responses (frequency of spikes) to current steps were measured (C). (Figures courtesy of N. Goodfellow and E. Lambe, University of Toronto).

Cued fear conditioning and extinction

Mice were fear conditioned using equipment set up similarly to that described by Drew et al., 2010. On day 1, mice were placed into the conditioning chamber ("context A"; ENV010MD, Med Associates, St. Albans, VT), allowed to explore freely for 180 s and then received three tone-shock pairings, in which 20 s, 80 dB, 2000 Hz tone (conditioned stimulus, CS) co-terminated with 2 s, 0.75 mA scrambled foot shocks (unconditioned stimulus, US) delivered via the grid floor. Tone-shock pairings were delivered with variable inter-trial intervals. On day 2, mice received a second day of cued fear conditioning in context "A" in which they received two tone-shock pairings. Mice then went two days of fear extinction training (days 3 and 4) in novel context "B" which differed significantly from conditioning context "A" in several ways including odor cues, wall color/composition, floor composition (grid floor vs. bedding) and room/chamber lighting. On day 5, mice were placed back into the extinction training context "B" and their response to 5 tones were measured. When indicated, % freezing values were normalized to freezing levels at the start of the extinction session (D3 start). Mice were recorded using a video camera mounted above the conditioning chamber and movement was monitored using FreezeFrame software from Actimetrics (Evanston, IL). Percent freezing was automatically calculated using FreezeView software (Actimetrics). Freezing was defined as the cessation of all movement excluding respiration and the freezing bout duration was defined as 0.5s. One-way analysis of variance (ANOVA) was used to analyze data, using percent (%) freezing was the main dependent variable.

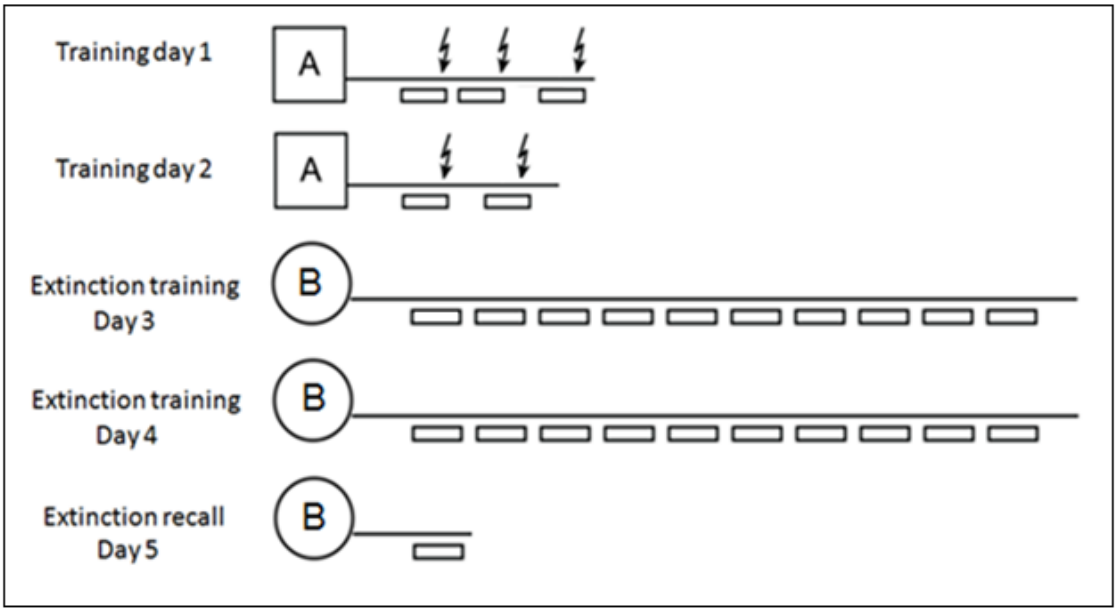


Fig 2.3 Cued fear conditioning and extinction protocol. Bars represent 20 s, 80 dB, 2000 Hz tones. Jagged arrows represent 2 s, 0.75 mA scrambled foot shocks. During training days, 1 bar = one tone, while on extinction training days, 1 bar = 3 tones. Orange bar represents the tones used to determine freezing to CS cue after fear conditioning session.

Infralimbic lesions

Lesion studies and behavioral tests and were conducted in accordance with the *Principles of Laboratory Animal Care guidelines* from the National Institute of Health (NIH) and the Institutional Animal Care and Use Committee guidelines. Adult (> P90) Male and female PN-Veh and PN-FLX mice were assigned to either sham- or IL-lesion groups. Post-natal treatments were carried out as previously described. Mice were anaesthetized using Isoflurane and underwent stereotactic surgery in which they received two bilateral injections of either ibotenic acid (0.25 µl of 5 mg/ml, 0.125 µl/min injection rate; Sigma Aldrich, St. Louis, MO) using a tapered glass micropipette with an internal

diameter of 12 μm (pulled from capillaries: 3-000-203-G/X, Drummond Scientific, Broomall, PA), if they were in the IL lesion groups or with vehicle (0.9% NaCl, 0.25 μl, 0.125 μl/min injection rate), if they were in the sham lesion group. Injection coordinates were 1.6 and 2.1 mm anterior to bregma, 2.8 mm ventral from the brain surface and +/- 0.3 mm lateral to the midline. After surgery, mice were returned to their cages and were monitored daily over the next week to ensure that they recovered without issue. Mice were given 2 to 4 weeks for recovery before the onset of behavioral testing. Behavioral testing on lesioned mice was conducted as previously described in this paper.

Behavioral testing of lesioned mice

Tests were administered in the following order: open field, novelty suppressed feeding, and shock avoidance with a minimum 7 day inter-test durations.

Open Field

Measurement of exploratory activity in response to a novel environment was assessed as described in Ansorge et al., 2008, using the open field test. Briefly, mice were allowed to explore brightly lit (800-900 lux) Plexiglass activity chambers (43.2 × 43.2 × 30.5 cm, length × width × height; model ENV-520; Med Associates, Georgia, VT) equipped with infrared beams located 1.5 cm above the chamber floor and spaced 2.5 cm apart as well as beams 6 cm above the chamber floor, spaced 2.5 cm apart. Beams were positioned such that both horizontal and vertical activity was detectable. Mice were placed in the center of the chamber and were allowed to freely explore for 60 minutes. Testing was done in bright light conditions (800-900 lux). Total ambulatory time, total

time in the center (defined as the central 15 ×15 cm region) and vertical activity (rearing) were measured as the dependent variables.

Novelty-suppressed feeding

The novelty-suppressed feeding test was performed similarly to previously described (Ansorge et al., 2008). Briefly, food deprived mice (24 hours, with water available *ad libitum*) were placed in a brightly lit (800–900 lux) open arena (51 × 35 cm) containing clean wood chip bedding. A white filter paper 125 mm in diameter which was attached firmly to the center of the arena (embedded in the bedding). One familiar food pellet weighing ~4 g was firmly attached to the paper such that the mice could not move the pellet from the center and thus the pellet remained in the center region throughout the experiment. Mice were removed from their home cage, placed in a holding cage for 30 min before the test, and then released in the southwest corner of the arena. The latency to approach the pellet and begin a feeding episode was recorded. Mice were given a maximum of 15 mins to complete the task. 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. As a control, mice were weighed pre and post-24 hr food deprivation and percent weight loss was calculated. Latency to approach food, home-cage food consumption and % weight loss were used as the dependent measures in this test and analyzed using one-way analysis of variance.

Shock Escape

The shock escape test was performed as previously described (Ansorge et al., 2008). Briefly, mice were tested in a Plexiglas box (model ENV 010MD; Med Associates, St. Albans, VT) located within a sound-attenuated chamber. The box was divided into two identical chambers (20 × 17 × 20 cm, length × width × height) and separated by an automated guillotine door. The apparatus was equipped with a grid floor made of stainless steel beams and connected to a shock generator and 8 infrared beams for detecting movement of the animal. Mice (one mouse per box) were placed into the chamber with the guillotine door raised and were allowed to freely explore both compartments for 5 min after which the guillotine door closed and the trials commenced. At the beginning of each trial, the door was raised and a mild scrambled foot shock (0.2 mA; 10 s maximum 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 ("escape") or after a lapse of 10 s. The test consisted of 30 trials each separated by a 30 s inter-trial interval. Latencies to escape for each trial were recorded. If the subject failed to make a transition during the 10 s duration of the foot shock, a maximum latency of 10 s was assigned. Locomotor activity was assessed during inter-shock intervals by counting total infrared beam interruptions during each session.

Histology

At the end of the study, mice were transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were excised and further fixed via incubation in 4% paraformaldehyde for 24hrs after which they were cryoprotected by means of incubation

in 30% sucrose for 3 days. A freezing microtome (Leica Microsystems, Buffalo Grove, IL) was used to cut the entire anterior-posterior extent of the IL region in 35um sections, which were mounted onto 3% gelatin-subbed slides and allowed dry completely so as to ensure they were well adhered to the slides. Sections were then stained with cresyl violet. Briefly, slides were immersed in 50% ethanol for 1 min, dipped in water, and then placed in the Nissl stain solution containing a 1:10 mixture of cresyl acetate (9-Amino-5-imino-5H-benzo[a]phenoxazine acetate salt) and neutral red, both from Sigma-Aldrich, St. Louis, MO) for 3 mins to allow for impregnation. Slides were then re-dipped in water, and dehydrated by submersion in progressively higher percentages of alcohol solutions (50%, 70%, 95%, 100%, each for 1 min) followed by placement in xylene for a minimum of 30mins. Processed slides were then coverslipped using DPX mounting media (Sigma-Aldrich, St. Louis, MO) and were allowed to dry for two days before being analyzed. To determine the location and extent of the lesion sections were visualized using light microscopy and by drawing out the regions of ibotenic-acid mediated neuronal loss that was visible on brain atlas figures (Comparative Cytoarchitectonic Atlas of the 129/Sv mouse brain by Hof et al., 2000). Animals with non-IL lesions were eliminated from the analysis.

Statistical analysis

Data were analyzed using Student's t test or one-way analysis of variance (ANOVA), with repeated measures where appropriate and as indicated. Post-natal treatment and surgery type (sham vs. lesion) were assessed as independent variables. The criterion for significance was $p<0.05$ whereas the criterion for a trend was $0.05 <= p <$

0.1. Datasets containing male and female mice were analyzed for main effects and/or interactions of sex with the dependent variables under study. Since none were found, male and female data were combined. No interaction or main effects of treatment were found, so data from PN-Veh and PN-FLX mice were collapsed to measure effect of lesion (primary dependent variable). All data are presented as the mean +/- standard error of the mean (S.E.M).

Results

Aim 1a (i). Altered neuronal cytoarchitecture in the infralimbic region of PN-FLX mice

Dendritic morphology of Golgi-stained pyramidal neurons in two key subregions of the mPFC, the infralimbic (IL) and prelimbic (PL) cortices from adult PN-FLX and PN-Veh mice was examined. We found a decrease in the complexity of the apical dendritic arbors of layer 2/3 IL neurons from PN-FLX mice, relative to controls, as indicated by a significantly reduced number of nodes ($F_{1, 22} = 7.376$; $p = 0.0126$, Fig. 2.4), intersections ($F_{1, 24} = 10.272$; $p = 0.0038$, Fig. 2.5) and total dendritic material ($F_{1, 24} = 7.599$; $p = 0.0110$, Fig 2.6). The reduced complexity of the IL apical arbor is throughout the entire extent of the arbor up to 240µm from the soma (repeated measures ANOVA up to 240 µm: $F_{1, 23} = 7.221$; $p = 0.0132$; Fig 2.7). Furthermore, the decreased dendritic complexity of IL apical arbors is in absence of any overall shrinkage of the dendritic tree as the average distance that the apical arbor extended from the center of the soma was not significantly different between treatment groups (Fig. 2.8). The finding of decreased dendritic complexity was also limited to the apical arbor of IL neurons as no changes were detected in IL basilar trees (Fig. 2.9). Representative models of IL layer 2/3 neurons from PN-Veh and PN-FLX mice are presented in Fig. 2.10. In the layer 2/3 of the PL cortex, we found no differences in morphology in neither the apical nor the basilar dendritic trees (Fig. 2.11, apical; Fig. 2.12, basilar). Additionally, no significant differences between PN-FLX mice and controls were observed in layer 5 pyramidal neurons in either the IL (Fig. 2.13, apical; Fig. 2.14, basilar) or PL (Fig. 2.15, apical; Fig.

2.16, basilar) region. Thus, we find a specific ~20% decreased dendritic complexity in the IL layer 2/3 sub-region of the mPFC (Fig 2.17).

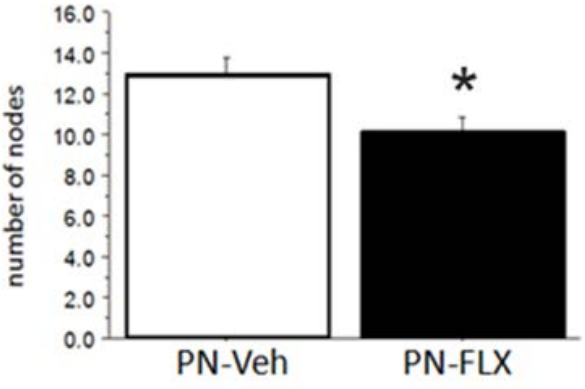


Fig 2.4. Apical dendrites of IL layer 2/3 pyramidal neurons from PN-FLX mice have significantly less total number of nodes relative to controls. N = 3 mice/group, 6-9 cells/mouse, * $p<0.05$. Data represent mean ± SEM

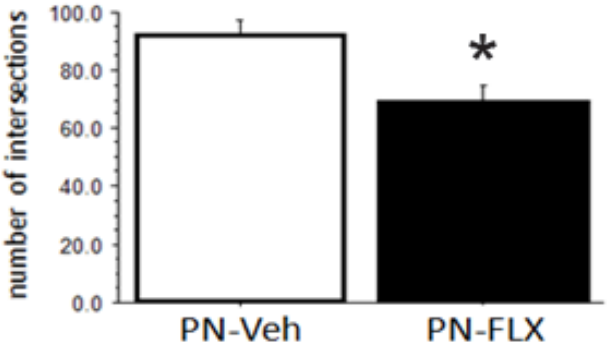


Fig. 2.5. Apical dendrites of IL layer 2/3 pyramidal neurons from PN-FLX mice have significantly lower total number of intersections relative to controls. N = 3 mice/group, 6-9 cells/mouse, * $p<0.05$.

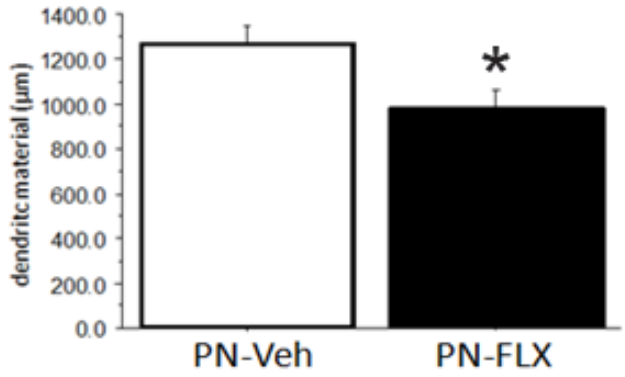


Fig. 2.6. Apical dendrites of IL layer 2/3 pyramidal neurons from PN-FLX mice have significantly less total dendritic material relative to controls. N = 3 mice/group, 6-9 cells/mouse, * $p<0.05$.

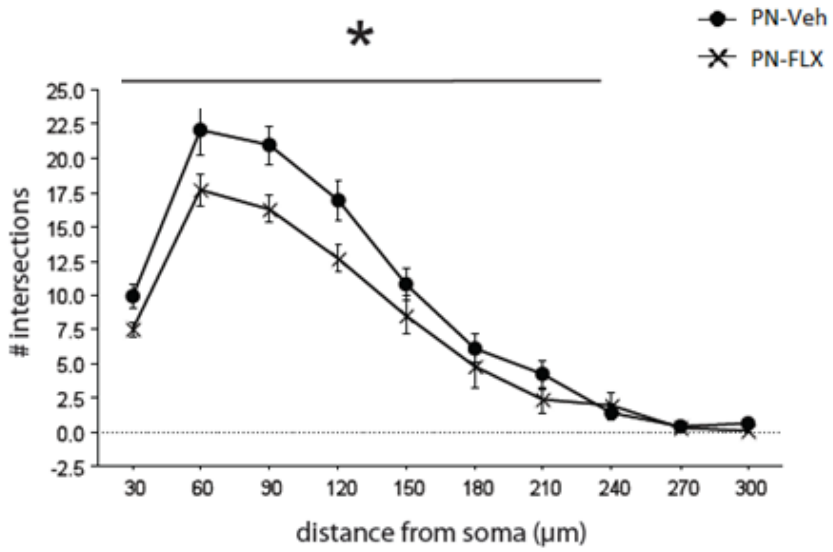


Fig. 2.7. The increased complexity (as captured by increased number of intersections) of PN-FLX apical layer 2/3 IL arbors are most prominent in the first 240µm proximal to the soma. N = 3 mice/group, 6-9 cells/mouse, * $p<0.05$.

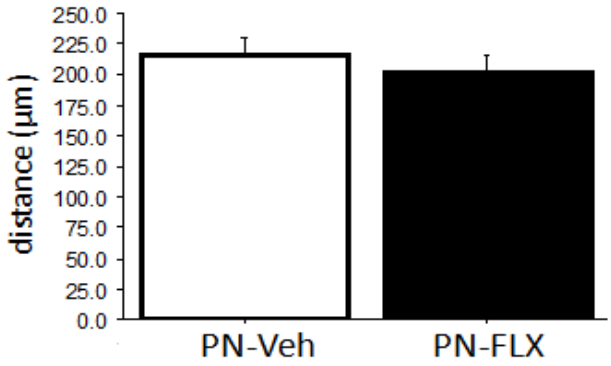


Fig. 2.8. No significant differences detected in the average distance that apical dendrites of IL layer 2/3 neurons extend out from the soma, between PN-FLX and PN-Veh mice. N = 3 mice/group, 6-9 cells/mouse.

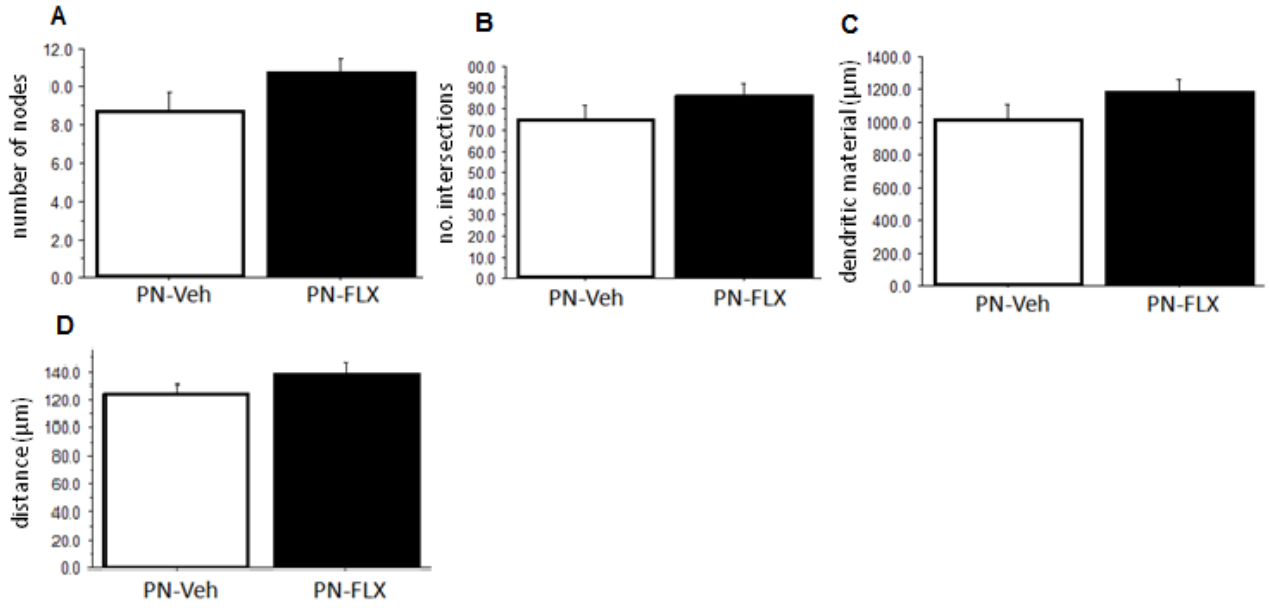


Fig. 2.9. No differences in total number of nodes (A), intersections (B), dendritic material (C) and distance from soma (D), in basilar dendrites of IL 2/3 neurons from PN-FLX and PN-Veh mice. N = 3 mice/group, 6-9 cells/mouse.

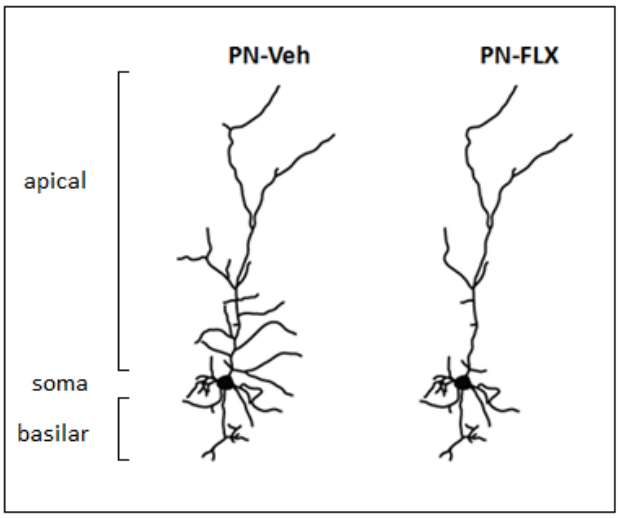


Fig. 2.10. Models of representative IL layer 2/3 neurons from PN-Veh and PN-FLX mice, depicting the decreased complexity of the apical arbor in PN-FLX mice.

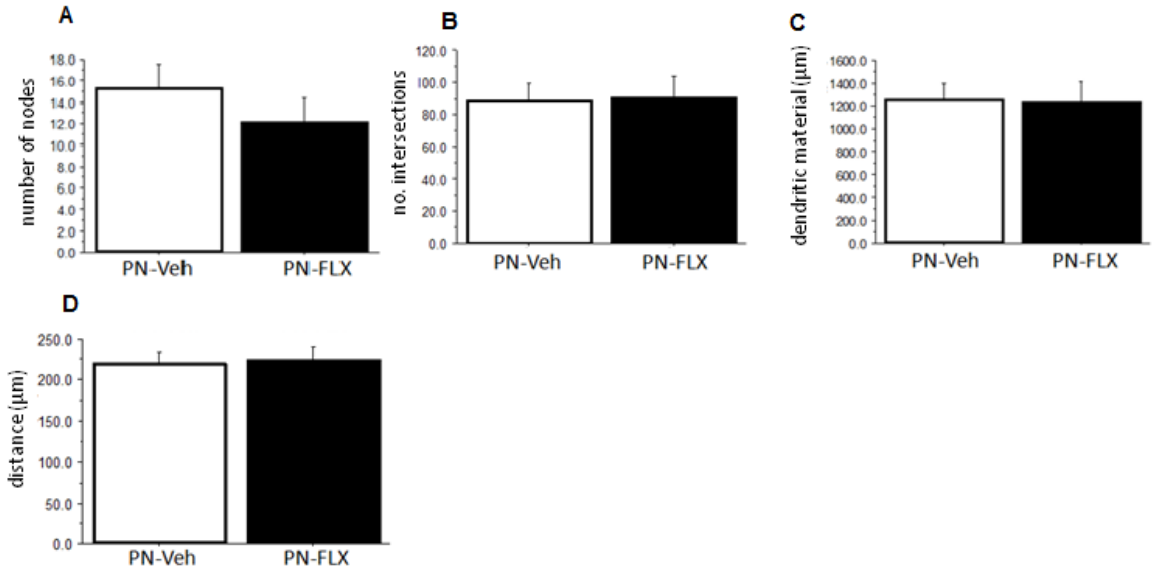


Fig. 2.11 No differences in total number of nodes (A), intersections (B), dendritic material (C) and distance from soma (D), in apical dendrites of PL layer 2/3 neurons from PN-FLX and PN-Veh mice. N = 3 mice/group, 6 cells/mouse.

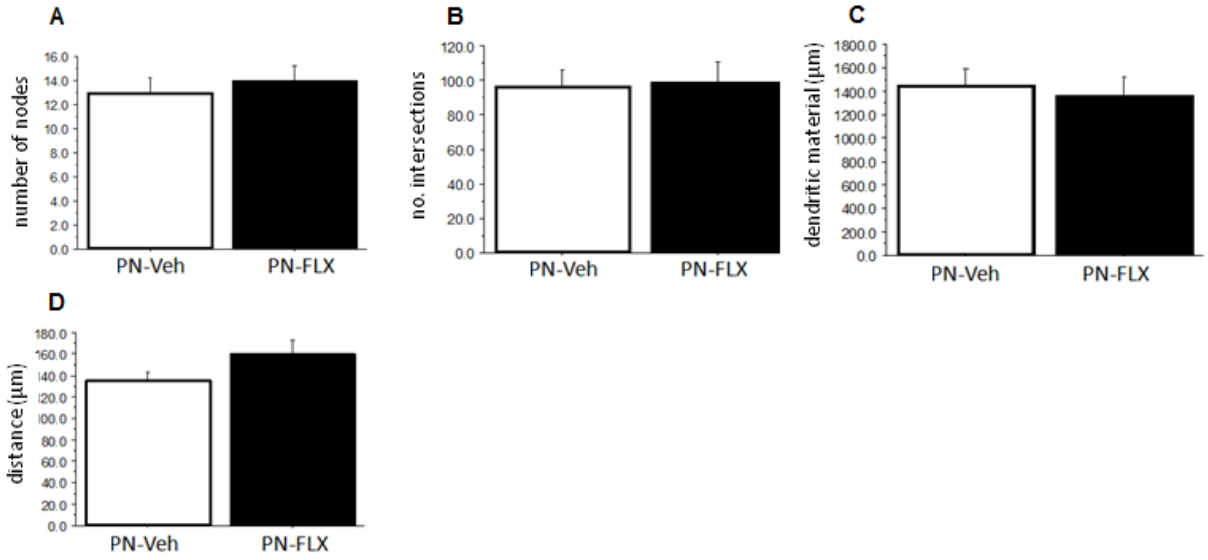


Fig. 2.12. No differences in total number of nodes (A), intersections (B), dendritic material (C) and distance from soma (D), in basilar dendrites of PL layer 2/3 neurons from PN-FLX and PN-Veh mice. N = 3 mice/group, 6 cells/mouse.

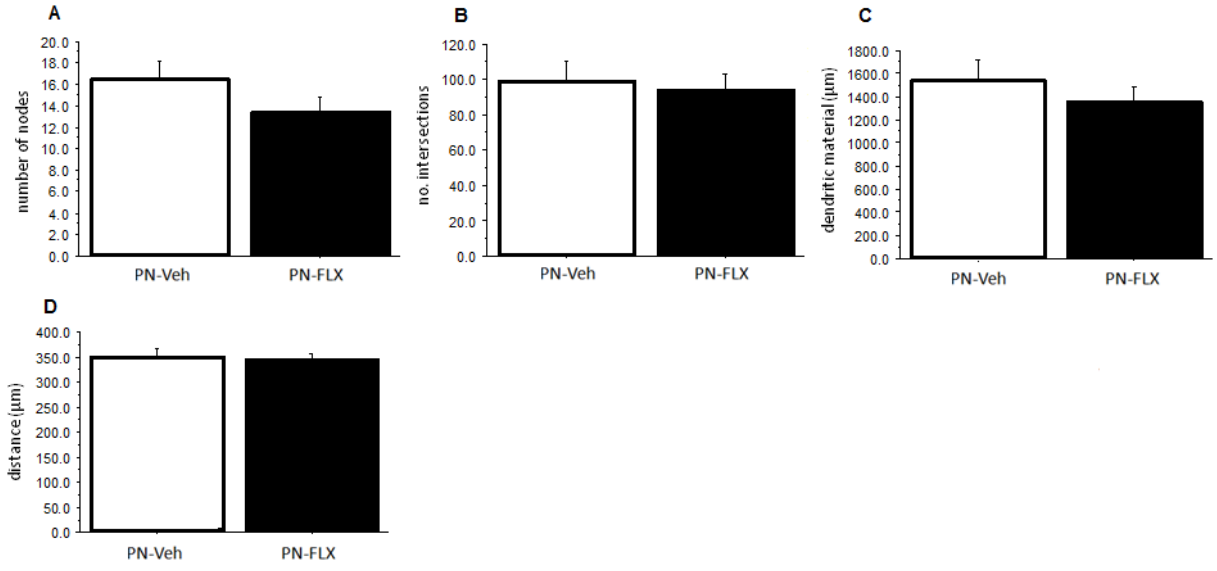


Fig. 2.13. No differences in total number of nodes (A), intersections (B), dendritic material (C) and distance from soma (D), in apical dendrites of IL layer 5 neurons from PN-FLX and PN-Veh mice. N = 3 mice/group, 6-7 cells/mouse.

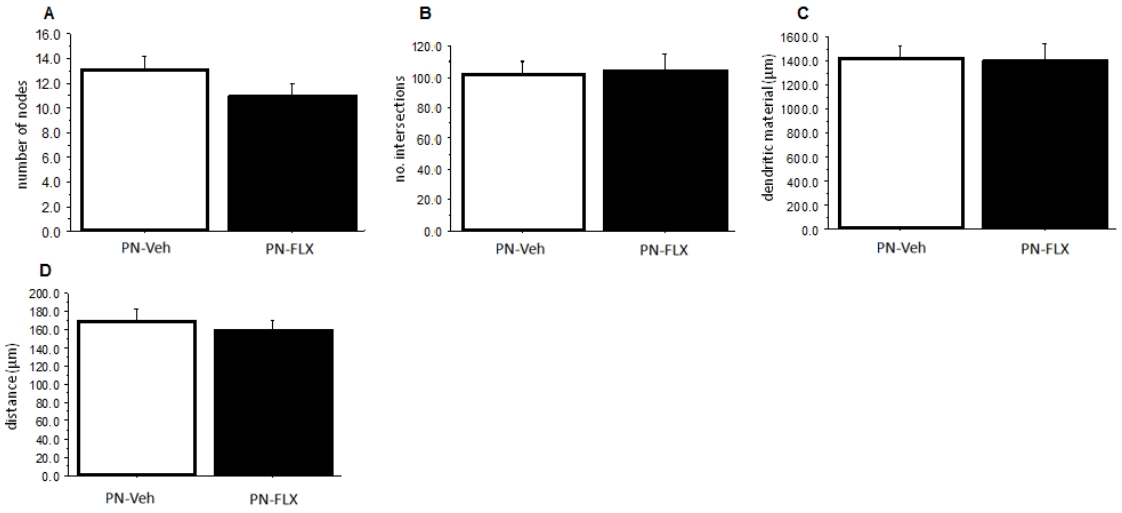


Fig. 2.14. No differences in total number of nodes (A), intersections (B), dendritic material (C) and distance from soma (D), in basilar dendrites of IL layer 5 neurons from PN-FLX and PN-Veh mice. N = 3 mice/group, 6-7 cells/mouse.

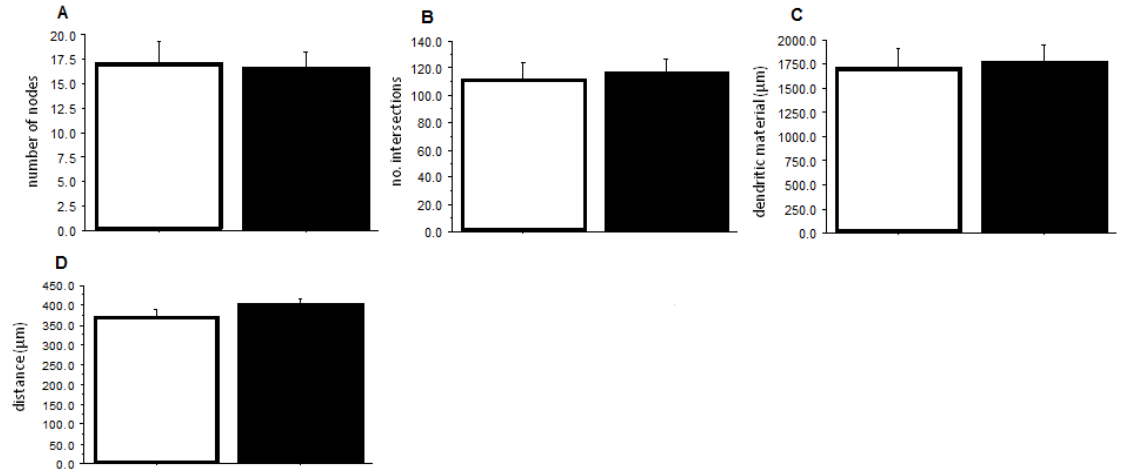


Fig. 2.15. No differences in total number of nodes (A), intersections (B), dendritic material (C) and distance from soma (D), in apical dendrites of PL layer 5 neurons from PN-FLX and PN-Veh mice. N = 3 mice/group, 5-6 cells/mouse.

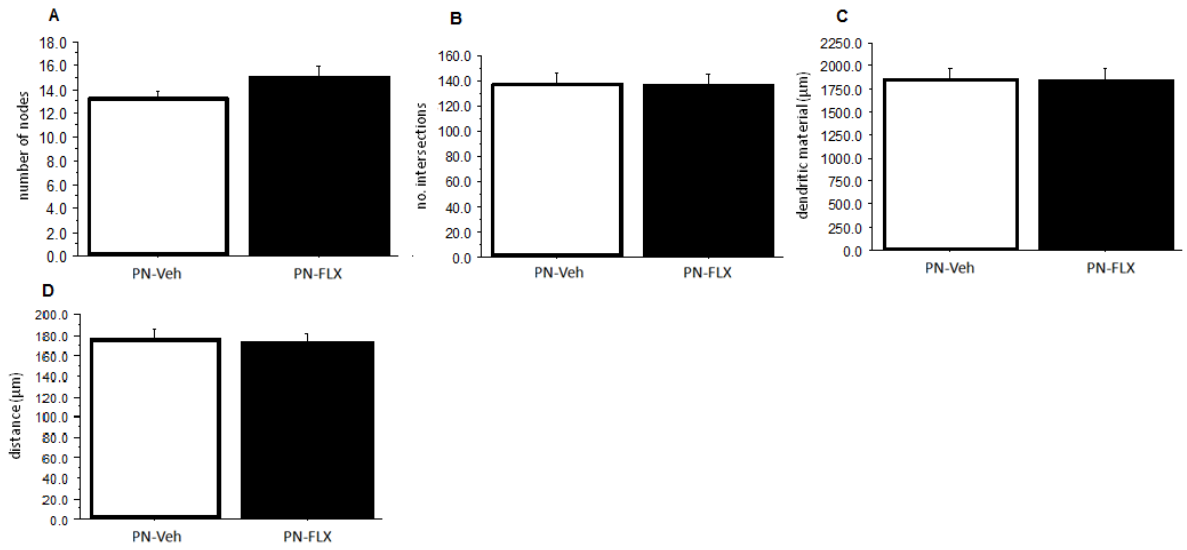


Fig. 2.16. No differences in total number of nodes (A), intersections (B), dendritic material (C) and distance from soma (D), in basilar dendrites of PL layer 5 neurons from PN-FLX and PN-Veh mice. N = 3 mice/group, 5-6 cells/mouse.

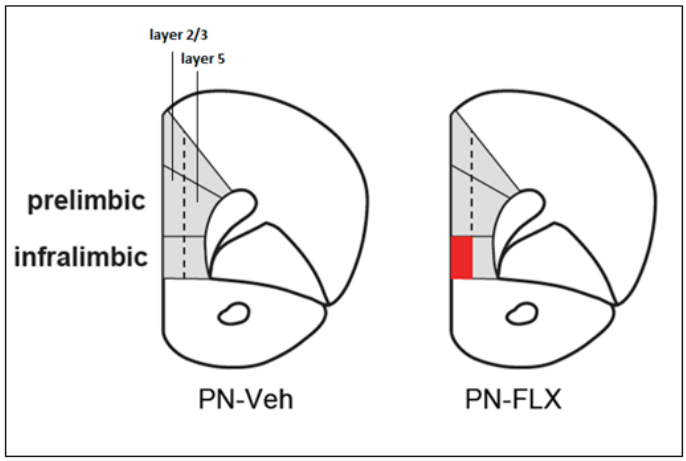


Fig. 2.17. Summary of morphological findings in mPFC region in PN-Veh and PN-FLX mice. The only region where changes (decreased complexity) were observed was in superficial layer 2/3 of the IL cortex (indicated by the red box).

Aim 1a (ii). **Concurrent decrease in infralimbic, and increase in prelimbic excitability in neurons from PN-FLX**

To determine whether the morphological changes we found in the mPFC were accompanied by changes in electrophysiological properties of the IL and PL pyramidal neurons, we performed whole-cell patch clamp recordings in acute brain slices. We investigated changes in both intrinsic physiological properties of neurons and also in their excitability, as gauged by their responses to varying current injections.

We found no significant differences in the intrinsic properties (membrane potential, rheobase, spike amplitude, input resistance or capacitance) of IL pyramidal neurons between treatment groups (Table 2.1). However, when spike frequencies in response to current injections were measured for IL pyramidal neurons, from both resting membrane potential or when held at -75 mV, a main effect of post-natal treatment was found using a two-way ANOVA, dependent variables: current and spike frequency (from rest: $p<0.0001$, $N_{PN\text{-}Veh} =13$; $N_{PN\text{-}FLX} = 19$; from -75mV: $p<0.0001$, $N_{PN\text{-}Veh} =13$; $N_{PN\text{-}FLX} = 19$) indicating that PN-FLX mice display significantly fewer spikes to current injections. Surprisingly, neurons from the PL region of PN-FLX mice responded with significantly more spikes to current injections, relative to controls when held at rest (two way ANOVA: $p < 0.01$, $N_{PN\text{-}Veh} =10$; $N_{PN\text{-}FLX} = 20$) or at -75 mV ($p < 0.05$) . PL neurons also exhibited significantly reduced rheobase values ($p = 0.04$) and a decrease in mean spike amplitude ($p =0.04$).

Our electrophysiological measurements were limited to the layer 2/3 where the morphological changes were found. Although preliminary data suggests that there are no

significant changes in intrinsic properties or input-output curves of layer 5 pyramidal neurons in either the IL or PL. Inclusively, our electrophysiological data indicate a significant decrease in excitability of IL neurons, concomitant to an increase in PL neuron excitability in PN-FLX mice.

	Resting potential (mV)	Spike amplitude (mV)	R_{in} (MΩ)	C_m (pF)	Rheobase (pA)
Infralimbic Layer II/III					
PN-Veh (*n*=13)	-85.5 ± 2.8	87.3 ± 1.5	165.0 ± 25.9	86.5 ± 5.0	134 ± 26
PN-FLX (*n*= 19)	-83.6 ± 1.8	84.9 ± 1.2	130.9 ± 13.2	91.6 ± 9.0	150 ± 16 (n=17)
P	0.6	0.2	0.2	0.7	0.6
Prelimbic Layer II/III					
PN-Veh (*n*=10)	-86.6 ± 2.4	88.5 ± 1.1	77.9 ± 15.4	99.8 ± 7.5	255 ± 31
PN-FLX (*n*= 20)	-88.9 ± 1.6	83.7 ± 1.4	115.7 ± 13.7	87.1 ± 4.9	173 ± 21 (n=17)
P	0.4	0.04*	0.1	0.1	0.03*

Table 2.1. Intrinsic properties of layer 2/3 pyramidal neurons in the IL and PL of adult PN-FLX and PN-Veh mice. Data are shown as mean ± SEM and includes *p* values for unpaired *t*-tests. N = 3-6 mice/treatment group. *p <0.05. (Data courtesy of N. Goodfellow).

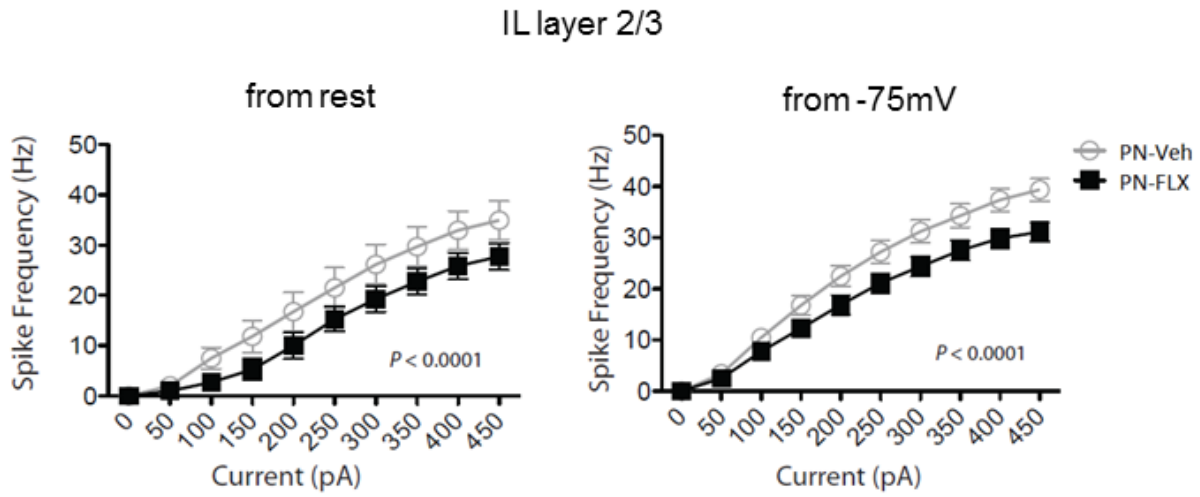


Fig 2.18. Input-output curve depicting frequency of spikes fired in response to varying current steps, from resting membrane potential (left) or from -75mV (right) for IL layer 2/3 neurons from PN-Veh and PN-FLX mice. PN-FLX mice fire significantly fewer spikes at higher currents (main effect of treatment, two-way ANOVA, for both panels: $p < 0.0001$). N = 3-6 mice/treatment group 10-20 cells/group. (Data courtesy of N. Goodfellow).

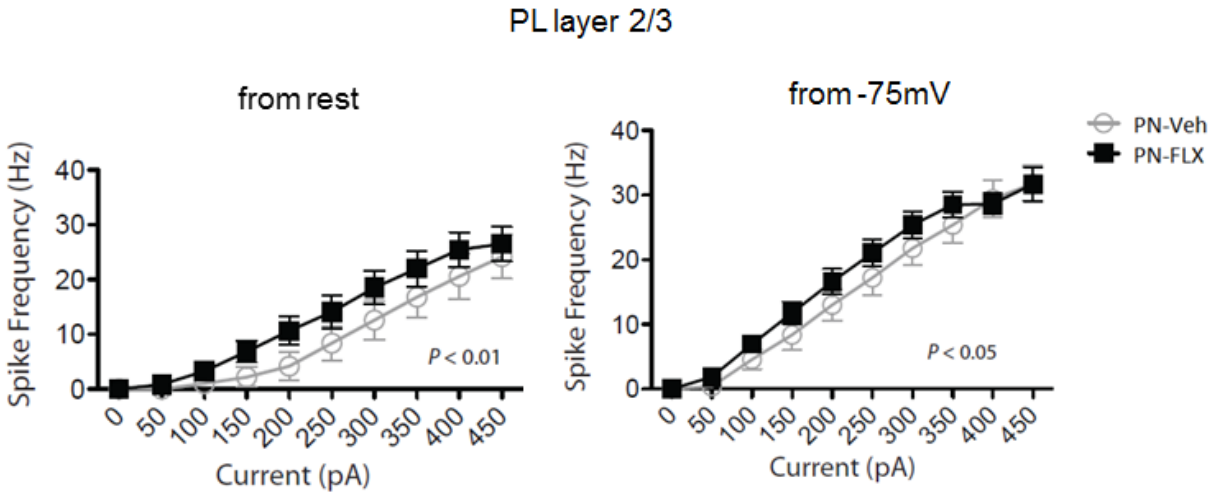


Fig 2.19. Input-output curve depicting frequency of spikes fired in response to varying current steps, from resting membrane potential or from -75mV, for PL layer 2/3 neurons from PN-Veh and PN-FLX mice. PN-FLX mice fire significantly more spikes at higher

currents (main effect of treatment: from rest, $p < 0.01$; from -75 mV $p < 0.05$). N = 3-6 mice/treatment group 10-20 cells/group. (Data courtesy of N. Goodfellow).

Aim 1a (iii). **PN-FLX mice display pronounced deficits in fear extinction learning and recall**

To gauge changes in mPFC output, PN-FLX mice underwent classical Pavlovian cued fear conditioning and subsequent extinction. During the conditioning phase, we detected no significant differences between post-natal treatment groups in either baseline freezing to training context "A" (2.20), in response to tone-shock pairings or post-training freezing levels (Fig. 2.21). On the following day, mice were placed in context "B", where they received extinction training. No significant differences in baseline freezing to novel context "B" between post-natal treatment groups were observed (Fig. 2.22). However, in response to the first three tones on extinction day 1 (24 hours after cued fear conditioning), PN-FLX mice have significantly reduced % freezing relative to controls ($F_{1,45} = 12.480$; $p = 0.0010$, Fig 2.23).

As visible in Fig. 2B, during the extinction training session (D1), PN-Veh controls exhibited a robust extinction learning phenotype as indicated by a progressive decrease in freezing over time (with exposure to each subsequent set of tones in absence of the noxious shock). PN-FLX mice, on the other hand, exhibit a pronounced deficit in extinction learning as indicated by the absence of a decrease in freezing levels over the extinction training session (post-natal treatment x time effect, repeated measures ANOVA: $F_{5, 240} = 3.204$; $p < 0.0001$). Post-tone freezing is also significantly higher in PN-FLX mice relative to controls ($F_{1, 48} = 21.474$; $p < 0.0001$).

To control for the differences in initial freezing to tone, % freezing values for all bins were normalized to each post-natal treatment group's respective initial freezing levels (at D1 start, Fig 2.25). As we previously saw in Fig. 2.24 (un-normalized data), PN-FLX mice display a deficit in extinction learning when compared to PN-Veh controls, even when % freezing is normalized to baseline response to tone (D1 start vs. end: treatment x time interaction $p < 0.001$). Between D1 and D2, neither PN-Veh nor PN-FLX mice displayed extinction recall (D2 start versus D1 start not statistically significant). On D2, both PN-Veh and PN-FLX mice displayed pronounced and comparable levels of extinction learning ($p < 0.001$ for effect of time; no treatment effect or interaction). However, on D3, despite learning to extinguish the CS-US memory to the same extent on D2, only PN-Veh displayed extinction recall, whereas PN-FLX do not (D3 start versus D2 start statistically significant only for PN-Veh; $p < 0.01$).

Overall, our findings suggest that mice experiencing increased serotonergic tone from P2-11 display deficits in both the learning and memory components of fear extinction and are congruent with our hypothesis that there exists a decrease in IL/PL output ratio in PN-FLX mice.

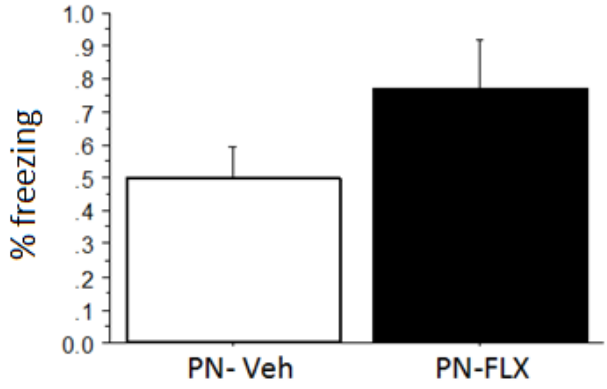


Fig 2.20. PN-FLX and PN-Veh mice have comparably low levels of baseline freezing to fear conditioning context "A". N = 23-24 mice per treatment group.

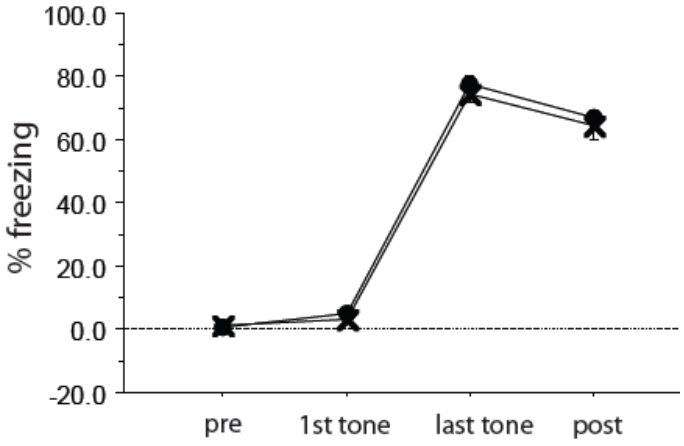


Fig. 2.21 PN-FLX mice exhibit comparable levels of freezing at baseline, in response to the first and last tones of the training session and in the post-training period, as PN-Veh controls. N = 23-24 mice per treatment group.

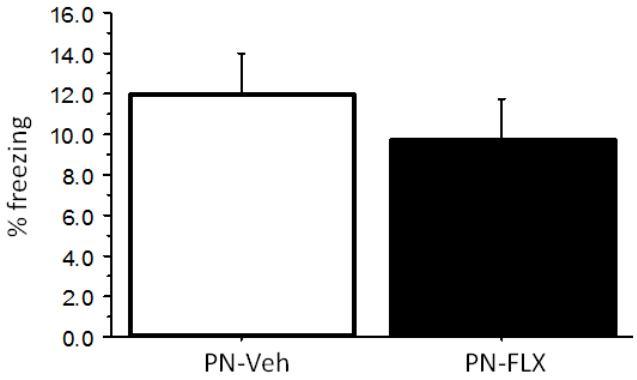


Fig. 2.22 PN-FLX and PN-Veh mice have comparably low levels of baseline freezing to the novel training context. N = 23-24 mice per treatment group.

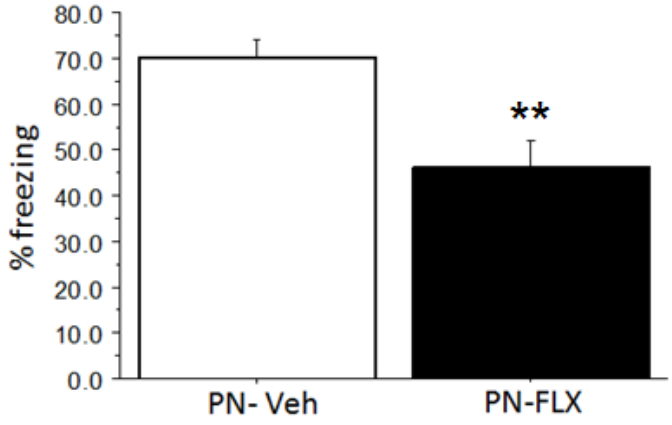


Fig. 2.23 PN-FLX mice freeze significantly less to the first tone on extinction day 1, relative to controls. **p < 0.01. N = 23-24 mice per treatment group.

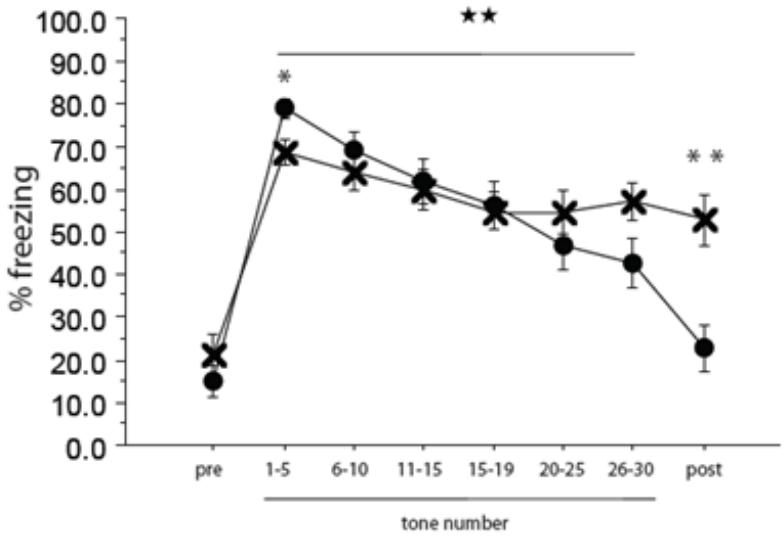


Fig. 2.24 PN-Veh mice exhibit a progressive decrease in freezing to tone over the entire extinction training session (day 1, D1) while PN-FLX mice do not (★★ treatment x time interaction effect : $p = 0.0081$). PN-FLX mice also exhibit significantly higher post-training freezing levels (** $p < 0.0001$). N = 24-25 mice per treatment group.

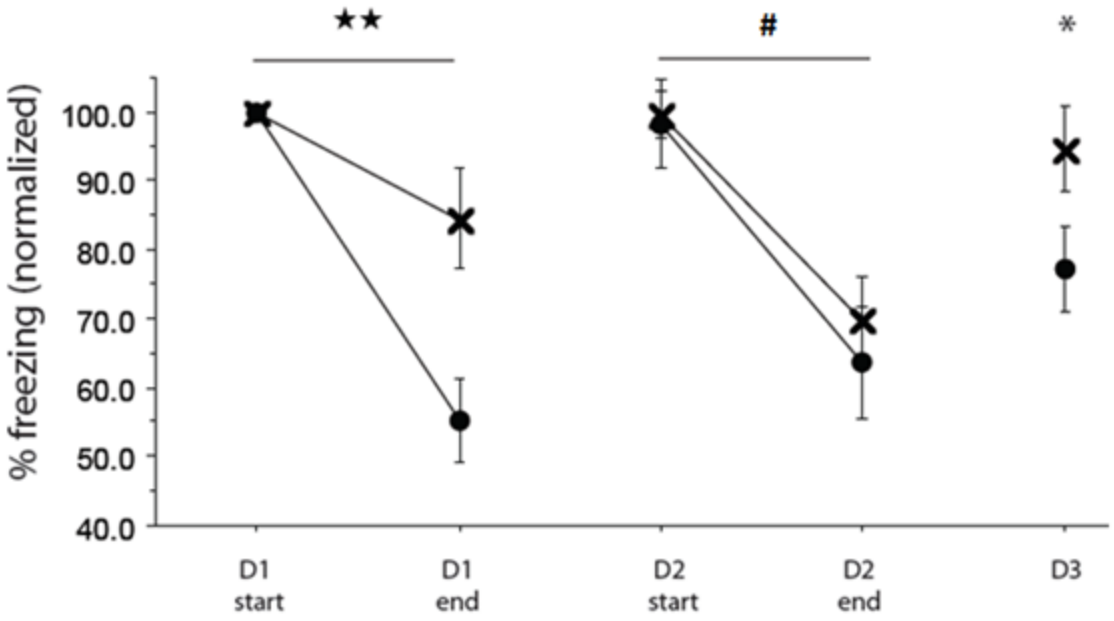


Fig 2.25. Freezing levels of PN-FLX and PN-Veh mice, normalized to their starting freezing levels at the start of the first extinction training (day 1, D1). On D1 of extinction training, PN-FLX treated mice displayed a deficit in extinction learning, when compared to PN-Veh controls (★★ treatment x time interaction $p < 0.001$). Between D1 and D2, neither PN-Veh nor PN-FLX mice displayed extinction recall (D1 start versus D2 start not statistically significant). On D2, PN-Veh and PN-FLX mice displayed extinction learning (# effect of time: $p < 0.001$, no treatment or interaction effects). Between D2 and D3, only PN-Veh displayed extinction recall (* D2 start versus D3 start statistically significant only for PN-Veh; $p < 0.01$). N = 24-25 mice per treatment group.

Aim 1b. **IL lesions result in a partial phenocopy of the PN-FLX mouse behavioral phenotype**

Next we addressed the question of whether the changes in the mPFC were causal to the behavioral phenotype of PN-FLX mice (Figs. 1.2-1.4). To mimic the decreased IL/PL output of PN-FLX mice, mice underwent stereotactic surgery in which they received two bilateral injections of the neurotoxin, ibotenic acid, resulting in cell death and gliosis of the IL cortex. We histologically verified the location of the lesions and excluded subjects that had non-IL lesions. Figure 2.26 displays the location and extent of the lesions in control mice. After a minimum 2 week recovery period, mice underwent behavioral testing. In the open filed test, one-way ANOVA demonstrated a significant effect of surgery on % time in center in which IL-lesion mice spent a significantly reduced amount of time exploring the central, more anxiogenic region of the arena ($F_{1,45}$ = 4.722; p = 0.0351, Fig. 2.27), relative to sham lesion controls, suggesting an enhancement of anxiety-like behavior in IL-lesion animals. IL-lesion mice also spent significantly less time engaging in exploratory vertical activity, relative to sham-lesion controls ($F_{1,42}$ = 4.154; p =0.0479, Fig. 2.28). Total ambulatory time and distance were not affected by surgery (Fig 2.29). IL lesions also resulted in an increase in the time to approach food (latency to feed) in the novelty suppressed feeding test in both PN-Veh and PN-FLX mice ($F_{1,45}$ = 4.667. p = 0.0361, Fig. 2.30). In the familiar home-cage environment, PN-FLX mice do not differ from controls in amount of food consumed (Fig. 2.31A). Effects of 24hr food deprivation between groups was also not significantly different (Fig. 2.31B) In the shock escape test lesioning the IL was not sufficient to phenocopy the increased latency to escape phenotype of PN-FLX mice as no differences

in latency to escape the shocks (Fig 2.32A) or baseline ambulatory activity during the habituation phase (Fig. 2.32B) between IL- and sham-lesion mice were detected.

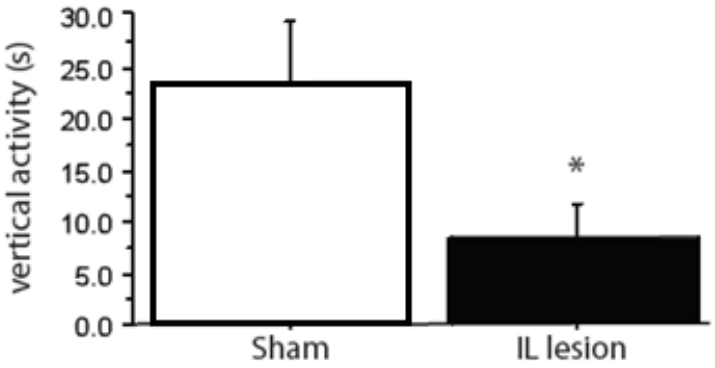


Fig 2.26. IL lesioned mice spend significantly less time engaging in vertical activity (time rearing) in the open field test, relative to Sham lesioned controls (* p = 0.0479). N = 23-24 mice per treatment group.

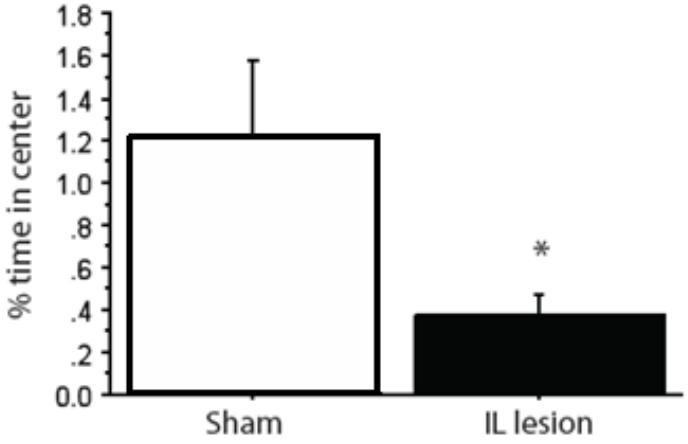


Fig 2.27. IL lesioned mice spend significantly less % time in center, relative to Sham lesioned controls (* p = 0.0351). N = 23-24 mice per treatment group.

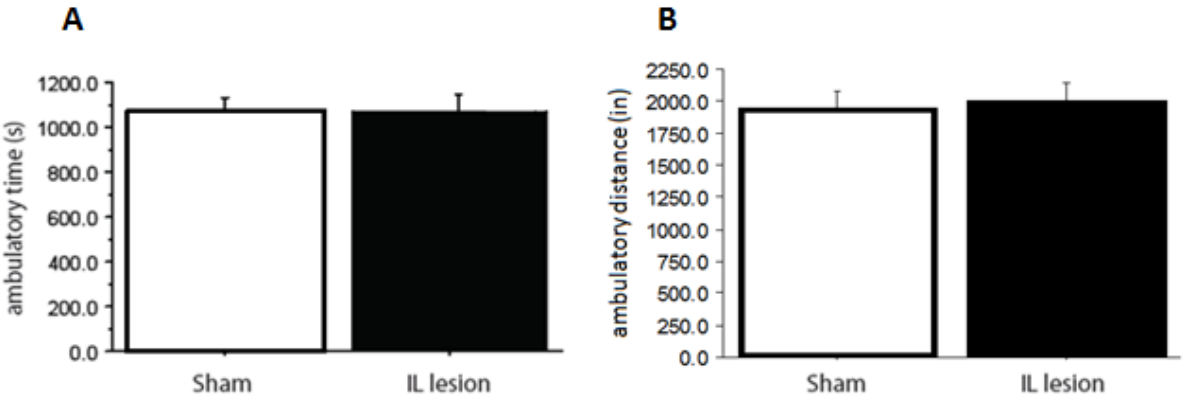


Fig 2.28. No differences in total ambulatory time (A) or distance (B) between PN-Veh and PN-FLX mice in the open field test. N = 23-24 mice per treatment group.

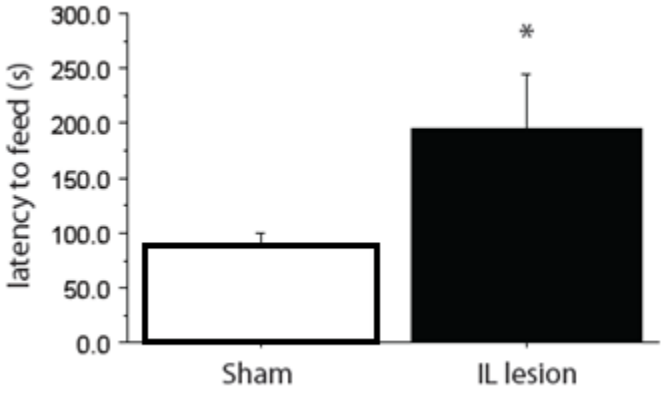


Fig 2.29. PN-FLX mice take significantly longer to approach the food pellet relative to PN-Veh mice, in the Novelty suppressed feeding test (* p = 0.0361). N = 23-24 mice per treatment group.

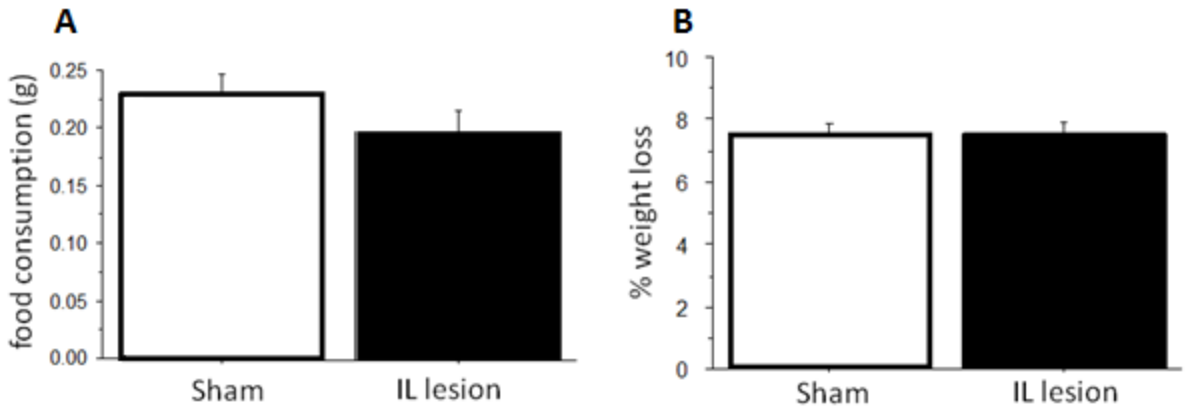


Fig 2.30. No significant differences between Sham- and IL-lesion animals in home-cage food consumption (A) or % weight loss after 24hr food deprivation (B).

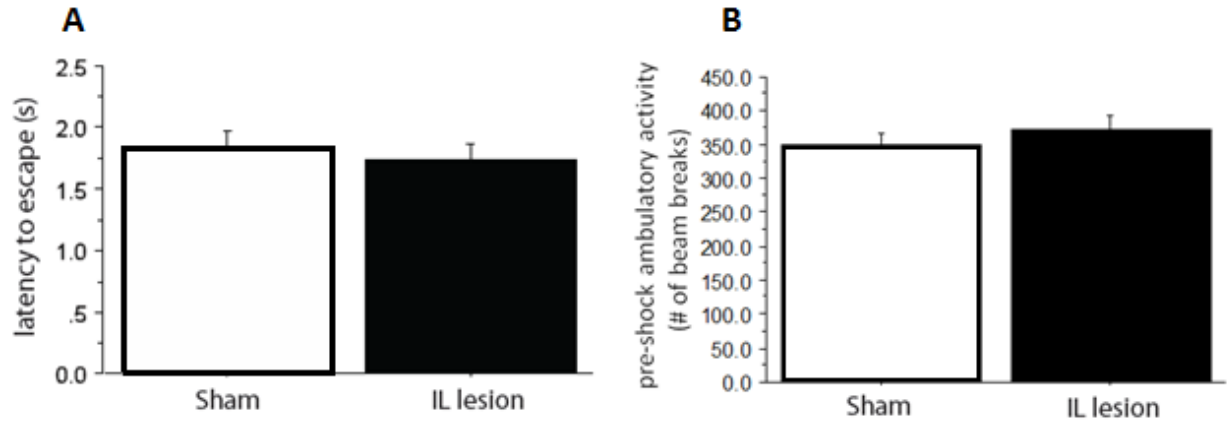


Fig. 2.31. No significant differences between Sham- and IL-lesion animals in latency to escape (A) or in baseline activity levels (B) in the shock escape test.

Discussion

Aim 1a (i). Structural changes

We have found pronounced cytoarchitectural changes in the mPFC of PN-FLX mice, suggesting that the structural development of the mPFC is sensitive to 5-HT levels in the early post-natal period (Figs. 2.4-2.7). Moreover, these changes are present specifically in the apical dendritic trees of layer 2/3 pyramidal neurons in the IL sub-field of the mPFC, a region in which volume reductions are also found in human "*s*" allele carriers (Pezawas et al., 2005). Thus, the altered volume in human "*s*" allele carriers may also have developmental roots. *5-htt -/-* mice also display changes in the IL region of the mPFC, however, the layer-specificity and directionality of the changes do not exactly mirror those found in PN-FLX mice (Appendix B, Fig.B.1-B.2). This is unsurprising as the constitutive loss of 5-HTT may generate an arborization phenotype quite dissimilar from that of PN-FLX mice who experience elevated 5-HT strictly during development, due to compensatory changes or due to persistently elevated 5-HT in adulthood.

The fact that arborization changes are limited to the IL region, and not the PL, may suggest that IL arbors are particularly sensitive to 5-HT levels during development. The increased sensitivity of IL neurons could be due to an enhancement of specific 5-HT receptors that mediate the cytoskeletal changes that affect dendritic arborization. Interestingly, the regions in which structural and functional changes are observed in "*s*" allele carriers have been shown to have especially high levels of 5-HTT (Varnas et al., 2004). A similar enrichment in 5-HTT may be present in the IL which could account for the most prominent effect of 5-HTT inhibition in this region. PL and IL arbors may also develop at different time points. Perhaps during the P2-11 period, the primary elaboration

processes, such as node formation and branch elongation are underway in the IL, while these processes may occur at an earlier, or later, time window in the PL region. This in turn could explain the IL-specific arborization changes in PN-FLX mice.

The enhanced sensitivity of the IL region is not limited to early-life FLX treatment as IL hypertrophy is also generated by other anxio- and depresso-genic paradigms. For instance, both chronic mild stress and brief uncontrollable stress, in adulthood, cause a retraction in the apical trees of IL pyramidal neurons, as evidenced by significantly reduced branch numbers and length, relative to unstressed animals (Radley et al., 2004; Cook and Wellman, 2004; & Radley et al., 2005; Liston et al., 2006; Izquierdo et al., 2006; Farrell et al., 2010). One study of the effects of chronic stress on IL arborization demonstrated a specific effect on neurons that projected to the entorhinal cortex and not in those projecting to the basolateral nucleus (BLA) of the amygdale (Shansky et al., 2009). It would be interesting to know whether similar dissociations exist in PN-FLX mice as it would provide insight into the specific corticolimbic circuits that are altered with early-life disruption of serotonergic tone. Together, these findings demonstrate that IL dendritic arbors are particularly sensitive to manipulations of either 5-HT or to factors that elicit emotional dysfunction. Indeed, stress itself has been shown to increase 5-HT levels in various brain regions, including the mPFC (Mokler et al., 2007; Mo et al., 2009). Thus the stress-induced and PN-FLX-generated hypotrophy of the IL may occur via similar mechanisms. Furthermore, while adult stress-induced changes in mPFC morphology are reversible (Radley et al., 2005; Goldwater et al., 2009), *early-life* stress has long-term and stable consequences on mPFC dendritic arbors (Murmu et al., 2006; Martinez-Tellez et al., 2009; Bock et al., 2011; Mychasiuk et al., 2011). This

fortifies the existence of critical time windows in the development of the structural properties of neurons in the mPFC, during which manipulations affecting 5-HT can result in permanent changes to mPFC structure.

Why the structural changes are observed specifically in layer 2/3 is a question of note that may shed light into the putative mechanism by which 5-HT affects dendritic arbor formation in cortical neurons. Early-life 5-HT-mediated signaling via the 5-HT3A receptor (5-HT3AR) is extremely important in proper maturation of the dendritic arbors of cortical layer 2/3 neurons (Chameau et al., 2009, Smit-Ritger et al., 2011a). The mechanism by which this occurs is dependent on Cajal-Retzius (CR) cells that release the glycoprotein reelin in a 5-HT3AR-dependent fashion. Reelin in turn acts to inhibit the extent to which dendritic elaboration occurs. Thus, 5-HT3AR genetic loss, pharmacological inhibition or anti-reelin antibody administration, all result in hypercomplexity of cortical neurons in layer 2/3 (Chameau et al., 2009). Conversely, increased activation of the 5-HT3AR due to increased 5-HT levels, for instance, would result in reduced complexity of apical arbors, and would be congruent with the dendritic phenotype of PN-FLX mice, especially in light of the fact that CRs are present and active in the first two postnatal weeks. Indeed, it has been shown that the decreases in dendritic arborization of mice treated with FLX in early (embryonic) development, can be reversed by pharmacological inhibition of the 5-HT3AR, and this hypotrophy does not occur in FLX-treated 5-HT3AR-/- mice (Smit-Rigter et al., 2011). Since CR cells reside in the superficial layers of the cortex (marginal zone/layer 1) it is understandable why its dendritic effects, via soluble reelin release, are limited to the near-by layer 2/3 neurons. Additionally, the level of expression of ($\alpha 3\beta 1$) integrin receptors, through which reelin is

thought to mediate its effects on dendritic arborization (Chameau et al., 2009), may be higher in IL region. This too may account for the fact that changes in PN-FLX mice are limited to the IL layer 2/3 region.

Taken together, our morphological data suggest that, as hypothesized, 5-HT acts during development to affect the maturation of mPFC structure. Furthermore, our findings underscore the neurotrophic properties of 5-HT, and demonstrate that the effects 5-HT on dendritic growth cannot be generalized, but rather are dependent on various factors such as location (layer-specificity) of the neuron, type of arbor (basilar vs. apical), which are likely reflective of differences in receptor types and responsivity of each region to 5-HT mediated trophic effects.

Aim 1a (ii). Electrophysiological changes

Concomitant to the changes in cytostructure, we find alterations in physiological properties of mPFC pyramidal neurons from PN-FLX mice. In the IL, for example, we see a marked reduction in the frequency of spikes generated in response to current injections (Fig. 2.18) indicating that IL neurons from PN-FLX mice are significantly less excitable than those of controls. Conversely, we find that PL pyramidal neurons from PN-FLX mice are more excitable, as they yield an increased number of spikes in response to current injections (Fig. 2.19). Thus, the electrophysiological footprint of early life perturbations of serotonergic tone is a change in the balance between IL and PL excitability resulting in a putative decrease in the IL/PL output ratio.

The question that arises next is how does 5-HT, acting during development, lead to persistent changes in mPFC excitability? In order to address this question a keen

understanding of the mechanisms underlying excitability are needed. Spike responses to current injections are influenced by passive electrical properties of neurons such as their resting membrane potential (RMP) and input resistance ($R_{in)}$ (Kandel et al., 2000; Part II, Chapter 8). Thus, excitability changes in PN-FLX mice could be due to 5-HT-mediated changes in these parameters. However, in IL neurons we find no differences in any of the intrinsic properties measured [RMP, R_{in}, membrane capacitance (C_m), spike amplitude and rheobase] (Table 2.1). Whereas in the PL we find a reduction in rheobase values signifying that PL neurons from PN-FLX mice have lower thresholds for spike generation, which is congruent with their enhanced excitability. The spike amplitude of PL neurons of PN-FLX mice is also significantly reduced and likely reflect changes in the cellular mechanisms underlying the currents that generate spikes, such as variations in number of VG sodium channels or receptor activation/inactivation properties (Kandel et al., 2000; Part II, Ch. 9), which may be influenced by 5-HT.

The lack of difference in R_{in} and C_m in both IL and PL neurons is particularly interesting as it may reveal the relationship between the morphological and physiological changes in PN-FLX mice. The morphological properties of a neuron can potentially influence its physiology, by affecting surface-area (SA) dependent intrinsic properties such as R_{in} and C_m, which in turn can affect the neuronal responses to current pulses (Kandel et al., 2000; Part II, Ch. 7-9). The lack of changes in these SA-dependent parameters, may suggest that the cytoarchitectural changes caused by early-life perturbations to 5-HT may be concomitant, but not causal, to the physiological phenotype of PN-FLX mice. A major caveat to this interpretation is that the technique we employed (whole-cell patch clamping), which primarily measures currents at the cell body, may be

unable to capture changes in dendritic area, despite them being prominent in the region proximal to the soma (Fig. 2.7). Moreover, the effect of morphological changes on physiology, may not be related to changes in intrinsic properties, but may impact the SA over which synapses, receptors and channels are expressed. This in turn could affect firing properties of a neuron and could be addressed experimentally by measuring spine density and channel/receptor expression via staining studies. An alternate means by which 5-HT could influence mPFC physiology is by affecting the repertoire of receptors and channels expressed at the surface of IL neurons later in adulthood. Variations in the balance between voltage-gated (VG) sodium, potassium and chloride channels on IL neurons could affect their excitability, without affecting resting neuronal properties such as R_{in} and RMP. For instance, various receptors, such as the metabotropic glutamate receptor 5 (mGluR5), N-methyl-D-Aspartate (NMDA) receptors, M-type potassium (K+) channel or the β-adrenergic receptor (β-AR) influence the intrinsic properties and/or excitability of mPFC neurons (Burgos-Robles et al., 2007; Mueller et al., 2008; Santini and Porter, 2010; Fontanez-Nuin et al., 2011). Although this has not been addressed experimentally, early-life 5-HT levels may impact the expression of these receptors in a stable and long-lasting manner such that their adult levels are altered, thereby affecting IL and PL neuronal physiology. Indeed, there is evidence that PN-FLX may in fact have such long-lasting effects on the expression of specific receptors and signaling molecules in adult neurons in the dorsal raphe nucleus (Neil Gray, unpublished results). Thus it is plausible it may similarly change affect receptor expression in the mPFC.

Indeed, the ability of circumscribed changes in 5-HT levels during development to leave a cellular fingerprint that affects physiology is not unheard of. For instance, it

has been shown that modulating 5-HT levels in embryonic development, alters receptor expression and ligand-binding well after birth (Azmitia, 1987). Early life stress, which has been shown to alter 5-HT levels in various limbic structures, also has long term effects on receptor mRNA editing and expression of intracellular signaling molecules such as G-proteins (Englander et al., 2005; Bhansali et al., 2007). Moreover, the impact of early-life changes in 5-HT is not limited to cells in the mPFC, as neurons in other regions, such as in the dorsal raphe nucleus, also have altered physiology after PN-FLX treatment (Emanuela Morelli, unpublished results). These data support the idea that physiological properties of neurons in the mPFC, and other regions, are under the influence of 5-HT during the early post-natal period.

Preliminary data reveals that a similar IL and PL physiological phenotype is found in *5-htt -/-* mice (Goodfellow et al., unpublished data). Although such comprehensive physiological studies have not been conducted in humans, imaging studies have revealed similar alterations in the activity of the mPFC in *"s"* allele carriers and depressed/anxious patients. For instance, diminished activity, as measured by glucose metabolism, cerebral blood flow and blood oxygen level dependence (BOLD) based fMRI studies suggest that there indeed are activity and physiological changes in the human PFC in patients with emotional dysfunction (Price and Drevets 2010). Additionally, post-mortem studies in depressed patients show reductions in mPFC neuronal activation as evidenced by reduced expression of immediate early genes such as *sif268, egr1, c-fos* and *arc* in regions analogous to the IL in rodents (Covington III et al., 2010). Thus, altered mPFC physiology may be an endophenotype of depression and anxiety disorders. Finally, based on our findings in PN-FLX mice, who share the

structural and functional changes in the mPFC with the *5-htt -/-* and "*s*" allele carriers, but who only experience altered 5-HT in development, it is likely that the physiological changes observed in the animals/humans experiencing constitutive 5-HTT hypofunction result as a consequence of developmental changes.

An important caveat is that although we see differences in *intrinsic* excitability of mPFC neurons, our experiments do not assess network effects or extent of input onto these neurons, which would influence their *in-vivo* physiology. For example, although IL neurons may display blunted intrinsic excitability, they may receive enhanced excitatory input which may compensate for their diminished firing propensity. Thus, the extent of excitatory and inhibitory input on these cells is also an important consideration when characterizing their physiological phenotype. This excitatory/inhibitory balance could be gauged experimentally, by measuring the number of mini excitatory vs. inhibitory post-synaptic potentials (mEPSPs and mIPSPs) in these neurons. Moreover, although we know that given a certain input, IL and PL neurons from PN-FLX mice have blunted and enhanced responses, respectively, our experiments to do not directly address the afferent projections that recruit these regions during specific behavioral paradigms. For instance, afferent input from the hippocampus (Farinelli et al., 2006; Garcia et al., 2008) and medio-dorsal thalamus (Herry et al., 1999; Li et al., 2004; Hugues and Garcia, 2007) are recruited during fear learning and extinction, and the extent to which these afferents communicate with the mPFC may predict how well an animal learns to extinguish learned fear. As an example, long-term potentiation at HC-mPFC synapses and BDNF release from hippocampal afferents in the mPFC are both important in acquisition of fear extinction (Farinelli et al., 2006; Peters et al., 2010). Thus, we cannot rule out that there

may be changes in afferent input that could compensate for, or perhaps enhance, the effect of the physiological changes in the mPFC of PN-FLX mice.

Finally, the directionality of the changes in mPFC neuron firing are also revealing and provide a putative mechanism for the etiology of the affective and fear-related phenotypes of PN-FLX. The importance of mPFC neuronal firing in fear-related behaviors, particularly in the fear extinction learning and recall has been well-characterized (summarized in Table 2.1). Extinction recall, for example, is associated with a pronounced increase in burst firing in the IL region of the mPFC (Milad and Quirk, 2002; Santini et al., 2008; Rabinak et al., 2008; Santini and Porter, 2010). As such, manipulations that that dampen IL excitability produce fear extinction deficits (Sierra-Mercado et al., 2006; Farrell et al., 2010; Santini and Porter, 2010; Sierra-Mercado et al., 2011). Conversely, enhanced PL excitability is associated with deficits in fear extinction, and in order for successful extinction learning to take place the PL needs to be silenced (Vidal-Gonzalez et al., 2006; Santini et al., 2008; Burgos-Robles et al., 2009; Sierra-Mercado et al., 2011).

These data suggest that IL recruitment and PL suppression is required for suppression of the fear-conditioning memory, and for recall of the extinction training. This fact is congruent with the known connectivity of these mPFC subregions to the amygdala—another region whose output is shown to modulate fear-related behaviors (Phillips and LeDoux, 1992; LeDoux, 2003). The IL sends excitatory projections to inhibitory GABAergic neurons that reside in the intercalated cell layer and to the basolateral amygdala (BLA) itself (Fig 2.33). Thus, enhanced IL output increases inhibitory tone in the amygdala, which through its projections to regions such as the

hypothalamus, bed nucleus of the stria terminals (BNST) and periacqueductal gray (PAG) mediates the physiological effects of fear such stress-hormone release, autonomic changes and freezing responses. Conversely, PL output increased excitation of key nuclei within the nucleus, such as the BLA and the central nucleus, which in turn enhance fear responses. Thus, this change in IL/PL excitability, would affect the mPFC-amygdala circuitry in a manner that would enhance fear-related behaviors in PN-FLX mice. This hypothesis was directly tested in the subsequent section.

	Infralimbic	**Prelimbic**
Lesions/Inhibition	Blunted extinction learning and recall	Decreases fear expression
Stimulation	Attenuates fear, enhances extinction learning and recall	Enhances fear, Blunts extinction learning and recall
During extinction	Increased output	Decreased output

Table 2.2. Summary of work in the field that demonstrates the opposite influence of infra- and pre-limbic regions on activation of the amygdala on fear behaviors (Quirk et al., 2000; Santini et al., 2003; Vidal-Gonzalez et al., 2006, Farrell et al., 2010; Sierra-Mercado et al., 2010).

Aim 1a (iii). mPFC functional output

Although PN-FLX mice display pronounced structural and physiological changes in the IL and PL, these changes may not necessarily affect mPFC output, if, for instance, there are compensatory changes in afferent input or local network activity in this region. To address this, we directly probed mPFC function by using a well-characterized mPFC-dependent fear extinction behavioral paradigm (Milad and Quirk 2002; Quirk et al.,

2006). We found that PN-FLX mice display pronounced deficits in both fear extinction learning and its subsequent recall (Figs. 2.24, 2,25), demonstrating that the structural/physiological changes in the mPFC are associated with altered mPFC function, as hypothesized. Based on our electrophysiological findings and the circuitry presented in Fig. 2.33, the blunted extinction learning and recall phenotype of PN-FLX mice is consistent with a decreased IL/PL output. For instance, pharmacological inactivation and anatomical lesions of the IL, which essentially replicate, in an extreme manner, the IL hypofunction observed in PN-FLX, phenocopy the PN-FLX fear extinction deficits (Sierra-Mercado et al., 2006; Farrell et al., 2010; Santini and Porter, 2010; Sierra-Mercado et al., 2011) . Likewise, norepinephrine, via the β-adrenergic receptor (β-AR), enhances IL intrinsic excitability, thus, inhibition of β-ARs in the IL decreases its output and consequently results in pronounced extinction deficits (Mueller et al., 2008). It was also shown that IL excitability is positively correlated with extinction recall (Santini et al., 2008). Interestingly, the retraction of the IL apical arbors that occurs with chronic stress is also associated with extinction deficits (Miracle et al., 2006; Izquierdo et al., 2006) and a negative correlation between extent of IL hypertrophy and extinction learning/memory was found (Izquierdo et al., 2006). Enhancements of PL activity, via microstimulation, which mimic the hyperfunction of the PL neurons from in PN-FLX mice result in extinction recall deficits (Vidal-Gonzalez et al., 2006; Sierra-Mercado et al., 2011). Administering the M-type K+ channel agonist, flupirtine, directly into the IL region, reduces its intrinsic excitability, mimicking the PN-FLX phenotype and also phenocopies the extinction recall deficits of PN-FLX mice (Santini and Porter, 2010). Taken together, these data suggest that the structural/physiological changes in the mPFC

of FLX mice are causal to their fear-extinction phenotype and indicated changes in mPFC output resulting from the morphological/physiological changes in this region.

Congruently, deficits in fear recall have also been characterized in *5-htt -/-* mice that share both the morphological and physiological mPFC phenotypes of PN-FLX mice (Wellman et al., 2007) and also exhibit blunted fear extinction behaviors. A similar correlation exists between changes in mPFC structure/function has been described in human "*s*" allele carriers and a higher vulnerability to developing PTSD (Lee et al., 2005; Kilpatrick et al., 2007) an anxiety disorder in which the prominent characteristic is the inability to extinguish traumatic memories. Our data suggest that the diminished ability to quench traumatic memories may be due to dysfunctional mPFC output due to altered serotonergic tone during development. Thus, drugs that may correct the putative imbalance in output ratio of specific substructures of the mPFC may provide benefits as therapeutic agents or adjuncts, in treatment of PTSD.

An important consideration is that other brain regions also play a role in extinction learning and recall, although their involvement have not been as thoroughly, nor reliably, characterized. For instance, the HC has been shown to affect extinction and learning behaviors (Corcoran et al., 2005; Milad et al., 2007; Peters et al., 2010). Thus, although we are using this behavioral paradigm to test for changes in mPFC function, we cannot rule out that the deficits in extinction behaviors we see are also due to other brain regions. Indeed, the crosstalk between regions such as the mPFC and HC will be discussed in detail in the last chapter of this thesis.

Interestingly, PN-FLX mice also show deficits in fear expression itself, as evidenced by significantly reduced freezing to tone ("cue" or "conditioned stimulus")

following cued fear conditioning (Fig.2.24). The expression of fear in response to the cue is classically thought to be dependent on amygdala function (Phillips and LeDoux, 1992), which suggests that PN-FLX mice may also have alterations in amygdala activity. Indeed, structural and imaging findings in *5-htt -/-* and *"s"* allele carriers suggest that the amygdala might be another locus of dysfunction in mice and humans that experienced altered serotonergic tone during development (Hariri et al., 2002; Pezawas et al., 2005; Wellman et al., 2007). Although this hypothesis merits further investigation, we have not addressed these question directly in the current body of work for several reasons. First, the deficits in freezing to cue lack replicability, as they were not present in a subsequent cohort tested while the pronounced extinction deficit phenotype were still observed (Appendix C, Fig. C.1-C.2). Secondly, cued freezing deficits may not necessarily indicate amygdala dysfunction. For instance, it was shown that altering the excitation/inhibition (E/I) balance in the mPFC results in potent deficits in freezing to cue (Yizhar et al., 2011). Thus, the changes in IL/PL output, or rather as yet uninvestigated changes in the excitation vs. inhibitory input into these regions, could potentially result in the cued deficits that are observed independent of any changes in the amygdala. Similarly, microstimulation studies have shown that enhancing output from the IL or PL can differentially modulate the expression of conditioned freezing to cue (Milad et al., 2004; Vidal-Gonzalez et al., 2006) and tetradotoxin mediated inhibition of the ventral HC also produces cued freezing deficits (Bast et al., 2001). To address these questions one could better characterize the E/I ratio in mPFC neurons of PN-FLX mice, by, for instance quantifying the frequency and number of mini excitatory and inhibitory post-synaptic potentials (mEPSCs and mIPSCs) in the IL vs. PL. Characterization of the morphology

and physiology in the amygdala to determine whether early-life 5-HT alterations also affect the functional development of this region is also warranted.

In this section, we have shown that the structural and morphological properties of mPFC neurons are set during a critical time window in post-natal development, and are under direct influence of 5-HT. As such, perturbing normal serotonergic tone during these critical time windows, permanently alters their structure/morphology which in turn affects mPFC output resulting in changes in mPFC-mediated behaviors such as extinction learning and recall. As the mPFC is involved in emotional regulation, in the next section we test whether the change in mPFC output we have characterized can also account for the affective phenotype of PN-FLX mice.

Aim 1b. Testing the link between the mPFC and behavioral changes

In the final part of our work, we investigated whether the changes in the mPFC are causal to the anxiety and depression-like phenotype of PN-FLX mice. There is copious correlative data implicating the mPFC in anxiety and mood regulation (Manji et al., 2001; Davidson 2002; Krishnan and Nestler, 2008; Price and Drevets, 2010). Altered volume, activity, and connectivity of this region have been characterized in patients with anxiety and/or depression and in mouse models of these conditions (Mayberg et al., 2003; Hariri et al., 2006; Wellman et al., 2007; Adhikari et al., 2010; Price and Drevets, 2010; Sotres-Bayon and Quirk, 2010). Despite their high correlative value, these data leave several questions unanswered. For instance, are the mPFC changes observed in depressed/anxious patients merely endophenotypes of the emotional dysfunction? Or do they arise as a consequence of disease progression? Or rather, are they causal to the

emotional behaviors? A substantial amount of data indicate that the mPFC is a primary locus of dysfunction in humans and rodents with emotional disorders and may be causal to their affective dysfunction. For instance, prevention of components of the mPFC morphological and/or physiological phenotype precludes the affective dysregulation in mouse models of anxiety/depression (Zoladz et al., 2008; Smit-Rigter et al., 2011). Moreover, therapeutic regimes used to treat affective dysfunction alter some of the mPFC phenotypes of depressed/anxious patients (Mayberg et al., 2003; Drevets et al., 2008; Moore et al., 2009; Covington III et al., 2011). Treatment with the SSRI class of anti-depressant/anxiolytic drugs, for example, can reverse the structural/physiological changes in the mPFC (Mayberg et al., 2000; Seminowicz, 2004; Bessa et al., 2009). Whereas DBS, a technique that has been shown to have efficacy in patients with treatment-resistant depression, suppresses the pathological activity within the mPFC network (Mayberg et al., 2003; Mayberg et al., 2005; Lozano et al., 2008; McNeely et al., 2008, Hariri et al., 2006). Additionally, the extent of mPFC volume reduction and physiological responses has predictive value in risk for anxiety and depression in humans and mice (Coryell et al., 2005; Izquierdo et al., 2006; Boes et al., 2010; Farb et al., 2011). Inclusively, these data indicate that the mPFC changes are likely causal to anxiety/depressive phenotypes.

Relating these findings back to the PN-FLX mice, who also display similar structural, physiological and functional changes in the mPFC, we asked whether their affective phenotype occurred as a result of focal mPFC alterations. Since the prime footprint of PN-FLX treatment is a decrease in the IL/PL output ratio, we recapitulated these changes in control mice, by specifically lesioning the IL region. We find that

changing this IL/PL balance in control mice is sufficient to phenocopy several (enhanced anxiety in novel open field, Fig 2.28; and suppressed feeding behavior in NSF test, Fig 2.30), but not all aspects (no change in helplessness behavior in SE test, Fig. 2.32) of their anxiety and depression-like behavior. In a somewhat complementary experiment, it was shown that optogenetic stimulation of the mPFC results in improvement in some depression-like behaviors in a rodent stress-model of enhanced anxiety/depression (Covington III et al., 2010). In particular, stimulation of the mPFC prevented the stress-induced reduction in anhedonic responses and social exploration, both of which are components of the depression. The fact that we cannot exactly replicate the phenotype of the PN-FLX mice, suggests that there are other brain regions, whose developmental trajectories are influenced by 5-HT, that may also be altered in PN-FLX mice, and may also contribute to the emotional phenotype of PN-FLX mice.

Inclusively, our data suggest that the structural/physiological changes in the mPFC of PN-FLX mice arise as a consequence of early-life manipulations of 5-HT levels. These changes in turn alter corticolimbic circuits such as those between the mPFC and amygdala and possibly other behaviorally relevant structures such as the hippocampus (discussed in next chapter). As corticolimbic circuits mediate affective behaviors, their dysregulation result in an enhancement in anxiety and depression in PN-FLX mice. This model is presented in Fig 2.33, which an emphasis on amygdala connectivity as this is the best characterized and has been probed directly in this body of work.

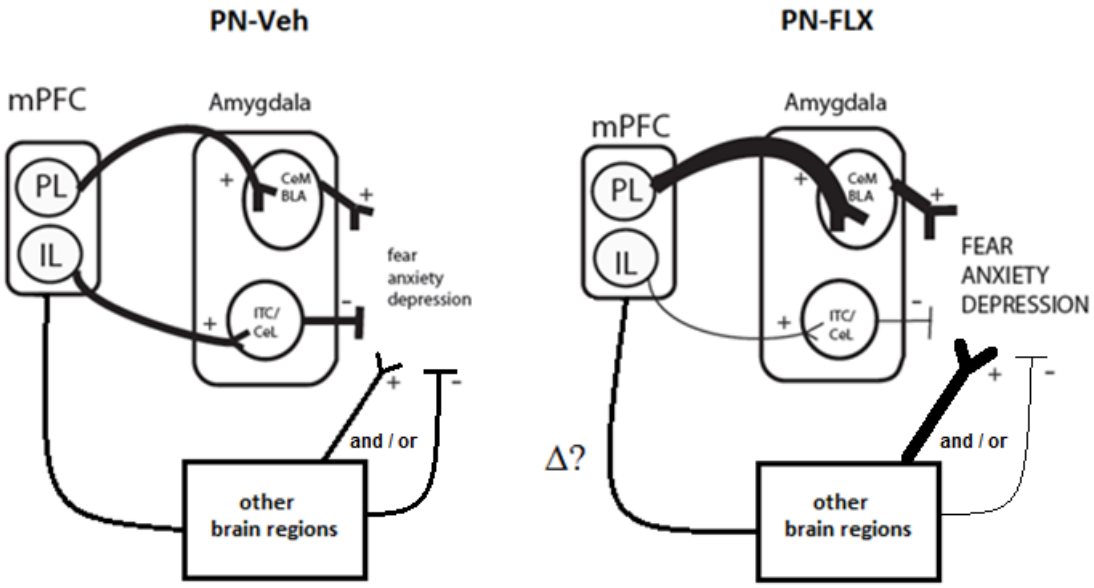


Fig. 2.32. Model depicting how changes in connectivity between mPFC the amygdala and other brain regions may result in the enhanced fear, anxiety and depressive behaviors in PN-FLX mice.

Concluding remarks

To return to our initial hypothesis, we have found that 5-HT affects the development of both structural and physiological properties of the mPFC and that this region is a major locus of dysfunction in mice that have experienced altered serotonergic tone during development. Moreover, we have demonstrated that the altered maturation of the mPFC is directly associated with its dysfunctional output, and thus provides a mechanism by which early-life alterations in serotonergic tone impact adult behaviors. Our data can be related to findings in "s" allele carriers, who display similar structural and functional changes in the mPFC. The altered function of the mPFC may act as a liability and may interact with further environmental stressors to enhance the vulnerability of an individual for affective dysfunction. Our findings thus provide a

model by which genetic or environmental factors that alter serotonergic tone during development can influence adult affective behaviors.

CHAPTER 3: Serotonin modulates the structural and functional development of the hippocampus

Introduction

In this chapter we explore the second part of our principal hypothesis, by investigating whether early-life manipulations of 5-HT levels yield changes in the structure and function of the hippocampus (HC).

Although best known for its effects on cognitive processes (Scoville and Milner, 1957; Moser and Moser, 1998; Burgess et al., 2002; Bannerman et al., 2004; Bars and Gage, 2007), the HC has been well-characterized as a modulator of affective state and is a major locus of change in humans and rodents displaying anxiety and depression-like behaviors (Moser and Moser, 1998; Pothuizen and Feldon, 2004; McHugh et al., 2004; Andersen et al., 2006; Fanselow and Dong, 2010). Data in support of this dual role of the HC comes from lesions studies that indicate a functional dichotomy along the septo-temporal axis of the HC. Cognitive behaviors are primarily affected by dorsal HC lesions (Moser et al., 1993; Hock and Bunsey, 1998; Matus-Amat et al., 2004; Hunsaker and Kesner, 2008) while ventral lesions affect emotional behaviors (Kjelstrup et al., 2004; Bannerman et al., 2004; Nascimento and Carobrez, 2007; Yoon and Otto, 2007; Trivedi and Cooper, 2009). As such, HC-dysfunction can result in both the affective and cognitive phenotypes of anxiety and mood-disorders. Indeed, changes in the HC are observed in human patients and mouse models displaying dysregulated affective behaviors. Such changes occur at the level of gene expression (neurotransmitters, stress-hormones, receptors, growth factors, signaling proteins), neuronal morphology,

neurogenesis and cellular viability (Gould et al., 1992; Boldrini et al., 2009; Yau et al., 2001; McKittrick et al., 2000; van der Hart et al. 2002; Alfonso et al., 2004; Czeh and Lucassen, 2007; Cai et al., 2008; Molteni et al., 2010; Weaver et al., 2010; Ivy et al., 2010; Andrus et al., 2010).

Although there are diverse changes in the HC, the most replicable and robust findings correlated with emotional dysregulation, to date, are alterations in HC structure. In particular, significant reductions in HC volume have been characterized in human patients, non-human primates and rodent models exhibiting depression and anxiety-like behavior (McEwen et al., 1997; Margarinos et al., 1996; Sheline et al., 1996; Sapolsky, 2001; Rusch et al., 2001; Bremner et al., 2006; Lee et al., 2009) and the degree of these changes are directly correlated with severity of symptoms and/or illness duration (Sheline et al., 1996; MacQueen et al., 2003; Bremner et al., 2006; Taylor Tavares et al., 2007). A prime contributor to the reduced volume is thought to be a retraction in neuropil (Reagen and McEwen, 1997; Stockmeier et al., 2004; Czeh and Lucassen, 2007) as dendritic material of HC neurons is reduced in patients and mouse models with affective disorders (Stockmeier et al., 2004; McEwen et al., 1997; Magarinos et al., 1995; Soetanto et al., 2010). For instance, *5htt -/-* mice and those lacking the brain derived neurotrophic factor (BDNF), display altered dendritic complexity of HC neurons (Appendix B, Fig. B.3, B.4 & B.7; Chen et al., 2006), fortifying the correlation between structural changes in the HC and emotional dysregulation. In *5-htt -/-* mice, changes are found in the cornu ammonis 3 (CA3) and dentate gyrus (DG) sub-regions of the HC (Appendix B, Fig. B.3, B.4 & B.7). A recent immunohistological study on human post-mortem brain slices showed an association between reduced HC dendritic material and enhanced trait anxiety/higher

depression scores, suggesting a similar correlation between HC structure and emotional dysfunction exists in humans (Soetanto et al., 2010). Congruently, pro-depressive and anxiogenic factors, such as stress-exposure cause dramatic reductions in the complexity of the dendritic arbors of HC neurons (Watanabe et al., 1992; Magarinos et al., 1995; Vyas et al., 2002; McLaughlin et al., 2007; Martinez-Tellez et al., 2009). Conversely, interventions that protect against depression/anxiety, such as environmental enrichment, antidepressant agents and anxiolytic drugs prevent/reverse these structural alterations, and concurrently preclude the associated negative impact on emotional behaviors (Watanabe et al., 1992; McEwen et al., 1997a; Magarinos et al., 1999; van der Hart et al., 2002; Czeh et al., 2001; Czeh et al., 2002; Santarelli et al., 2003; Hutchinson et al., 2010; Price and Drevets, 2010).

In conjunction to the changes in dendritic architecture, altered density of dendritic spines (regions of synaptic input) are also associated with pro-depressive/anxiogenic interventions such as restraint stress, learned helplessness (inescapable shock exposure) and social defeat stress (Sunanda et al., 1995; Magarinos et al., 1997; Sousa et al., 2000; Kole et al., 2004; Hajszan et al., 2009; Magariños et al., 2011). Congruently, interventions that protect against emotional dysfunction, such as antidepressant treatment, often reverse/prevent these changes in spine density (Watanabe et al., 1992; Hajszan et al., 2010), bolstering the correlative relationship between the structural changes in the HC and affective and cognitive dysfunction. Given the demonstrable impact of the HC in regulating affective state, and since PN-FLX mice have robustly dysregulated affective behaviors, we hypothesized that they display alterations in HC dendritic architecture and spine density, similarly to other models of depression and anxiety.

During the early post-natal period, development of the HC is highly influenced by 5-HT. Most of HC development occurs post-natally in rodents, at a time when serotonergic innervation to this structure is already present (Swann et al., 1989; Segal et al., 1990; Tansey et al., 2002; Gaspar et al. 2003; Andersen et al., 2006). Additionally, many 5-HT receptors are greatly up-regulated during this period suggesting that in the first few weeks of life, the ability of the HC to sense, and be influence by, 5-HT is both prominent and growing (Grove and Tole, 1999; Gross et al., 2002; Sodhi and Sanders-Bush, 2004). Indeed, early-life modulation of 5-HT has been shown to alter the expression of key genes know to regulate development and growth of HC neurons, such as those regulating the expression of growth factors, including BDNF (Molteni et al., 2010; Zhou et al., 2008), receptors (Sibille et al., 2000; Sodhi and Sanders-Bush, 2004), synapses (Gaspar et al., 2003; Wellman et al., 2007) and cytoskeleton-associated proteins (Yuen et al., 2005; Bonnin et al., 2007; Chameau et al., 2007). In summary, as the HC is a modulator of emotional state and because its development is influenced by 5-HT, we hypothesized that the early-life dysregulation of serotonergic tone in PN-FLX mice would impact the proper development of the HC, resulting in altered dendritic complexity and spine densities. In this regard, we addressed the following aims:

Aim 2: Does *development-specific disruption of serotonergic tone alter the maturation of the hippocampus?*

- ***i.*** *Are there structural alterations within the hippocampus?*
  - *a. Are there changes in dendritic arborization?*
  - *b. Are there changes in dendritic spine density?*

Furthermore, to probe whether the potential structural changes in the HC would impact its output, we used HC-sensitive behavioral tests, specifically those that probe cognitive functioning in PN-FLX mice. The use of these tests was contingent on the fact that apart from its validated role in the regulation of the affective component of depression and anxiety, the HC has also been implicated in the cognitive phenotypes of these psychiatric disorders (Sapolsky 2001; Fanselow and Dong, 2010;). For instance, the deficits in learning, memory, and spatial cognition described in humans with anxiety, depression, or those experiencing prolonged stress, can be generated via HC manipulations (Moser et al., 1993; Phillips and LeDoux, 1992; Burgess et al., 2002). HC lesions or altering HC-output via external inhibition/stimulation have a direct impact on learning and memory, especially those requiring the acquisition and recall of contextual and spatial information (Moser et al., 1993; Phillips and LeDoux, 1992). Similar deficits in learning and memory are well-characterized in human patients under chronic stress and those that have depressive symptoms and pathological levels of anxiety (Austin et al., 2001; Lupien et al., 2007; Bossini et al., 2008; Withall et al., 2010). Patients with mood disorders also often display altered spatial cognition. For instance, patients with unipolar depression fare worse in tasks that require spatial recognition (Gould et al., 2007; Taylor Tavares et al., 2007). Interestingly, the aforementioned changes in volume, dendritic complexity and spine density in the HC are correlated to the cognitive deficits in some humans and rodent studies (Bremner et al., 2007; Sheline et al. 2003, O'Brien et al., 2004), suggesting that changes in HC structure can affect HC function and, thus, may be probed using cognitive behavioral paradigms. As such, we ran PN-FLX mice through a battery of HC-

sensitive cognitive tests to probe both HC function and to characterize potential changes in PN-FLX cognitive function. Specifically, we asked:

Aim 2 ***ii****. Is the functional output of the hippocampus altered?*

a. Are there changes in spatial learning and memory?

b. Are there changes in acquisition of contextual information?

Methods

Dendrite morphology

Three male mice from each post-natal treatment group were anaesthetized and sacrificed in adulthood (P90-P100). Brains were excised and processed with the Golgi stain as previously described (Rosoklija et al., 2003). Golgi-stained sections were visualized using light microscopy. Pyramidal neurons in the CA3(ventral CA3c) and DG granule cells, whose cell bodies were in the stratum granulosum (ventral DG, both inner and outer blades) and were identified based on morphology and location using the "Comparative Cytoarchitectonic Atlas of the C57BL/6 and 129/Sv Mouse Brains" (Hof et al., 2000) as a guide. Fully impregnated cells with intact arbors that lay in the middle of the z-plane were selected as candidates for analysis. From this candidate pool, 6-10 cells per region/mouse were manually traced using Neurolucida (MBF Bioscience, Williston, VT). Neuroexplorer software (MBF Bioscience) was used to perform Sholl analysis on the tracings to determine dendritic complexity, in which number of nodes, intersections and total dendritic material were designated as the dependent measures. To gauge how compact or extend arbors were, the distance dendritic trees extended out from

the soma was measured using the maximum Sholl radius as a measure of the length of dendritic trees. One-way analysis of variance (factorial or repeated measures, as indicated) was used to analyze data.

Dendritic spine analysis

A subset of cells used in the dendritic complexity study were chosen for analysis of spine number and density (n = 2-5 cells/mouse; 3 mice per treatment group). Neurolucida software was used to trace the apical dendrites of neurons and to manually specify the location of spines. Spines of all major classes (mushroom, thin and stubby and thorny excrescences) were included in the analysis and not distinguished from each other. Neuroexplorer was used to analyze traces and determine total number of spines. Total spine density was calculated by dividing total number of spines by total amount of dendritic material present. Only the first 200 μm of the apical trees were analyzed as past this point spines were increasingly harder to visualize which made the tracings and designation of spines above 200 μm unreliable. This proximal region was also of special interest as it receives input from the DG via the main processing circuit in the HC, the trisynaptic pathway.

Morris Water Maze

Male PN-FLX and PN-Veh mice were tested in a water maze procedure consisting of the following stages: 1.) habituation 2.) visible platform training 3.) hidden platform training and 4.) probe trials.

Stage 1: Habituation

The habituation phase consisted of two days, with mice undergoing two trials per day. On day 1, behaviorally naïve mice were placed into a bucket (38 x 35 x 26 cm) that contained a clear, plexiglass platform in its center (height: 20 cm), and was filled with water to about 0.5 cm above the platform. Water temperature was maintained between 23-25 °C. Mice were placed, one at a time, into the corner of the bucket (trial 1 = southeast corner; trial 2 = northwest corner) and allowed to freely swim and explore within the bucket in order to find, and mount, the platform. The trial was designated as over when a mouse had remained on the platform for 30 consecutive seconds. The clock was reset whenever the mouse jumped off the platform. Day 2 of habituation was the same as day 1 except that the bucket was filled with water that was made opaque using non-toxic white gesso, and in that the criteria used to end a trial was that the mouse had been on the platform for 20 consecutive seconds.

Stage 2: Visible platform training

After habituation mice were tested to determine their ability to find a platform in a larger water maze which consisted of a circular, white plastic reservoir (1.7 m diameter, approximately 2 feet high) filled to a depth of approximately 20 cm (1 cm above platform) with water that was maintained between 21-25 °C. The water was made opaque using white, non-toxic gesso. Water was stirred at the start and middle of the day, to prevent settling of the gesso and to ensure opacity of the water. Black curtains surrounded the pool and each curtain panel had distinct patterns placed on them to act as visual (extra-maze) cues. The cues included: 1.) green stripes across the wall 2.) fuchsia squares in a symmetrical pattern 3.) a large pink cross with curlicues drawn on it 4.) blue nitrile gloves taped to the wall and 5.) yellow circles in a random pattern. A ceiling-

mounted digital camera was positioned directly above the pool and the focus/zoom was adjusted to ensure the entirety of the pool was captured. Images from the camera were captured and stored by a tracking computer, hidden behind the curtains. The pool was divided into four quadrants (North, West, South and East). A clear plexiglass platform (14.6 cm in diameter) was placed in the NW quadrant (15cm away from the pool edge) of the pool and whose location was indicated by a tall visible, multi-colored flag. The location of the platform was fixed for the entire experiment. Each mouse underwent three trials per day for two days. During a trial, a mouse was placed into the water (with their heads facing the pool wall) at one of the four designated release sites each roughly equidistant from the platform location. All mice were released at the same start location for a given trial, but release sites were different between trials. Mice were allowed to swim and find the location of the submerged platform. If a mouse found, and remained on the platform for a 15 consecutive seconds, the trial was considered over and the mouse was retrieved. Each trial lasted for a maximum of 120s. If mice were unable to find the location of the visible platform in that time, they were gently guided towards it by the experimenter. After each trial, mice were directly placed into a warmed cage containing tissue paper, to help dry them off and to keep them warm. After an entire cage of mice was run, all mice were transferred from the warming cage to their home cage. During the trials, mice were recorded and tracked using the AnyMaze video tracking system from Stoelting (Wood Dale, IL). AnyMaze was also used to analyze video files and determine the latency to find the platform and swim speed. Heat-maps generated from AnyMaze were also obtained to visualize the swim trajectory of mice during a trial.

Stage 3: Hidden platform training

This stage was performed using the same experimental set up as for visible platform training. In this case, however, the location of the platform was not indicated by any visible marker and was thus was hidden from view as it was fully submerged under opaque, white water. Mice underwent 3 trials per day for a period of eight days with about a 60 minute inter-trial interval. At the start of each new trial/session, pool water was stirred using a squeegee to ensure homogenous opacity of the water. Mouse release sites were selected in a random manner and were different for each trial within a day; however, all mice had the same start location within a session.

Mice were recorded for a maximum of 120 s after which data collection terminated. However, if mice were unable to find the platform in that time, they were given an additional two minutes of unrecorded time to search for the platform. If a mouse was still not able to find the platform in the additional 2min period, the experimenter manually guided the mice towards the platform. In order to end a trial, a mouse had to remain on the platform for 15 consecutive seconds. Mice swim trajectories were tracked and analyzed as described in the previous section. Mice received 9 days of hidden platform training.

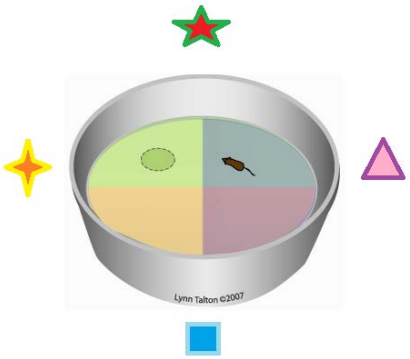


Fig. 3.1 Cartoon of Morris Water Maze set up. Platform is represented by the smaller grey circle in the NW (green) quadrant. Distinct extra-maze cues were placed on the walls surrounding the pool. *Modified image from Lynn Talton©, UCLA behavioral testing facility.*

Stage 4: Probe trials

During probe trials, the platform was removed and mice were released into the pool and allowed to search for the platform for 90 s. Probe trial 1 was done on the morning of day 10, 24 hours after the last day of training, and all extra-maze cues used during the training session were left intact (P1, "full-cue" probe). After completion of P1, the platform was replaced in its initial position and mice were given three more hidden platform trials so that they would retain their memory of the platform's location. On day 11, the 2nd probe trial (P2, "partial-cue" probe) was conducted, in which 3 out of 5 of the extra-maze cues were removed from the wall/curtain, leaving only two (blue nitrile gloves and the fuchsia squares in the NE and SE quadrants, respectively). On day 12, all extra-maze cues were removed and the final "no-cue" probe trail was conducted.

AnyMaze software was used to calculate the percent of time spent in the target (NW) quadrant, in which the platform was located during the training sessions.

Contextual fear conditioning

Conditioning was performed on male and female PN-Veh and PN-FLX mice between the ages of PN90-PN100, using equipment set up similarly to that previously described by Drew et al., 2010. Briefly, the conditioning context or context "A" consisted of conditioning chambers (ENV010MD, Med Associates, and St. Albans, VT) in which the metal grid floors and metal chamber walls were left exposed and unaltered, the interior chamber fan and house lights were turned on. Three drops of lemon scent (oil/essence) were applied to the interior of chambers using a cotton Q-tip at the start of each round. Mice were habituated for 5-10 mins in a separate room (a distance away from the room housing the conditioning chambers so as to prevent auditory or olfactory cues emanating from the shocking chambers from reaching the mice that were to be subsequently run). Habituation took place in plastic cages containing familiar corn bedding (same as that in the home cage). On the conditioning day (day 1), mice were placed into context "A" and allowed to explore freely for 180 s after which they received three scrambled foot shocks (duration: 2 s, intensity: 0.75 mA) delivered via the grid floors of the chambers. As depicted below, shocks were delivered with variable inter-trial intervals and mice remained in the chamber for an additional 30seconds before being removed and placed back in their home cage. On day 2, mice were placed back into context "A" and their freezing responses over 180 s were measured.

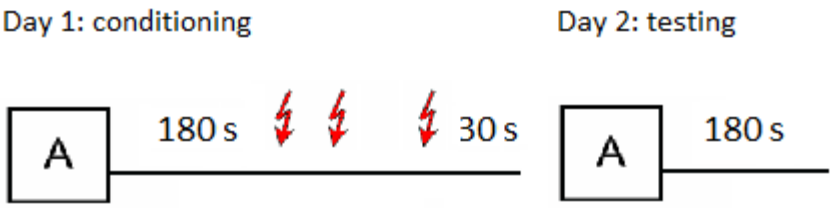


Fig 3.2 Contextual fear conditioning protocol. Red, jagged arrows represent 2ms, 0.75 mA scrambled foot shocks delivered via the grid floor of conditioning chamber. "A" represents training context.

Results

Aim 2 i a. Pronounced changes in dendritic structure of CA3 neurons from PN-FLX mice

In the CA3, we found that, relative to PN-Veh controls, PN-FLX mice have significantly more condensed apical dendritic trees ($F_{1, 43} = 7.359$; $p = 0.0096$, Fig. 3.3) suggesting early-life exposure to FLX results in a retraction of the dendritic arbor. Despite having arbors that do not extend out as far as those of PN-Veh mice, the apical dendritic trees from PN-FLX mice are more branched as evidenced by their significantly higher total number of nodes, relative to controls ($F_{1, 40} = 6.111$; $p = 0.0178$, Fig. 3.4). To determine whether this increased branching was uniformly present throughout the extent of the arbor, or whether it was limited to a specific region along the tree, we plotted spine density versus distance from soma. From this analysis, it is clear that the increased

number of nodes in PN-FLX mice is present specifically in the region proximal to the soma, within the first 100μm from the soma (repeated measures one-way ANOVA: $F_{1,31}$= 4.669; p = 0.0385, Fig. 3.5). The increased branching of CA3 apical trees in the proximal arbor, was accompanied by increased number of intersections (trend effect: $F_{1, 43}$ = 4.030; p = 0.0510, Fig. 3.6) and dendritic material ($F_{1,41}$ = 6.120; p = 0.0176, fig. 3.7). However, when the total number of intersections and dendritic material (over the entire extent of the arbor) were measured, we found no significant differences between treatment groups (Figs. 3.8 and 3.9). The lack of an overall difference in total number of intersections and dendritic material despite there being a significantly higher number of total branch points in PN-FLX mice suggests that either i.) the mean length of the apical branches of PN-FLX mice are shorter or that ii.) there is a higher frequency of shorter length branches in the apical arbors of CA3 neurons from PN-FLX mice. To broach this question directly, we looked at the mean length of the branches and found no significant differences between PN-Veh and PN-FLX mice (Fig. 3.10). However, when we look at the % of short (<500μm) vs. long branches (>500μm), we find that PN-FLX mice have a significantly higher number of shorter branches and a significantly lower number of long branches ($F_{1,46}$ = 6.733; p = 0.0127, Fig. 3.11). Thus, our morphological data indicate that the PN-FLX mice have more compact, but bushier CA3 apical arbors relative to their PN-Veh counterparts.

Unlike the apical arbors, the basilar CA3 dendritic trees of PN-FLX mice do not differ in how far they extend out from the soma, relative to controls (Fig 3.12). However, the basilar CA3 arbors of PN-FLX are more branched than controls, as indicated by their significantly higher number of nodes ($F_{1,28}$ = 4.990; p = 0.0337, Fig 3.13). The increased

number of branching seems to be higher specifically within the first 150 μm of dendritic tree proximal to the soma (60 μm to 150 μm, effect of treatment: $p = 0.0092$, Fig. 3.14). This increased branching is also accompanied by significantly higher total intersections ($F_{1,26} = 5.413$; $p = 0.0280$, Fig.3.15) and total dendritic material ($F_{1,27} = 3.719$; $p = 0.0644$, Fig.3.16). The increase in intersections is uniform throughout the entire extent of the dendritic arbor (Figs 3.17). These data suggest that unlike the apical trees that are stubbier, the basilar arbors of CA3 neurons from PN-FLX mice are more branched and more expansive than those of PN-Veh mice. This fact is corroborated by the finding that the mean branch length of CA3 basilar arbors from PN-FLX mice are significantly larger relative to PN-Veh mice ($F_{1,35} = 6.094$; $p = 0.0186$, Fig. 3.19). A model encapsulating the changes in both the apical and basilar CA3 arbor in PN-FLX mice is presented in Fig 3.20.

A second region in which we detected alterations in dendritic complexity in SERT KO mice was the DG. Thus, we sought to determine whether similar changes were observable in PN-FLX mice. In the DG we looked at alterations in dendritic complexity in the primary DG cell type, the granule cells, which solely possess apical arbors. We found no significant differences in dendritic complexity of the apical arbors of granule cells between PN-FLX and PN-Veh mice, as they had comparable number of nodes (Fig. 3.21), intersections (Fig. 3.22) and total amount of dendritic material (Fig. 3.23). The mean distance to which DG arbors extended from the soma were also comparable between treatment groups (Fig. 3.24).

Informed from the morphological analysis of *5-htt -/-* mice, who do not display changes in the CA1 region (Appendix B, Fig. B.8, B.9), we did not predict any changes in the

CA1 region in PN-FLX mice, and thus our analysis was focused exclusively on the CA3 and DG regions. Morphological findings in the HC are summarized in Fig. 3.25.

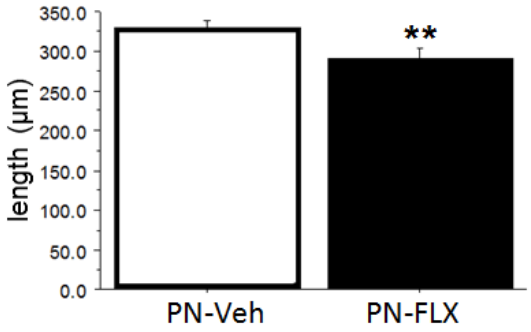


Fig. 3.3. PN-FLX mice have significantly reduced length of CA3 apical dendritic trees, indicating that their arbors do not extend out as far as those of controls (** p = 0.0096). N = 22-23 cells/treatment, 3 mice/group.

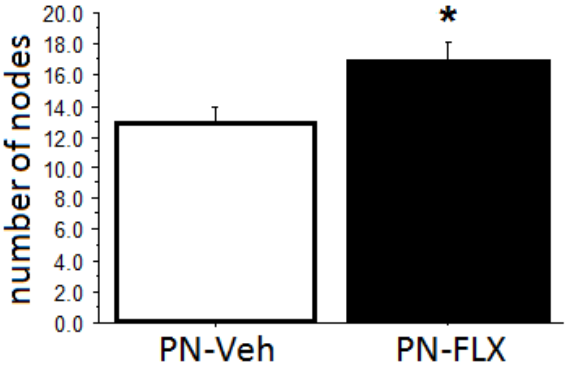


Fig. 3.4. PN-F LX mice have significantly higher number of nodes in the CA3 apical arbor, relative to controls (*p = 0.0178). N = 19-20 cells/treatment, 3 mice/group.

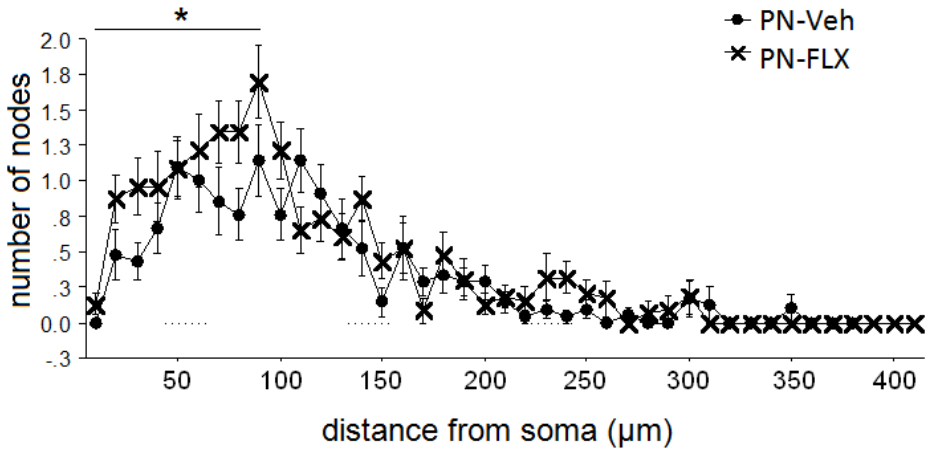


Fig. 3.5. The increased number of nodes in CA3 apical arbors of PN-FLX mice is primarily within the first 100µm proximal to the soma (*p = 0.0385). N = 19-20 cells/treatment, 3 mice/group.

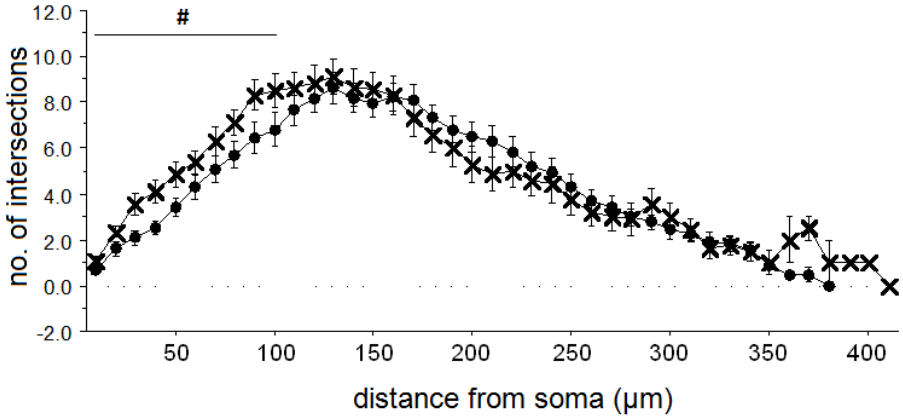


Fig. 3.6. Despite no overall differences in intersection number, PN-FLX mice display significantly higher number of intersections in the first 100µm proximal to the soma, the same region in which the pronounced increase in number of nodes is observed. (# p = 0.0510) N = 19-20 cells/treatment, 3 mice/group.

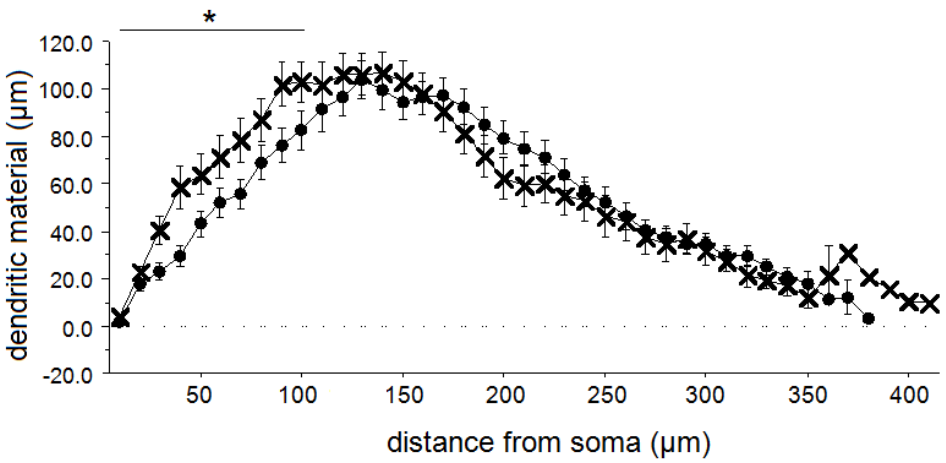


Fig. 3.7. Despite no overall differences in dendritic material, PN-FLX mice display significantly higher number of intersections in the first 100µm proximal to the soma, the same region in which the pronounced increase in number of nodes and intersections is observed. (*p =.0176)N = 19-20 cells/treatment, 3 mice/group.

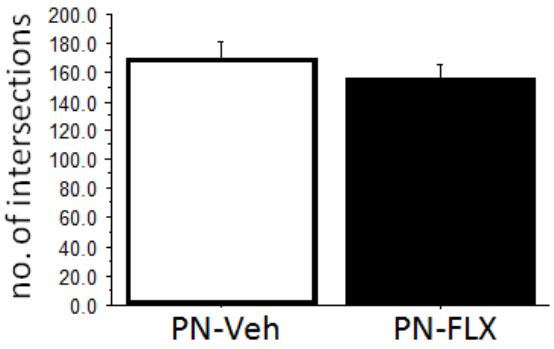


Fig. 3.8. No differences in the total number of intersections between PN-FLX and PN-Veh mice. N = 19-20 cells/treatment, 3 mice/group.

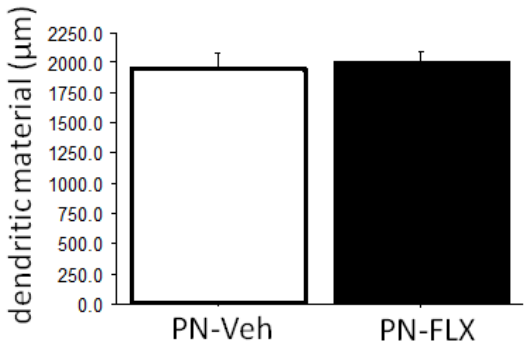


Fig. 3.9. No differences in the total dendritic material between PN-FLX and PN-Veh mice. N = 19-20 cells/treatment, 3 mice/group.

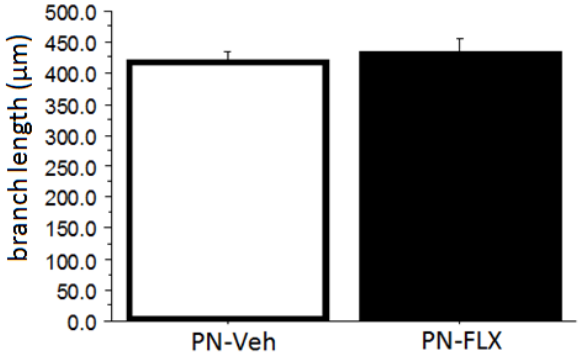


Fig. 3.10. Mean dendritic length in the apical CA3 arbor is not significantly different between PN-FLX mice and PN-Veh controls. N = 19-20 cells/treatment, 3 mice/group.

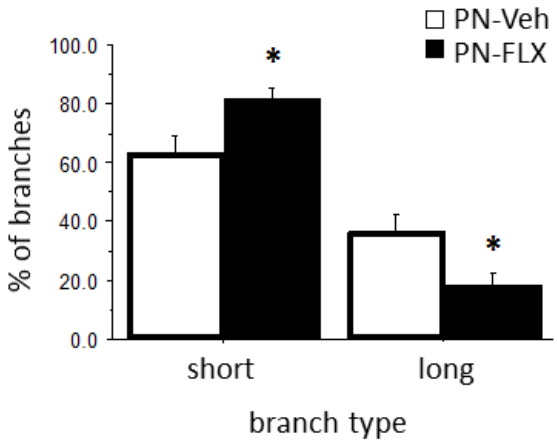


Fig. 3.11. Histogram displaying percentages of each branch type (categorized by length) in CA3 apical arbor. PN-FLX mice have a significantly higher % of medium length branches and a significantly lower % of long branches relative to controls. (* p = 0.0127) N = 19-20 cells/treatment, 3 mice/group.

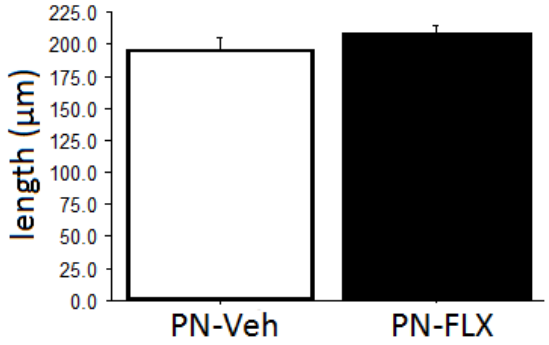


Fig. 3.12. No differences in how far arbors extend from the soma are observed between PN-FLX and PN-Veh mice. N = 19-20 cells/treatment, 3 mice/group.

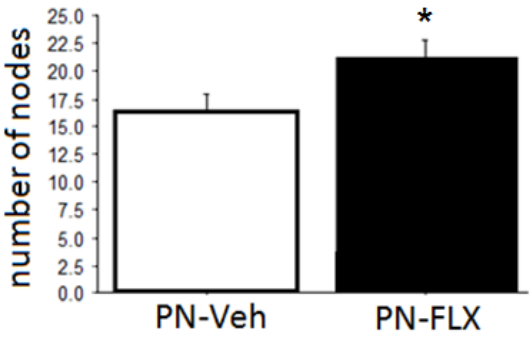


Fig. 3.13. PN-FLX mice have significantly higher total number of nodes relative to PN-Veh controls. (*p = 0.0337). N = 11-13 cells/treatment, 3 mice/group.

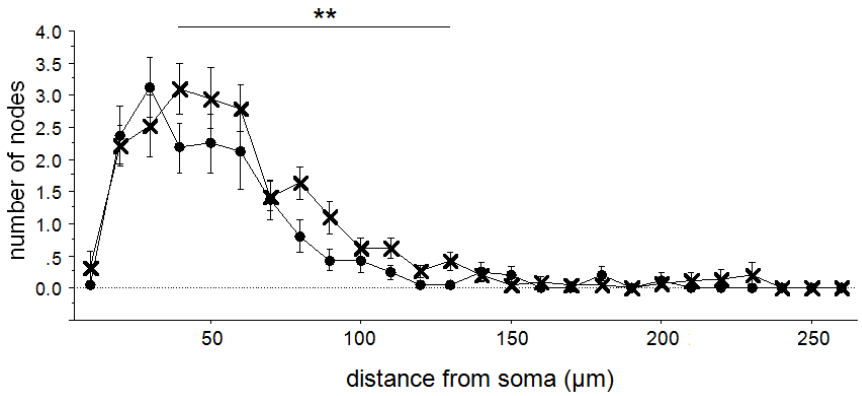


Fig. 3.14. The changes in total number of nodes in PN-FLX mice start in the proximal to medial region of the basilar CA3 dendritic tree. (**p < 0.001). N = 11-13 cells/treatment, 3 mice/group.

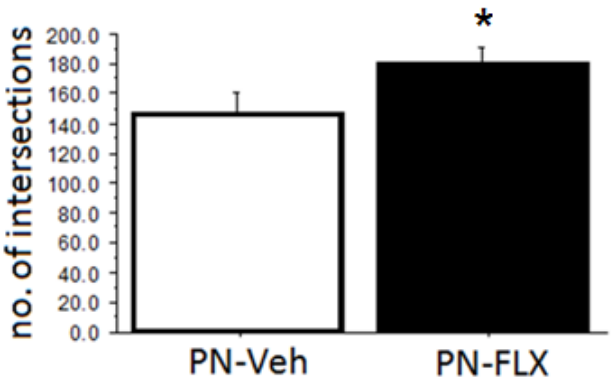


Fig. 3.15. PN-FLX mice have significantly higher total number of intersections relative to PN-Veh controls. (* p = 0.0280). N = 19-20 cells/treatment, 3 mice/group.

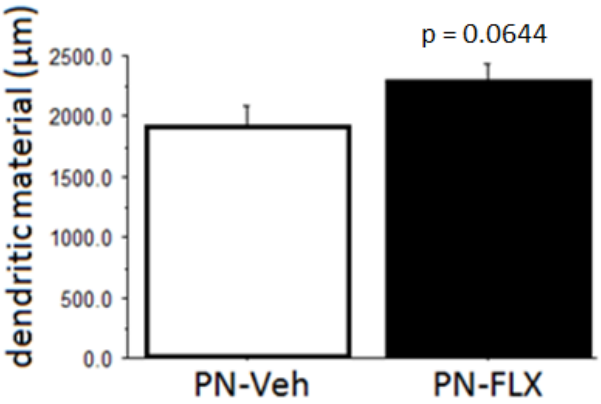


Fig. 3.16. PN-FLX mice have a trend increase (p = 0.0644) in total dendritic material relative to PN-Veh controls. N = 19-20 cells/treatment, 3 mice/group.

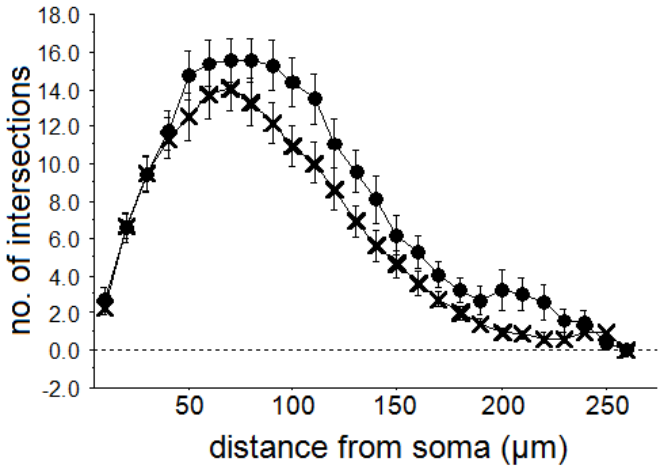


Fig. 3.17. PN-FLX have higher number of intersections from the proximal (starting at 50µm) to the very distal aspect of the basilar arbors. N = 19-20 cells/treatment, 3 mice/group.

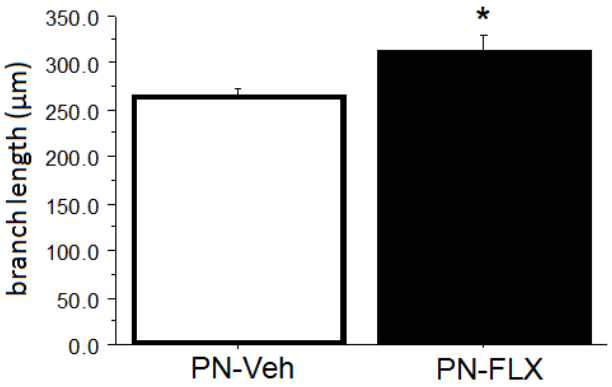


Fig 3.18. Branches in the CA3 basilar trees of PN-FLX mice are significantly longer than those of PN-Veh controls. (*p = 0.0186). N = 18 cells/treatment, 3 mice/group.

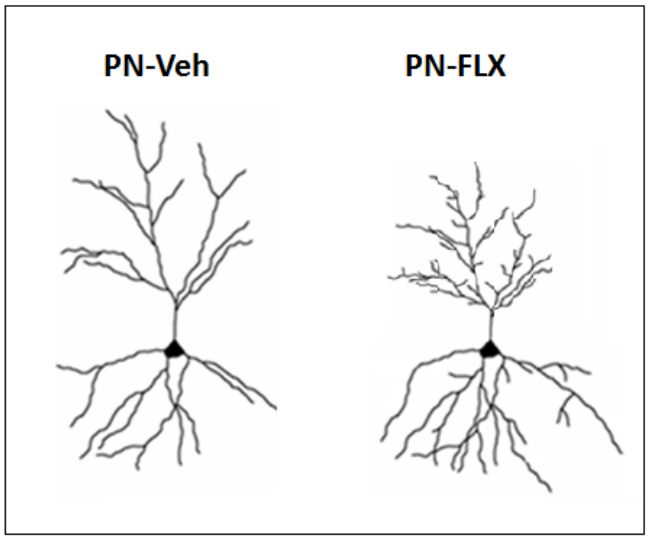


Fig 3.19. Model depicting the differences in both the apical and basilar arbors of CA3 neurons from each treatment group.

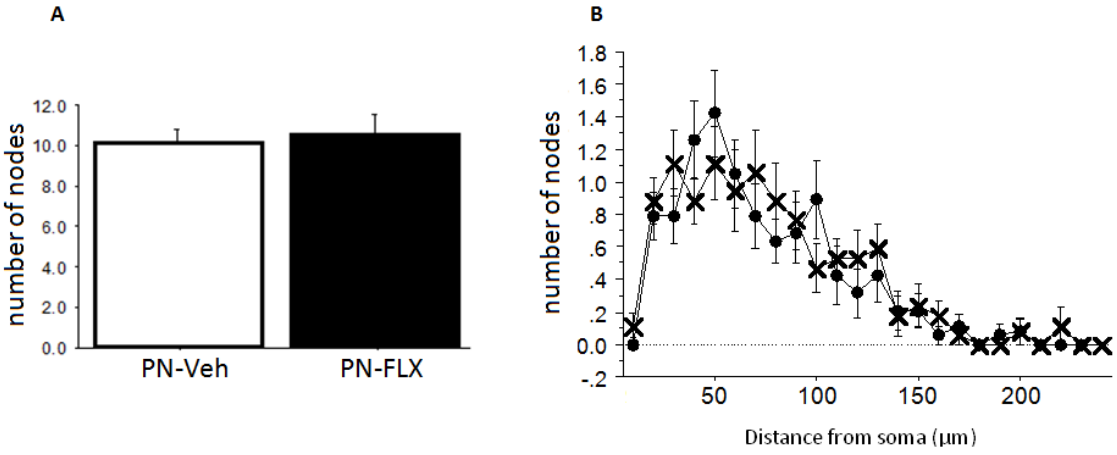


Fig. 3.20. No effect of treatment on total number of nodes (A) in DG granule cell arbors and no treatment effects on number of nodes at any point along the arbor (B). N = 18 cells/treatment, 3 mice/group.

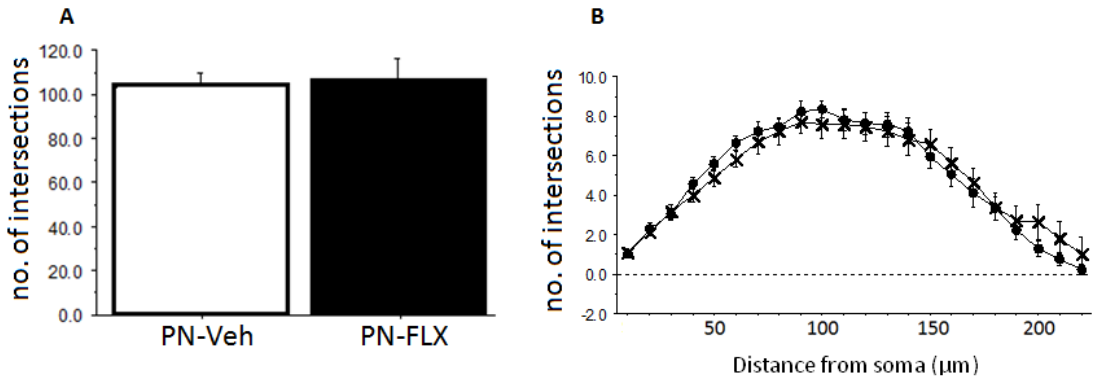


Fig. 3.21. No effect of treatment on total number of intersections in DG arbors (A) and no treatment effects on number of intersections at any point along the arbor (B). N = 18 cells/treatment, 3 mice/group.

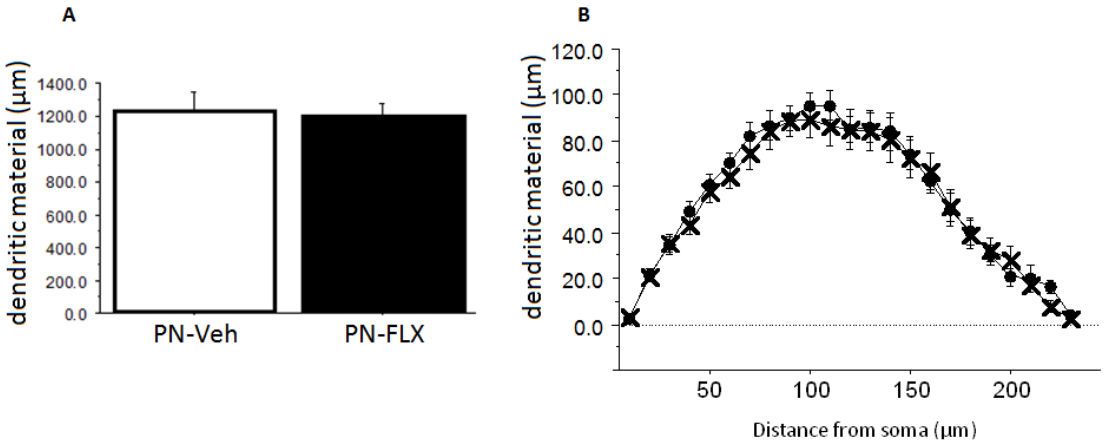


Fig. 3.22. No effect of treatment on total dendritic material of DG arbors (A) and no treatment effect on total dendritic material at any point along the arbor (B). N = 18 cells/treatment, 3 mice/group.

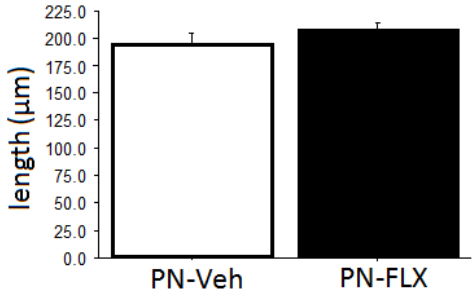


Fig. 3.23. The average distance that the apical arbors of DG granule cells extend from the soma, is not different between PN-FLX and PN-Veh mice. N = 18 cells/treatment, 3 mice/group.

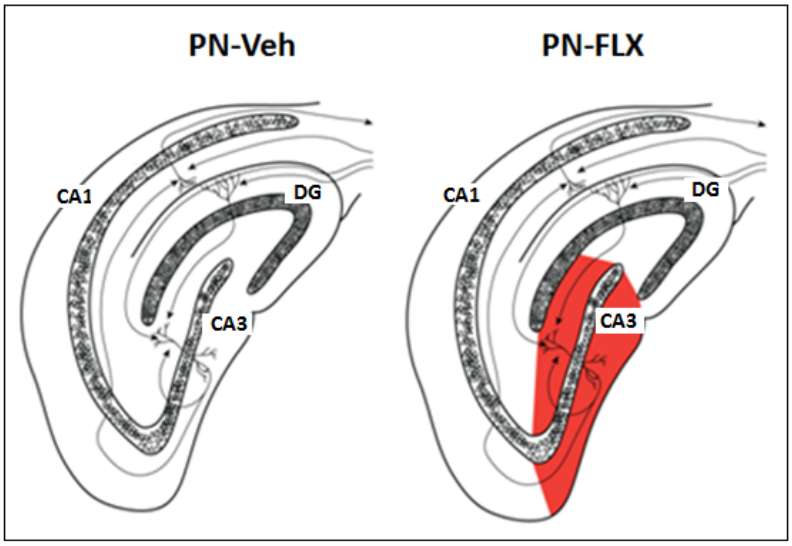


Fig. 3.24. Summary of morphological findings in the HC. Changes in dendritic complexity are found in the CA3 of PN-FLX mice, and not in the DG and likely not in the CA1 region either.

Aim 2 i b. **PN-FLX mice have fewer spines and reduced spine density in CA3 apical arbors**

As a preliminary readout for the extent of input to the CA3 region we examined the number and density of spines on the apical dendritic arbors. We limited our analysis to the CA3 region as this was the area in which we identified the most pronounced changes in dendritic arborization. In this initial 200µm segment of the apical arbor, we find that PN-FLX mice have a reduced total number of spines, relative to controls (overall trend effect: $F_{1,20}$ = 3.681; p = 0.0694, Figs. 3.26 and 3.27), with a significant reduction 130 to 150 µm from the soma ($F_{1,20}$ = 5.409; p = 0.0307). PN-FLX mice also display significantly reduced spine density (total number of spines/total amount of dendritic material) relative to PN-Veh controls ($F_{1,19}$ = 15.266; p = 0.0009, Fig. 3.28 and 3.29). As is visible from the spine number or density vs. distance from soma graphs (Figs. 3.27 and 3.29), the decreased number and density of spines observed in CA3 neurons from PN-FLX mice is uniform throughout the entire extent of the 200µm segment analyzed.

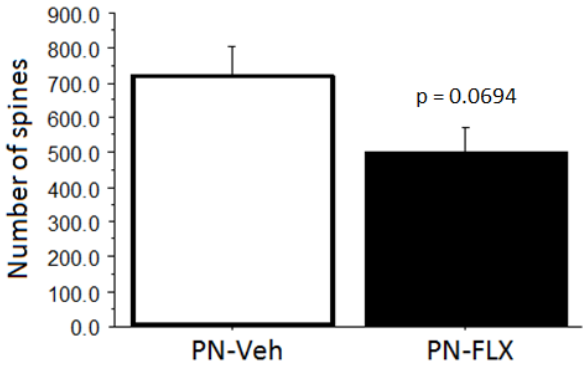


Fig. 3.25. PN-FLX mice have a decrease in the total number of spines in the CA3 apical arbor. (trend effect: p = 0.0694). N = 10-12 cells/treatment, 3 mice/group.

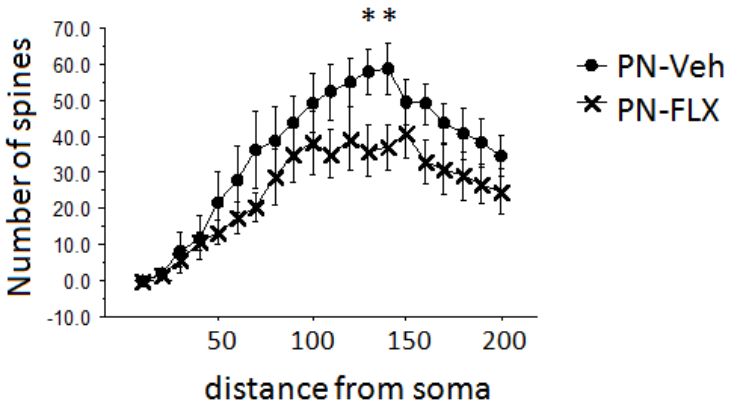


Fig. 3.26. The decrease in spine number in PN-FLX mice is throughout the 200µm region analyzed. (* p = 0.03307; # trend effect: p = 0.0694). N = 10-12 cells/treatment, 3 mice/group.

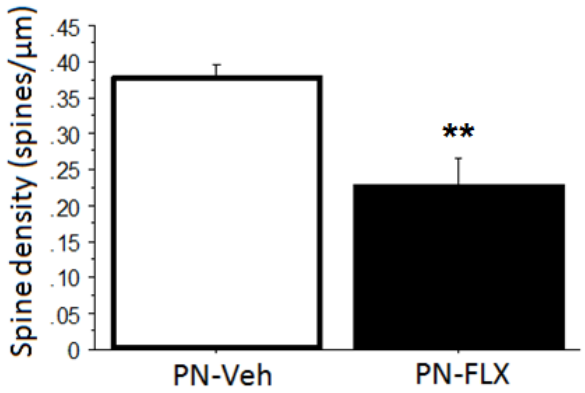


Fig. 3.27. PN-FLX mice have a significantly lower spine density in the CA3 apical arbor, relative to controls. (**p = 0.0009). N = 10-12 cells/treatment, 3 mice/group.

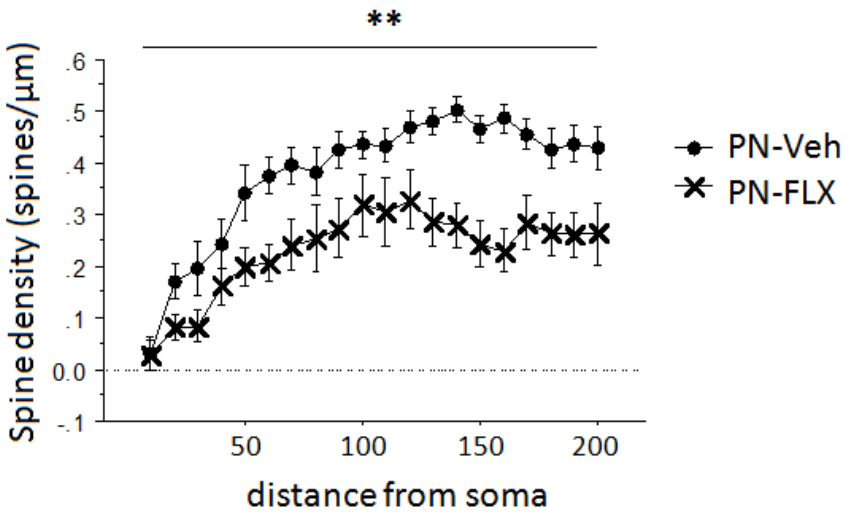


Fig. 3.28. The pronounced decrease in spine density in PN-FLX mice is throughout the extent of the 200µm region analyzed. (**p = 0.0009). N = 10-12 cells/treatment, 3 mice/group.

Aim 2 ii a. PN-FLX mice display pronounced deficits in spatial learning and memory

To determine whether the structural changes in the HC of PN-FLX mice affect its functional output, we utilized the HC-sensitive Morris Water Maze paradigm. During the habituation phase both PN-Veh and PN-FLX mice were able to find the location of the central platform and successfully mount it within 10s +/- 5s. Mice required a maximum of three attempts to remain on the platform for 20 consecutive seconds, and based on experimenter observations, no obvious differences in any of the aforementioned parameters were detectable between treatment groups.

In the visible training phase, the location of the platform, which was situated in the NW quadrant of the pool, was indicated by a tall, multi-colored flag. On day 1 of visible training, mice took an average of 62.036 +/- 5.078 seconds (averaged over two trials) to find the location of the platform and to mount it successfully, with no detectable effect of post-natal treatment on this parameter (Fig. 3.30). On day 2 of the visible platform task, the mean latency to find the platform was again comparable between groups and was significantly decreased relative to day 1 (effect of time: $F_{1,24} = 92.366$; $p < 0.0001$, Fig. 3.30) suggesting that mice learned and recalled the position of the platform from day 1's training session and/or established a faster swim trajectory to the platform. No differences in average swim speeds between groups on either day of visible training were observed (Fig. 3.31).

During the hidden platform training stage, the flag was removed such that mice could solely rely on extra-maze cues to navigate and determine the platform's location. On day 1 of hidden training, mice from both treatment groups took an average of 40.789s +/- 4.515s to find the platform, which was significantly more time relative to the second day of visible training (effect of flag removal: $F_{1,25} = 38.153$; $p < 0.0001$), suggesting that the removal of the flag made the task more challenging. Although a noticeable decrease in latency with each consecutive training day was observed in both groups (effect of time: $F_{1,15} = 6.627$; $p < 0.0001$), PN-FLX mice took significantly longer overall to locate the platform, compared to controls, over the first 8 days of the hidden training session ($F_{1, 15} = 6.697$; $p = 0.0206$, Fig 3.32). On the final (9th) day of training, however, PN-FLX mice had comparable latencies to platform as their PN-Veh littermates (Fig. 3.32). During the

hidden training session, PN-FLX mice had significantly increased swim speeds relative to controls ($F_{1,21}$ = 15.113; p = 0.008, Fig. 3.33).

We next looked at long term retention of the platform location by conducting a probe trial on day 10 (P1, full-cue probe). Despite being trained to the same degree on day 9, PN-FLX mice spent significantly less time searching for the platform in the target (NW) quadrant, relative to controls ($F_{1, 25}$ = 8.705; p = 0.0068, Fig. 3.34A and B). However, both groups did show a preference for the target region over the other 3 quadrants, spending significantly more that 25% of their time in this area (one tailed t-test, hypothesized mean $\leq$ 25: PN-Veh: t_{12} = 8.139; p < 0.0001; PN-FLX: t_{12} = 4.407; p = 0.0004, Fig. 3.34C).

To increase the difficulty of the task in order to determine if this would further separate the two groups in their ability to locate the platform, we removed three of the extra-maze cues, leaving solely the cues in the NE and SE corners of the room housing the pool. In this partial-cue probe trial (P2), a significant and comparable drop in % time spent in the target quadrant was observable in both treatment groups (effect of partial cue removal: $F_{1,24}$ = 49.648; p < 0.0001). A treatment effect on this parameter was also detected with PN-FLX mice spending significantly less time in the target region relative to controls ($F_{1,24}$ =5.748; p = 0.0246, Fig. 3.35A and B). With the removal of some of the extra-maze cues, the preference that both groups of mice previously showed for the target quadrant was no longer present as they did not spend more than 25% of their time in this region (Fig. 3.35C). Upon closer examination, we determined that PN-FLX mice seemed to have a selective preference for the two other quadrants in which the cues were still present (NE and SE) as they spent significantly more than 25% of their time in these

regions (one tailed t-test, hypothesized mean ≤ 25. NE quadrant: $t_{12} = 2.807$; $p = 0.0079$, Fig. 3.35C; SE quadrant: $t_{12} = 4.107$; $p = 0.0007$, Fig. 3.35C). PN-Veh mice did not show a preference for any quadrant, spending ~25% in each of the 4 regions.

On the final day of testing, mice were given a so-called "no-cue" probe trial (P3) in which all extra-maze cues were removed. With no cues present, both PN-Veh and PN-FLX mice do not show a preference for the target quadrant, spending only 19.897% +/- 2.066 of their time in this region (Fig. 3.36A and B). Instead, both groups of mice show a preference for the NE quadrant which contained a non-salient extra-maze cue (computer wire) spending significantly more than 25% of their time in this region (one tailed t-test, hypothesized mean ≤ 25. both groups combined: $t_{28} = 6.274$; $p < 0.0001$, Fig 3.36C). No significant differences in swim speeds were detected between PN-FLX and PN-Veh mice in any of the three probe trials (Fig. 3.37).

Thigmotactic behavior (as measured by percent time spent in the outer 10 cm periphery of the water maze) of both groups of mice, during each of the probe trials describe above, was measured. PN-FLX mice displayed a trend effect of higher levels of thigmotaxis during the full- and partial cue probe trials (effect of treatment: $p = 0.0790$). An increase in thigmotaxis was also observable in both treatment groups with increasing removal of extra-maze cues (effect of cue removal: $p < 0.0001$) suggesting that the periphery was the "default" region in the case when animals had difficulty locating the platform due to a lack of adequate extra-maze landmarks.

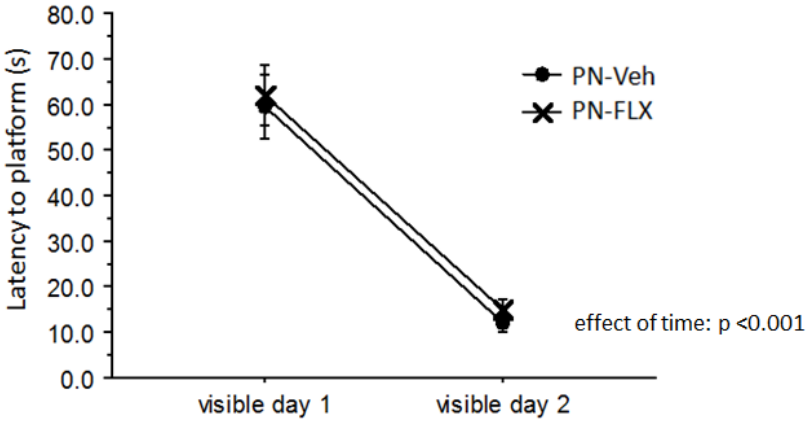


Fig. 3.29. No differences between groups in latency to platform during visible training session (in which platform is marked with a flag) on either day 1 or day 2 of training. Significant effect of time ($p < 0.001$) indicates that both treatment groups learn to find the visible platform faster on day 2. N = 11-12 mice per group.

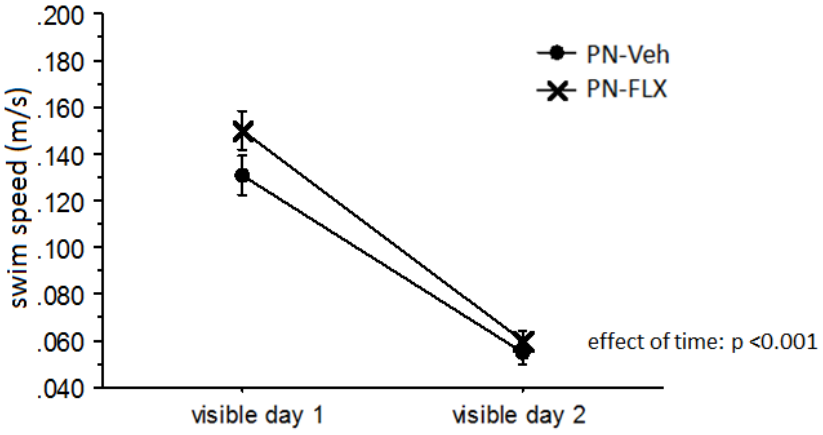


Fig. 3.30. No differences in latency to platform during visible training session (in which platform is marked with a flag) on either day 1 or day 2 of training. Significant effect of time indicates that both treatment groups learn to find the visible platform faster on day 2 (effect of time: $p < 0.001$). N = 11-12 mice per group.

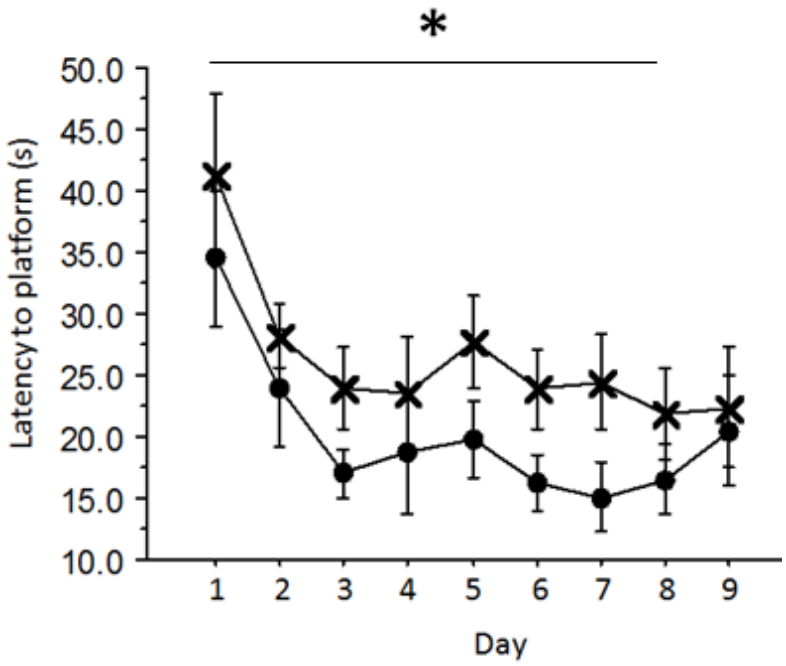


Fig. 3.31. PN-FLX mice have significantly longer latencies to locate the hidden platform over all 9 days of the training session. (* p = 0.0206). N = 7-8 mice/treatment.

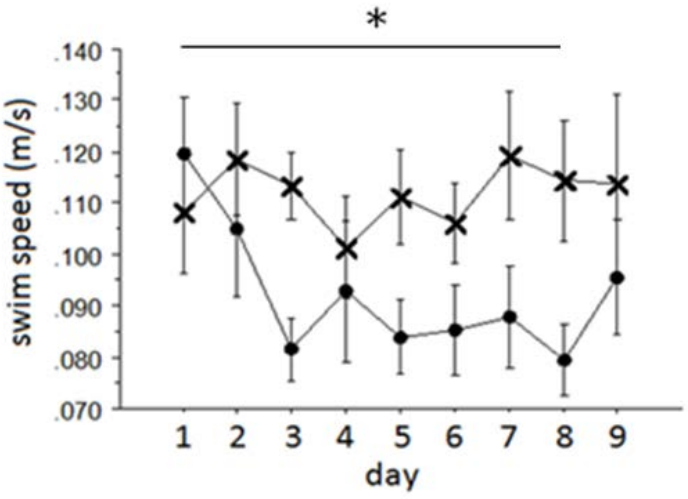


Fig. 3.32. PN-FLX mice have significantly higher swim speeds during the 9 day hidden platform training session (* p = 0.0008). N = 7-8 mice/treatment.

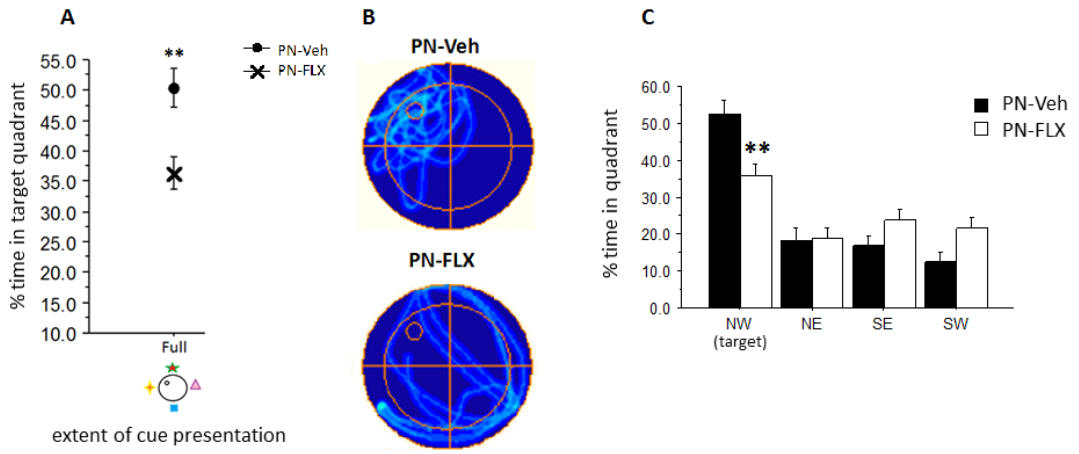


Fig. 3.33. Full-cue probe trial. (A) PN-FLX spend significantly less time in the target quadrant, relative to PN-Veh mice, when all extra-maze cues remain present (**p = 0.0068). (B) Heat maps displaying representative tracts of PN-Veh and PN-FLX mice swim tracts. Red circle within target (NW) quadrant indicates platform. (C) % time spent in each of the four quadrants. N = 12-13 mice/group.

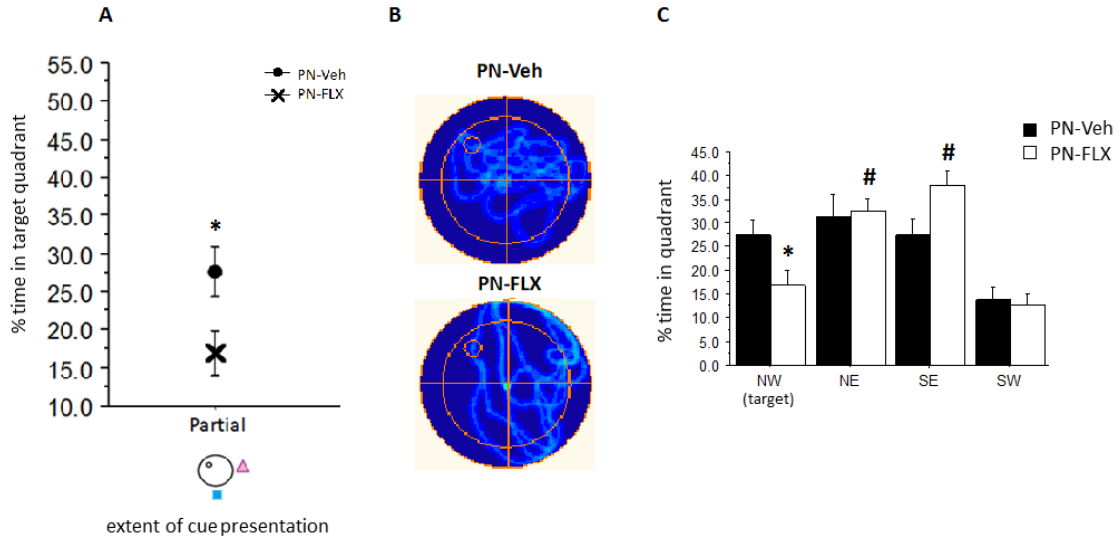


Fig. 3.34. Partial-cue probe trial. (A) PN-FLX spend significantly less time in the target quadrant, relative to PN-Veh mice, when all extra-maze cues remain present (*p = 0.0246). (B) Heat maps displaying representative tracts of PN-Veh and PN-FLX mice

swim tracts. (C) Graph displaying % time spent in each of the quadrants, indicating a preference for the NE and SW quadrants. N = 13 mice/group. (C) Graph displaying % time spent in each of the quadrants. PN-FLX mice spend significantly more than 25% of time in the NE and SE quadrant (# $p < 0.01$ for one-tailed t-test). N = 12-13 mice/group.

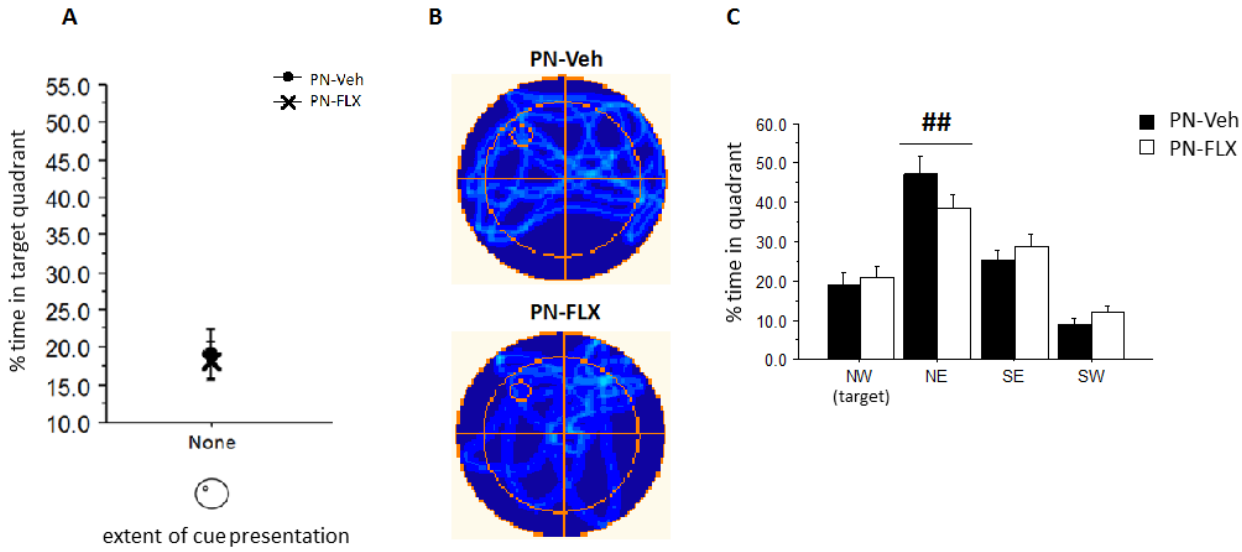


Fig. 3.35. No-cue probe trail. (A) PN-FLX and PN-Veh mice spend comparably little time in the target quadrant. (B) heat maps displaying representative swim tracts of PN-Veh and PN-FLX mice. (C) Graph displaying % time spent in each of the quadrants. Both PN-Veh and PN-FLX mice spend significantly more than 25% of time in the NE quadrant (## $p < 0.0001$ for one-tailed t-test). N = 12-13 mice/group.

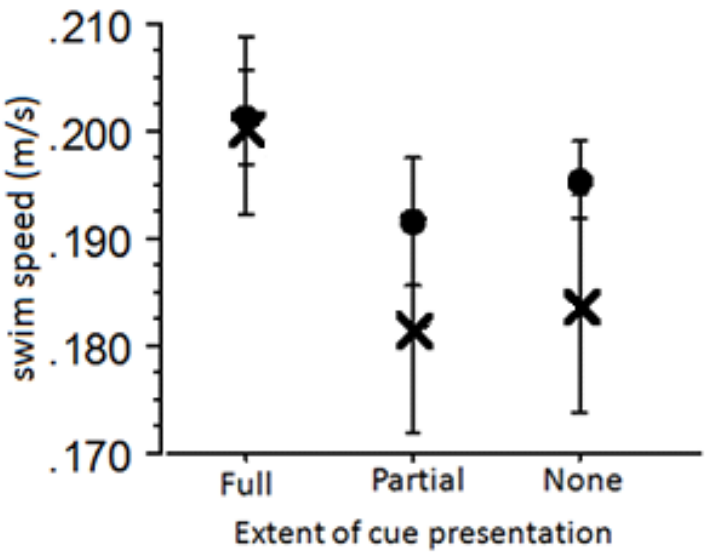


Fig. 3.36. No differences in swimming speed between treatment groups during all three probe trials. N = 12-13 mice/group.

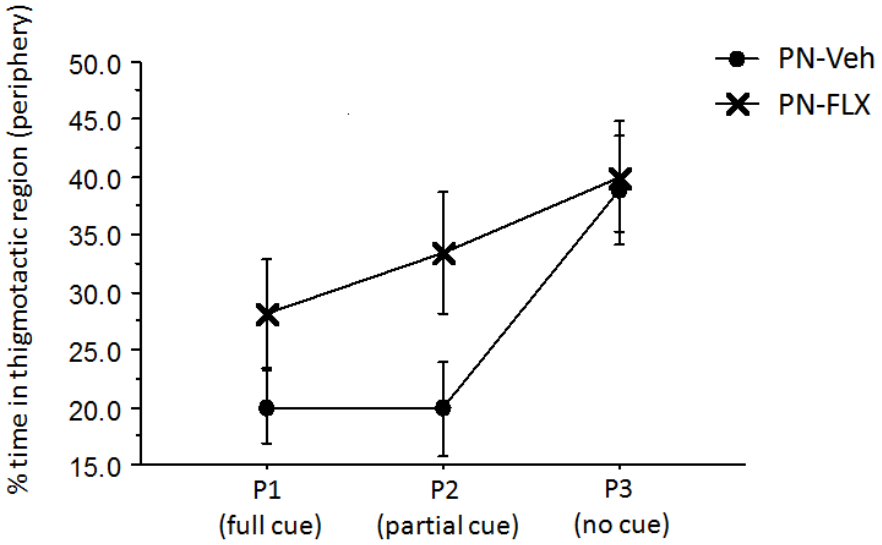


Fig. 3.37. Thigmotactic behavior during the three probe trials, PN-FLX vs. PN-Veh mice. Effect of cue removal $p < 0.0001$. PN-treatment x cue removal interaction $p = 0.0940$.

Aim 2 ii b. **PN-FLX mice have deficits in contextual fear conditioning**

To verify the changes in HC-mediated we behaviors PN-FLX mice underwent contextual fear conditioning. A specific HC-dependent version of this test, which involves adequate pre-exposure to the training context (180 s) was employed (Drew et al., 2010). PN-FLX mice display no significant differences, relative to PN-Veh controls, in either baseline freezing (Fig. 3.38) or in acute responses to the three shocks during the conditioning phase of the protocol (Fig. 3.39). An incremental rise in % freezing to each subsequent shock was observed for both groups ($F_{1, 2}$ = 188.319; $p < 0.0001$), with no main or interaction effects of/with treatment. However, 24 hours after conditioning, when mice were returned to the training context in which they received the shocks, PN-FLX mice have significantly reduced freezing relative to controls ($_{F1,48}$ = 32.488; $p < 0.0001$, Fig. 3.40) throughout the entire 180s session (Fig. 3.41).

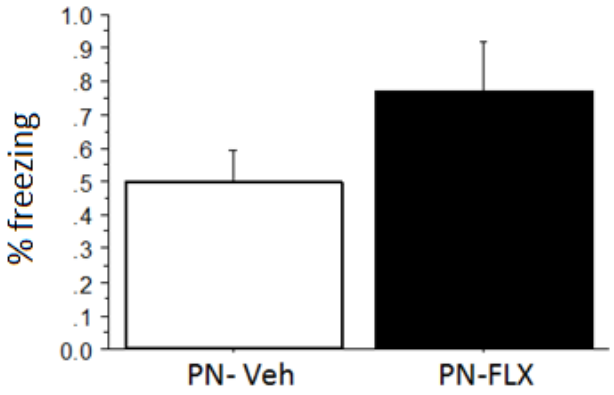


Fig. 3.38. PN-FLX and PN-Veh mice have comparably low levels of baseline freezing to the novel training context. N = 24-16 mice/group.

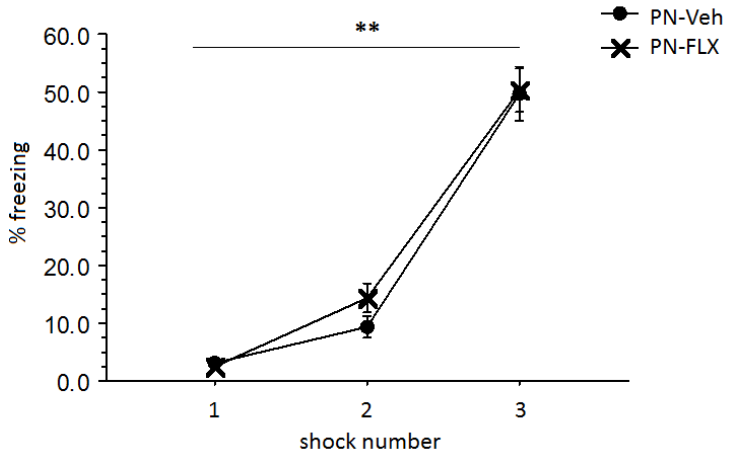


Fig. 3.39. No differences in acute freezing responses to shocks during fear conditioning session between treatment groups. N = 24-16 mice/group. (**effect of shocks: p < 0. 0001)

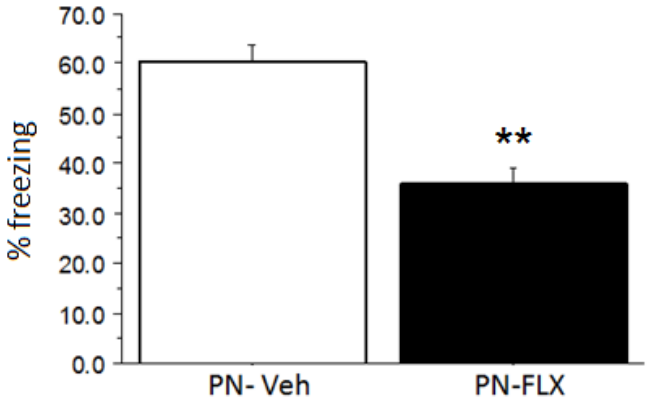


Fig. 3.40. PN-FLX mice have significantly lower % freezing to context 24 hours after fear condition training session. (**p < 0.001) N = 24-16 mice/group.

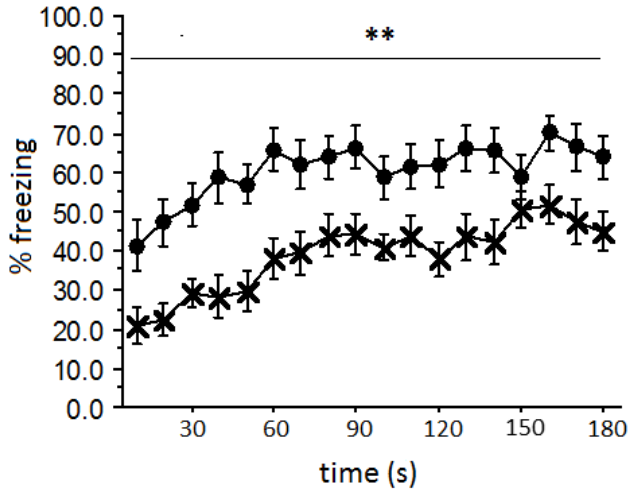


Fig. 3.41. PN-FLX mice have significantly lower % freezing to context 24 hours after fear conditioning training session during the entire 180s of exposure to context. (**p < 0.001) N = 24-16 mice/group.

Discussion

Structural changes

CA3 apical dendrites

We have found that early-life perturbation of serotonergic tone significantly impacts the cytoarchitectural development of HC neurons suggesting that, as hypothesized, the structural maturation of the HC is influenced by 5-HT levels in development. Specifically, mice that experienced elevated serotonergic tone in early post-natal life display altered dendritic complexity of both CA3 apical and basilar arbors (Fig. 3.20). Apical dendrites of PN-FLX mice do not extend out as far as those of PN-Veh mice (Fig. 3.3) but have increased branching in the first 100 µm proximal to the soma (Fig. 3.4, 3.5), although these branches are stubbier (Fig.

3.11). This region of the CA3 apical arbor is well-characterized as it receives the most pronounced afferent input from the DG via the mossy-fiber (MF) pathway (Amaral and Witter, 1989; Andersen et al., 2006), which comprises the first segment of the trisynaptic pathway (TSP), a term used to describe the serial connections from the DG→CA3→CA1 regions of the HC (Andersen et al., 2006). Thus, any changes in the complexity of dendrites in this proximal, apical segment of CA3 neurons could signify changes in the TSP connectivity, which in turn could influence HC function as the TSP is the primary processing circuit within the HC (Andersen et al., 2006). It is possible however, that despite there being more dendritic material in this region, MF input may itself be altered which could either compensate for, or potentiate, the PN-FLX dendritic phenotype. To address this question, we assessed dendritic spine number and density in this region proximal, apical region. Although we included all spine types in our studies, the primary class in this region had a thick and large morphology, characteristic of the so-called “thorny excrescences” which represent the post-synaptic aspect of mossy fiber (DG)-CA3 synapses (Gonzales et al., 2001; Andersen et al., 2006). We find that PN-FLX mice have a reduced number and density of spines in this region (Fig. 3.26-3.29), suggesting that DG input into the CA3, and thus TSP connectivity, has been diminished. The decreased spine number and density are likely due to synaptic-scaling, in which the enhanced branching of apical arbors elicits a compensatory decrease in number of synapses in order to maintain input equilibrium (Turrigiano, 2010). This finding also suggests that the number and density of spines along the dendritic arbor of HC neurons is under the influence of 5-HT in early development. Most likely, the dendritic arborization changes occur as a direct consequence of altered 5-HT signaling, followed by adaptive changes in the number of nodes.

The loss of synaptic input into the CA3 can potentially affect its electrophysiological properties, as it may reflect a shift in the excitatory/inhibitory balance within the CA3. In particular, the neurotransmitter released at mossy-fiber-CA3 terminals is glutamate, which, via primarily its ionotropic kainate receptors, results in an excitation of CA3 neurons (Contractor et al., 2000; Kamiya et al., 2002). Thus, decreased MF input into the CA3 would likely result in reduced excitation of CA3 neurons which could subsequently dampen their output. Thus, an important next step would be to fully characterize the effects of the morphological/synaptic change on the physiological properties of CA3 neurons from PN-FLX mice. These physiological experiments would inform us of whether structural changes in the CA3 have ramifications on the physiological properties of these neurons and whether they represent a loss- or gain of function phenotype.

In line with the above findings, *5-htt-/-* mice display a similar CA3 apical dendritic phenotype, but in their case the increase in total number of nodes is accompanied by significant changes in total intersections and dendritic material (Appendix B, Fig. B.3), implying that *5-htt-/-* mice have a higher number of branches with greater mean length than controls. Mechanistically, it may be that 5-HT during early post-natal development is important in setting the number of nodes (our data suggest it increases node number), but prolonged elevation in 5-HT levels, such as those present in *5-htt -/-* mice, may be responsible for continued branch elongation. This would explain why PN-FLX mice have a higher node number, but less extended branches. These data also suggest that certain morphological parameters, such as node number, are less plastic and set primarily during the early-post natal critical period, while changes in branch length can occur beyond this epoch, and can be retracted even in adulthood.

Changes in CA3 arbors have also been described in post-mortem studies in humans with emotional dysfunction. For example, depressive symptoms were correlated with decreases in CA3 dendritic and synaptic proteins such as microtubule-associated protein 2 (MAP-2), synaptopodin and spinophilin in mood-disorder patients (Law et al., 2004; Soetanto et al., 2010). These findings emphasize the strong association between the morphological/synaptic properties of the CA3 and affective dysfunction.

The apical CA3 dendritic arbor, is also particularly sensitive to pro-depressive and anxiogenic manipulations. For instance, chronic stress paradigms produce a pronounced retraction of CA3 apical dendrites and a decrease in CA3 spine density, similar to what we observe in PN-FLX mice (Watanabe et al., 1992; Magarinos et al., 1997; Sousa et al., 2000; Kole et al., 2004; McLaughlin et al., 2007; Magariños et al., 2011). In this regard, the PN-FLX phenotype, is identical to that of chronically stressed mice, who also display a stress-induced enhancement of depression and anxiety-like behaviors and cognitive deficits (Bowman et al., 2002; Conrad et al., 2003; Gregus et al., 2005; McLaughlin et al., 2007; Luine et al., 2007). This fortifies the idea that changes in the CA3 are strongly associated with affective dysfunction and suggests there is a causal relationship between them.

CA3 Basilar dendrites

The basilar arbors of PN-FLX mice are more complex than those of controls, as evidenced by the significantly higher number of nodes, intersections and dendritic length (Figs. 3.12-3.18). These findings imply that, during the post-natal development of these arbors, 5-HT has a hypertrophic effect on their growth and affects their structure in a long-lasting manner. The

CA3 basilar phenotype of PN-FLX mice is identical to that of *5-htt-/-* mice, who also display bushier, more expansive arbors (Appendix B, Fig. B.5). Additionally, our analysis was done on pyramidal neurons in the CA3c region, the area within the blades of the DG. Tracing and immunohistological studies have shown that the basilar arbors of CA3c neurons receive significant input form the mossy-fiber pathway (Andersen et al., 2006). This finding further fortifies the idea that TSP connectivity and function may be altered in PN-FLX mice. Along with mossy-fiber afferents, basilar arbors also receive input from other CA3 neurons in both the ipsi- and contra-lateral HC, in a fiber path termed the associational-commisural (A/C) pathway (Andersen et al., 2006). This fiber pathway composed of the recurrent collaterals from CA3 neurons is thought to form an autoassociative network regulating the retrieval of memories, based solely on a small part or single feature of that memory (Gold and Kesner, 2005; Cravens et al., 2006). In particular, the (A/C)-recurrent collateral network has been shown to be important in cognitive tasks such as pattern completion and encoding of place/space ("spatial mapping") (Leutgeb and Leutgeb, 2007; Nakashiba et al., 2008; Fellini et al., 2009). As such, this pathway is implicated in the acquisition of contextual and spatial information (Nakazawa et al. 2002). Thus, structural changes in the basilar CA3 arbors, may reflect disruptions in A/C connectivity and potentially alter the behavioral functions mediated by this pathway, such as spatial and contextual cognition (discussed in subsequent section).

Dentate Gyrus

PN-FLX mice do not exhibit any changes in morphology of granule cells within the DG relative to controls (Fig. 3.21-3.24). This is contrary to our findings in *5-htt-/-* mice that possess

significantly less complex granule cell arbors relative to WT controls (Appendix B, Fig. B.7). This suggests that the affective phenotype shared by both PN-FLX and *5-htt-/-* mice is not likely due to changes in the DG as they do not share the granule cell structural phenotype. Since 5-*htt-/-* mice exhibit altered DG neuronal morphology, it is clear that the development and/or maintenance of the dendritic arbors of DG neurons is dependent on 5-HT. However, the lack of changes in the DG of PN-FLX mice implies that DG neurons may mature at a different period in development (outside the P2-P11 time window), relative to the CA3. Alternatively, the 5-HT receptor(s) that mediated the effects of 5-HT on dendritic arborization may not be expressed (or have limited expression) on the DG during the post-natal period in which we have pharmacologically altered 5-HT levels in PN-FLX mice, which would explain the lack of changes in the DG of PN-FLX mice, relative to controls.

HC-mediated behaviors

Next we asked whether the structural changes within the HC of PN-FLX mice affect its function using HC-sensitive behavioral paradigms including the Morris Water Maze (MWM) test and an associative contextual learning task (contextual fear conditioning). The MWM test requires mice to locate a hidden platform within a pool ("the maze") using extra-maze cues. Lesions of the HC, especially those affecting the dorsal HC, result in pronounced deficits in this task as evidenced by longer latencies to find the platform (Moser et al., 1995; Cho et al., 1999; Broadbent et al., 2006). This is true only when mice are released at different locations along the pool edge, as was done in our protocol, which prevents the use of an egocentric strategy (in which the self is the main reference point) or elemental associations, both of which are non-HC

dependent strategies (Rudy and Sutherland, 1989). Instead, when salient extra-maze cues are present, and when mice are released at varying locations within the maze, they are forced to make complex configural representations of space in order to locate the platform ("allocentric" or configural strategy), which is contingent on HC-output (Rudy and Sutherland, 1989; Arns et al., 1999). In this specific task, we find that PN-FLX mice display pronounced deficits in learning the location of a hidden platform as evidenced by significantly longer latencies to find the platform, relative to controls (Fig. 3.32). As loss-of-function manipulations of the HC, such as lesions and pharmacological inhibition, result in similar spatial learning deficits, it is likely that the PN-FLX mice have diminished HC output, and that increasing 5-HT levels in early-life result in a hypofunction of the HC.

Genetic inhibition of specifically the CA3 region, where we find the structural changes in PN-FLX mice, is sufficient to yield deficits in spatial cognition in the MWM test, despite the DG and CA1 regions being intact. For instance, mice with genetically silenced CA3 regions, due to conditional loss of the NR1 subunit of the excitatory glutamatergic NMDA-R (CA3-NR1-KO mice), have deficits in the MWM task (Nakazawa et al., 2002). Moreover, in these CA3-NR1-KO mice, signaling in the DG-CA3 and CA3-CA1 synapses is intact, but the associational-commisural (A/C) fibers representing the recurrent collateral CA3-CA3 connections have diminished signaling. This indicates a specific role of the A/C pathway in meditation of spatial cognitive behavior in the partial-cue probe trial of the MWM task. As previously mentioned, the dendritic changes in PN-FLX mice include regions that receive input form the A/C pathway. Thus our morphological and behavioral data indicate that early-life 5-HT levels are important in the maturation of the A/C circuit. This hypothesis could be directly addressed electrophysiologically to detect changes in A/C-CA3 connectivity, as was done by Nakazawa et

al., by stimulating the stratum radiatum of the CA3 region (that contains the A/C fibers) and recording in CA3 neurons in the stratum pyramidale. Taken together, these findings suggest that the structural changes in the CA3 of PN-FLX may be causal to their spatial cognitive deficits.

To verify that the prolonged latencies in PN-FLX mice were indeed due to altered ability to make configural representations of space, and not due to changes in sensory acuity, motivation or swimming ability, we performed a non-HC version of the MWM, in which the platform's location is clearly indicated by a large flag. In this visible platform version of the MWM test, PN-FLX mice performed as well as PN-Veh controls, as indicated by comparable latencies to find the platform and similar swim speeds (Fig. 3.30-3.31). The lack of difference in the visible training segment of the MWM test, suggests that PN-FLX mice do not differ from controls in their visual acuity or in comprehension of the task. Moreover, PN-FLX mice have significantly higher swim speeds and cover more distance during the hidden platform training phase (Fig. 3.33), suggesting that their motivation to escape from the maze and onto the platform is intact, and may even be enhanced relative to PN-Veh controls. Despite this enhanced searching, however, PN-FLX mice still have longer latencies to find the hidden platform, validating the deficit in their ability to create spatial representations of the maze context in order to find the platform, using an allocentric strategy.

In line with their spatial learning deficits, during the full-cue probe trial, PN-FLX mice display pronounced spatial memory deficits as evidenced by significantly less time in the target/platform quadrant, relative to controls (Fig. 3.34). When the spatial configuration surrounding the maze was altered by partial removal of extra-maze cues PN-FLX mice again spend significantly less time in target quadrant relative to controls (Fig. 3.35). Locating the platform under the partial-cue condition requires a pattern completion or autoassociative spatial

memory in which a single or few elements of the spatial context are sufficient to recall the entire spatial context (Nakazawa et al., 2002; Leutgeb and Leutgeb, 2007; Nakashiba et al., 2008). The CA3 region has been shown to play a major role in pattern completion (Gold and Kesner, 2005; Cravens et al., 2006; Nakashiba et al., 2008; Fellini et al., 2009), and its A/C fibers composed of the recurrent collaterals of CA3 neurons are thought to form a network, in which positive feedback loops and Hebbian mechanisms, can support such complex processes as pattern completion (Gold and Kesner, 2005; Kesner, 2007; Gilbert and Brushfield, 2009; Myers and Scharfman, 2011). Indeed, the aforementioned CA3-NR1-KO mice have been shown to have specific defects in pattern completion that may underlie their deficits in ability to perform on the MWM task, especially under partial-cue conditions (Nakazawa et al., 2002). Thus, the poorer performance of PN-FLX mice, relative to controls, in the partial-cue trial, indicates diminished pattern-completion ability, likely related to the structural changes in their CA3 regions. Finally, in the "no-cue" trial, in which all extra-maze cues were removed, neither treatment group showed a preference for the target quadrant (Fig. 3.36), indicating that the extra-maze cues were necessary for the mice to correctly locate the target region which validates the hippocampal-dependent nature of our protocol.

Our behavioral findings suggest that PN-FLX mice display changes in HC-output as evidenced by their deficient spatial learning and recall abilities. Congruently, PN-FLX mice also display pronounced deficits in the ability to form rapid representations of space and context in an associative contextual conditioning task. Specifically, PN-FLX mice display deficits in freezing to the training context after a fear conditioning paradigm (Figs. 3.40-3.41) suggesting that they have difficulties in making rapid (with 180 s of context pre-exposure) configurations of novel contexts. This task is HC-dependent, as lesions of the HC cause pronounced deficits in freezing

to context (Phillips and LeDoux 1992; Chen et al., 1996; Corcoran and Maren, 2001; Bast et al., 2001; Anagnostaras et al., 2001; Bast et al., 2003). The importance of specifically the CA3 region in contextual freezing is also established, since mice with genetically inhibited CA3 regions (CA3 TetX mice), display pronounced deficits in contextual freezing (Nakashiba et al., 2008). The genetic technique used in the study by Nakazawa et al., allowed for reversibility of CA3 inhibition, and it was shown that restoring CA3 output rescued contextual deficits, suggesting a causal relationship between CA3 function and acquisition of contextual information. Similar deficits in contextual learning have been characterized in *5-htt-/-* mice, who share the structural changes in the CA3 region observed in PN-FLX mice (Muller et al., 2011). Thus, the context deficits observed in both *5-htt-/-* and PN-FLX mice likely result from the cytoarchitectural/synaptic changes in their CA3 regions.

Although freezing to context is primarily attributable to altered HC-function, other structures such as the mPFC, amygdala and periacqueductal gray also affect contextual freezing (Philips and LeDoux, 1992; Morgan and LeDoux, 1995; Amorapanth et al., 1998; Ponnusamy et al., 2007; Yizhar et al., 2011). Thus, one cannot rule out the involvement of other regions in the contextual freezing deficits of PN-FLX mice. However, given their poor performance in the MWM test, which is more specific to HC-manipulations, it is likely that the decreased contextual freezing of PN-FLX mice is truly reflective of altered HC function, though changes in the aforementioned brain regions may exacerbate their deficits.

A final point regarding the contextual fear deficits of PN-FLX mice is that they are somewhat hard to reconcile with the well-characterized enhancement in anxiety and depression behaviors displayed by these mice. If the response to freezing is solely dependent of emotional factors, we would perhaps expect PN-FLX mice to display increased contextual fear

conditioning due to a putative increased reactivity to the stressful context-shock association. However, given the MWM data, the contextual deficits in PN-FLX mice are likely due to a diminished ability to learn spatial/contextual information, and are not as reflective of their emotional dysfunction. The dissociability of the affective and cognitive components of contextual fear conditioning have been established (Fanselow and Dong, 2010) and other mouse models of anxiety and depression similarly have diminished freezing to context (Muller et al., 2011).

Conclusions and future directions

In line with our main hypothesis, we have shown that modulation of serotonergic tone in early post-natal life impacts the maturation of the structural properties of the HC and these changes are directly correlated with altered HC output. In particular, we find that the development of the dendritic arbors and the density of spines in the CA3 sub-field of the HC are directly under the influence of post-natal 5-HT levels. Our findings also demonstrate that that the circuits underlying spatial/contextual cognition develop early on and are influenced by serotonergic tone. Indeed, given the high co-morbidity of cognitive and affective symptoms in mood disorder patients (Zakzanis et al. 1998; Hasler et al., 2004;; Ludewig et al., 2005; Gualtieri et al., 2008; Gohier et al., 2009), it is likely that the circuits mediating these two realms of behavior greatly overlap in the HC. Thus alterations to HC structure/function could account for both the affective and cognitive deficits in PN-FLX mice, and potentially in *5-htt -/-* mice and *"s"* allele carriers as well. As PN-FLX mice display both anxiety/depression behavior and learning/memory dysfunction, this mouse model encapsulates both the affective and cognitive components of emotional disorders and thus has strong heuristic value.

The data presented in this chapter suggest that the altered maturation of the CA3 in PN-FLX mice likely contribute to their cognitive deficits. An important question that remains, however, is whether the altered structural/synaptic changes in the CA3, of PN-FLX mice are causal to (at least some component) of their affective phenotype? And,if so, by what mechanism? These questions will be the focus of future experiments in our laboratory, and some of the previously described mouse models could serve as useful tools in this regard. For instance, if the focal, loss-of-function changes in the CA3 are causal to the affective phenotype of PN-FLX mice, it is likely that CA3-TeTX and/or CA3-NR1-KO mice display similar anxiety and depression-like behaviors. This can be experimentally addressed using the same battery of tests employed in the characterization of the PN-FLX mice behavioral profile (OF, NSF, SE tests). Investigating the behavioral profile of CA3-TeTX or CA3-NR1 KO mice that were treated post-natally with FLX or Veh would also prove informative. Specifically, if the changes in the CA3 are causal to the emotional dysfunction observed in PN-FLX mice, it is likely that mice that already have genetically silenced CA3 regions may be immune to the effects of further PN-FLX-mediated dysregulation of this region.

Several lines of evidence suggest the CA3-specific changes would indeed impact the emotional behaviors of PN-FLX mice. First, the CA3 subfield, via its ubiquitous connections to other HC sub-regions, is an important modulator of the oscillatory theta activity in the HC (Vertes and Kocsis, 1997; Buzsaki, 2002; Villarreal et al., 2007; Yamaguchi et al., 2007). As mentioned in previous chapters, theta oscillations represent synchronized neuronal firing, indicative of network activity, and play an important role in the modulation of emotional behaviors. For instance, mice with enhanced anxiety and depression-like behaviors display increased theta in the HC when confronted with anxiety-provoking situations (Gordon et al.,

2005). The synchrony between theta oscillations in two different regions, or theta coherence, between the HC and mPFC is also enhanced in anxiogenic conditions, and dysregulated connectivity between these regions is observed in mice displaying emotional and cognitive deficits (Sigurdsson et al., 2010; Adhikari et al., 2010). Thus, changes in the CA3 of PN-FLX mice may affect theta rhythms and/or coherence and may provide a mechanism by which the morphological (and potential physiological/connectivity) changes in the CA3 result in altered anxiety-like behavior of PN-FLX mice.

Furthermore, the CA3 can be considered a central unit in the HC as it communicates with the CA1 via Schaffer collateral fibers, has back-projections to the DG, and also receives direct, monosynaptic input from the superficial layers of the entorhinal cortex via the temporoammonic pathway (Andersen et al., 2006; Scharfman, 2007). In addition, it also sends ubiquitous collaterals to other CA3 neurons on the both the ipsi- and contra-lateral sides via the A/C pathway and is part of the main processing circuit in the HC, the trisynaptic pathway (Andersen et al., 2006). Alterations in the CA3 could potentially affect many, or all, of these pathways, and thus a focal change in the CA3 can theoretically affect a great many behavioral processes mediated by these various circuits, including those that involve regulation of affective state. The ubiquitous connectivity of the CA3 with other regions may also explain why a seemingly small and limited change in one sub-field of the HC can result in a profound effect on behavior.

Finally, if the changes in the CA3 are common to *"s"* allele carriers and other patients with affective/cognitive dysfunction observed in depression and anxiety, this information could potentially inform clinical interventions. For instance, drugs that target specifically the CA3 region of the HC may have value in the treatment of some symptoms of depression and anxiety. Finally, as the stress literature demonstrates, the CA3 region retains plasticity into adulthood.

Thus, despite the post-natal period being a critical period in the development of CA3 arbors, it is likely that this window may be re-opened in adulthood and any pathological structural/physiological changes in this region could be corrected with adult interventions. Thus, pharmacological agents that increase CA3 plasticity may prove beneficial in reversing the CA3-mediated effects on anxiety, depression and cognitive behaviors. Indeed diminished plasticity of the brain is implicated in affective disorders, and agents that enhance plasticity have therapeutic potential (Duman, 2002; Pittenger and Duman, 2008).

CHAPTER 4: Discussion

Our data demonstrate that the structural and functional maturation of brain regions and corticolimbic circuits that underlie affective and cognitive behaviors are influenced by 5-HT signaling during a critical developmental epoch. Specifically, we find that increased 5-HT signaling from P2-11leads to morphological and physiological changes in the adult mPFC and HC that correlate with anxiety/depression-like behaviors, and cognitive impairment, in later life.

Our model

Our findings support the idea that emotional and cognitive components of anxiety and depression disorders, which manifest in adulthood, may have neurodevelopmental origins and their ontogeny is dependent on abnormal serotonergic tone during development.

According to our model (Fig. D.1), factors that alter 5-HT signaling from P2-11 in mice, or equivalent species-specific critical periods, whether genetic (mutations or functional polymorphisms in *5-htt*, *tph*, *maoa*, *5-ht1a* receptor etc.) or environmental (stress, exposure to serotonin-modulating agents such as SSRIs), permanently alter corticolimbic circuits that mediate adult affective and cognitive behaviors. Thus, if individual, or combinations of, modulatory factors lead to increased serotonergic tone during the critical period, they subsequently augment the vulnerability to anxiety and depression disorders later in life. Thus, the paradoxical enhancement in depression and anxiety behaviors displayed by *5-htt -/-* mice and human "*s*" allele carriers are likely caused by increased serotonin signaling during P2-11 and the third trimester of gestation (comparable to the P2-11 period in mice, in terms of developmental processes underway), respectively.

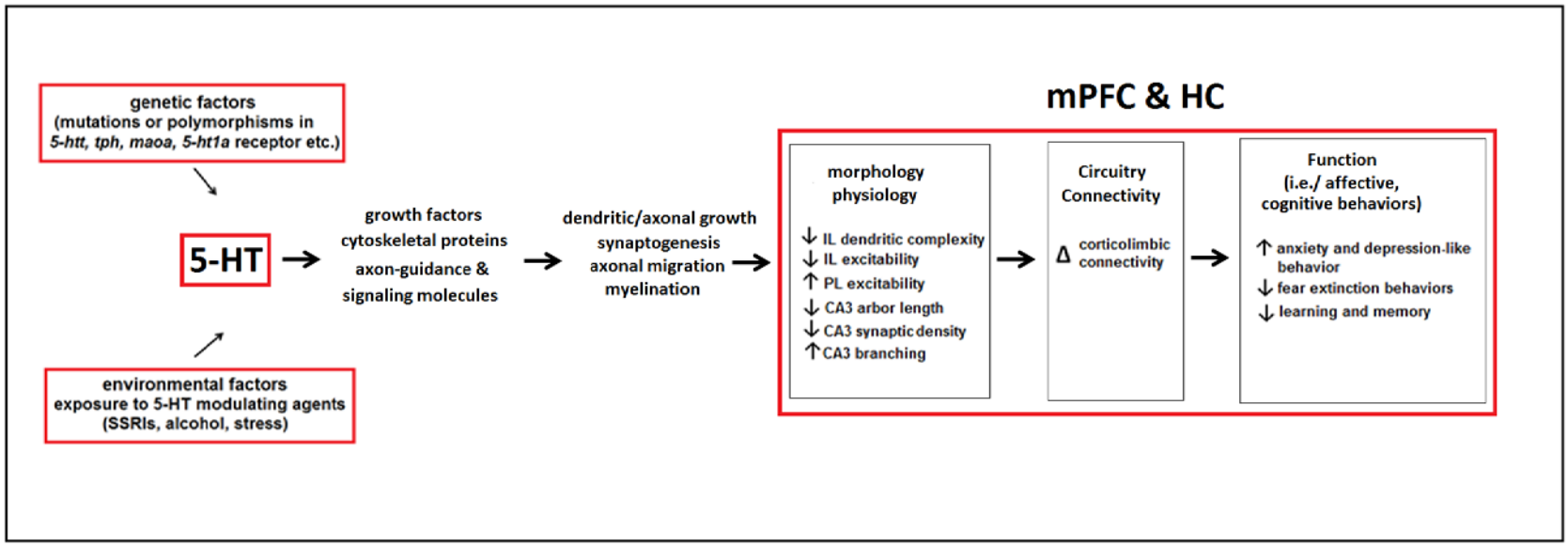


Fig.D.1 Model by which genetic and/or environmental factors that alter serotonergic tone during key critical periods in development impact adult affective, fear and cognitive behaviors.

mPFC-HC synergy

In the mPFC, the fingerprint of increased serotonergic tone during the critical post-natal epoch, is a decrease in IL/PL output ratio, which when mimicked in control mice, is sufficient to phenocopy some, but not all, of the anxiety and depression-like phenotype of PN-FLX mice. In the HC, augmentation of serotonin levels during development generate a CA3-hypofunction phenotype. Although we have not addressed this directly, we predict that the structural/synaptic changes in the CA3 of PN-FLX mice are also causal to some components of their emotional dysfunction and cognitive deficits. The fact that neither the mPFC of HC would contribute to the entirety of the PN-FLX phenotype, underscores the synergy between these two regions in the etiology of the anxiety and depression behaviors observed in PN-FLX mice. Indeed, corticolimbic connectivity has been shown to be paramount in behavioral responses during anxiety, and is dysregulated in mice models of emotional dysfunction (Price and Drevets, 2010; Adhikari et al., 2010). Thus, future work will investigate how mPFC-HC connectivity is affected by increased developmental serotonin signaling, and how potential changes in communication between these regions, act in concert to alter behavior.

Additional alterations

Although our focus has been primarily on the mPFC and HC, other behaviorally relevant structures may also be altered and warrant further investigation. For instance, our findings suggest that the structural and physiological alterations in the mPFC of PN-FLX mice, impact amygdala activity through altered top-down control. This is supported by our fear extinction data and is reviewed in the connectivity model presented in Chapter 2, Fig. 2.33. Our model may thus explain the increased amygdala activity seem in human *"s"* allele carriers (Pezawas et al., 2005;

Heinz et al., 2005; Price and Drevets, 2010). However, we have not directly assessed amygdala morphology and physiology and thus cannot exclude that developmental serotonin signaling also modulates the maturation of this region. Thus, future work will close this gap in knowledge and investigate the consequences of increased serotonin signaling during P2-11 on amygdala maturation.

Additionally, the mPFC and HC project to other brain structures which are also important in regulation of emotional state, and the physiological responses to stress and fear, such as the hypothalamus, nucleus accumbens and the ventral tegmental regions (Antoniadis et al., 2001; Davidson et al., 2002; Seidenbecher et al., 2003; Vertes, 2004; Quirk et al., 2006; Price and Drevets 2010). As such, it is imaginable that a primary dysfunction in the mPFC and HC may elicit secondary changes in these brain systems, thereby contributing to symptoms observed in patients with mood and anxiety disorders, such as dysregulated stress response, blunted affect and anhedonia.

Finally, the maturation of the 5-HT system itself is altered in mice that experienced disrupted serotonergic tone in development (Kinney et al., 1997; Maciag et al., 2006a and 2006b; Neil Gray, Emanuela Morelli and Tessa Hirschfeld-Stoler, unpublished results). This finding emphasizes the known auto-trophic properties of 5-HT (Gaspar et al., 2003; Sodhi and Sanders-Bush, 2004). For instance, PN-FLX mice display a hypotrophic serotonergic system with reduced serotonergic input into the mPFC and HC (Neil Gray, unpublished results). Thus, to fully understand the etiology of the affective and cognitive behaviors of mice or humans that experienced altered serotonergic tone during development, the contributions of both the presynaptic (5-HT) and post-synaptic (areas receiving 5-HT input, such as the mPFC and HC) structures need to be determined. This is especially important given the pivotal role of serotonin,

in the adult brain, in the modulation of anxiety, depression and cognitive behaviors. It is likely that the pre- and post- synaptic changes work collaboratively to generate the enhanced anxiety/depression and cognitive-deficit phenotype generated via early-life disruptions of normal serotonergic tone.

Considerations

Although our approach to disrupt normal serotonergic tone during development is to increase it via FLX administration, others in the field have used depletion methods to address the role of serotonin in affective/cognitive circuit formation (Yan et al., 1997; Popa et al., 2008; Hohmann et al., 2007; Zhou et al., 2008; Hamon et al., 2011; Fernandez and Gaspar, 2012). Evidently, both increasing and dampening 5-HT tone during development have deleterious effects on brain maturation and function. This suggests, that there is a normal range of serotonin that is required during development in order to ensure proper maturation of brain structures such as the mPFC and HC, and either abnormally high or low levels of serotonin during critical time windows in development may alter the structural and functional maturation of emotional/cognitive circuits in the brain.

Furthermore, the role of 5-HT in circuit formation does not seem to be limited to those mediating emotional behaviors, as a similar requirement for proper 5-HT signaling has been demonstrated for the circuits underlying sensorimotor gating, sexual and aggressive behaviors and sensory perception (Rebsam et al., 2002; Gaspar et al., 2003; Maciag et al. 2006a; Nakamura et al., 2006; Qinghui Yu, unpublished results), with differing critical time windows for each behavior type. These findings underscore the general neurotrophic properties of 5-HT in circuit formation and suggest that the impact of early-life modulation on diverse adult behaviors are

contingent on the specific epochs in which they are altered (Ansorge et al., 2007; Leonardo and Hen, 2009).

Future directions

Although we have shown that serotonin modulates the development of mPFC and HC neurons the exact mechanism by which this occurs remains to be investigated. Further characterization of the specific serotonin receptors and intracellular signaling pathways that mediate the effects of serotonin on dendritic morphology, synaptogenesis and receptor expression, and ultimately behavior, require further characterization.

Finally, an important question with clinical significance is whether we can reverse or rescue the changes in the mPFC-HC circuits that arise from early-life disruption of serotonin tone. If the focal changes in the mPFC and HC are causal to their affective phenotype, then by enhancing IL activity, dampening PL activity or increasing CA3 output (or perhaps a combination of these), we may be able to correct the emotional phenotype of mice and humans that experienced early-life alterations in serotonin signaling.

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APPENDIX A

This section contains the specific dendritic morphology data from serotonin transporter knockout (*5-htt* -/-) mice, (generated as previously described in Lira et al., 2003) referred to in the main text. Data were analyzed using one-way analysis of variance (ANOVA) using genotype and are presented as mean +/- SEM.

Medial prefrontal cortex (mPFC)

Infralimbic (IL) region

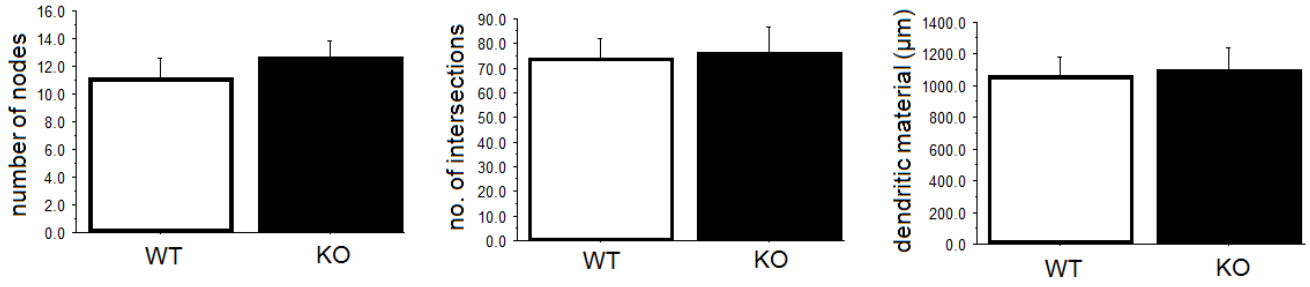

Fig. B.1. No observable differences in the dendritic complexity of apical dendrites of pyramidal cells in layer 2/3 of the IL of 5-htt -/- mice, relative to controls. N = 15-16 cells/treatment, 3 mice/group.

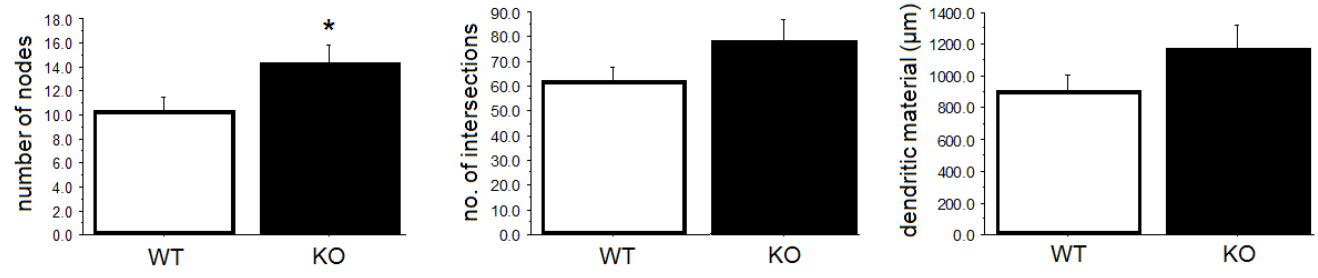

Fig. B.2. Apical dendrites of pyramidal cells in layer 5 of the IL of *5-htt* -/- mice display significantly increased number of nodes ($F_{1,31}$ = 4.631; p = 0.0393) with no changes in number of

intersections and total dendritic material, relative to controls. N = 15-16 cells/treatment, 3 mice/group.

Hippocampus (HC)

Cornu Ammonis (CA3) neurons

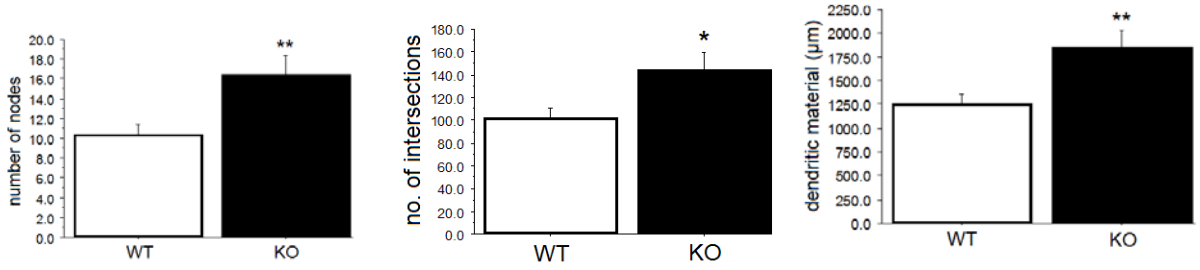

Fig. B.3. Apical dendrites of pyramidal cells in the CA3 of *5-htt -/-* mice display significantly increased number of nodes ($F_{1,32}$ = 8.434; p = 0.0066), intersections ($F_{1, 32}$ = 6.037; p=0.0196) and total dendritic material ($F_{1,32}$ = 8.125; p = 0.0076), relative to controls. N = 16-17 cells/treatment, 3 mice/group.

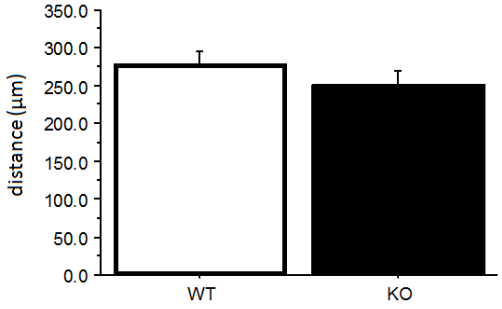

Fig B.4. No significant differences in how far the CA3 apical arbors of KO and WT mice extend out from the soma. N = 16-17 cells/treatment, 3 mice/group.

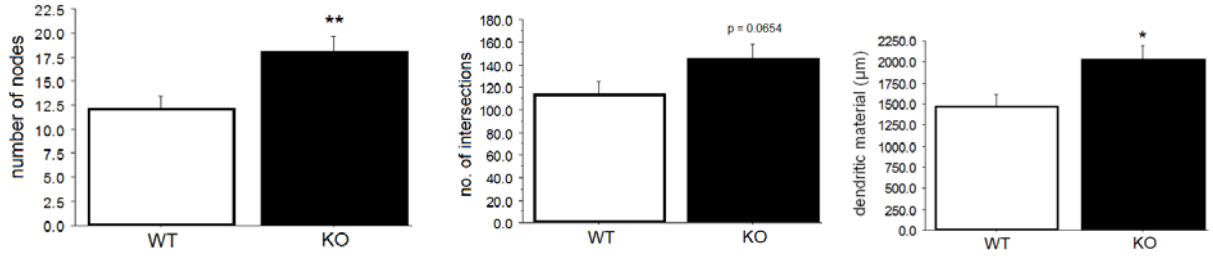

Fig. B.5. Basilar dendrites of pyramidal cells in the CA3 of *5-htt -/-* mice display significantly increased number of nodes, ($F_{1,30}$ = 8.780; p = 0.0069), intersections (trend effect: $F_{1,31}$ = 3.660; p = 0.0654) and total dendritic material ($F_{1, 31}$ =7.047; p = 0.0124), relative to controls. N = 15-16 cells/treatment, 3 mice/group.

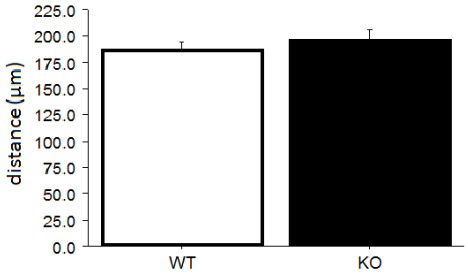

Fig B.6. No significant differences in how far the CA3 basilar arbors of KO and WT mice extend out from the soma. N = 15-16 cells/treatment, 3 mice/group.

Dentate gyrus granule cells

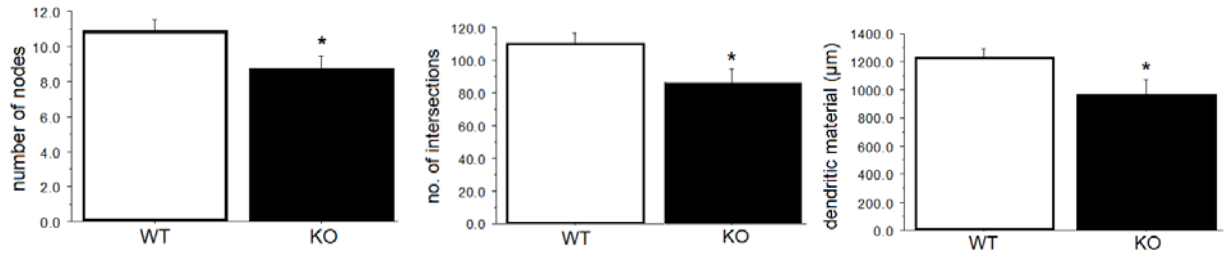

Fig. B.7. Apical dendrites of granule cells in the dentate gyrus from *5-htt -/-* mice display significantly decreased number of nodes ($F_{1,31}$ = 4.907; p = 0.0342), intersections ($F_{1,34}$ = 5.614;

p = 0.0236) and total dendritic material ($F_{1, 33}$ = 4.845; p = 0.0348), relative to WT controls. N = 16-17 cells/treatment, 3 mice/group.

Cornu Ammonis 1 (CA1) pyramidal neurons

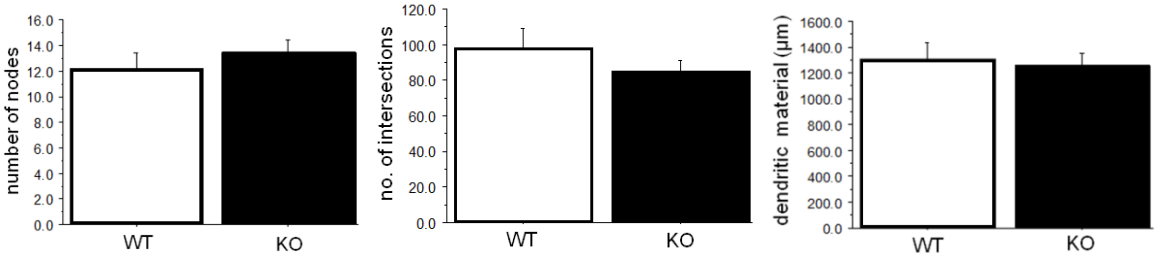

Fig. B.8. Apical dendrites of CA1 neurons of *5-htt -/-* do not significantly differ from those of WT controls. N = 18 cells/treatment, 3 mice/group.

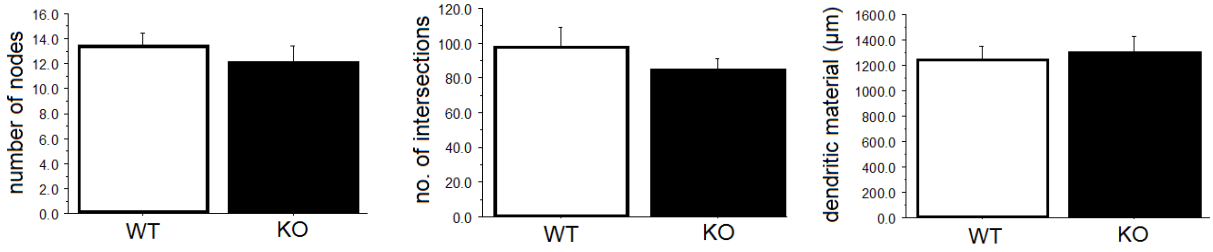

Fig. B.9. Basilar dendrites of pyramidal cells in the CA1 of *5-htt -/-* do not significantly differ from WT controls. N = 18 cells/treatment, 3 mice/group.

APPENDIX B

These data display the results from the cued fear conditioning and extinction paradigms done on a second group of PN-FLX and PN-Veh mice ("cohort 2"), referred to in chapter 2. No cued deficits were found in cohort 2, but they did display pronounced extinction learning deficits as was characterized in the cohort described in the main text. This demonstrates that the cued deficits are not replicable, whereas the extinction deficits are.

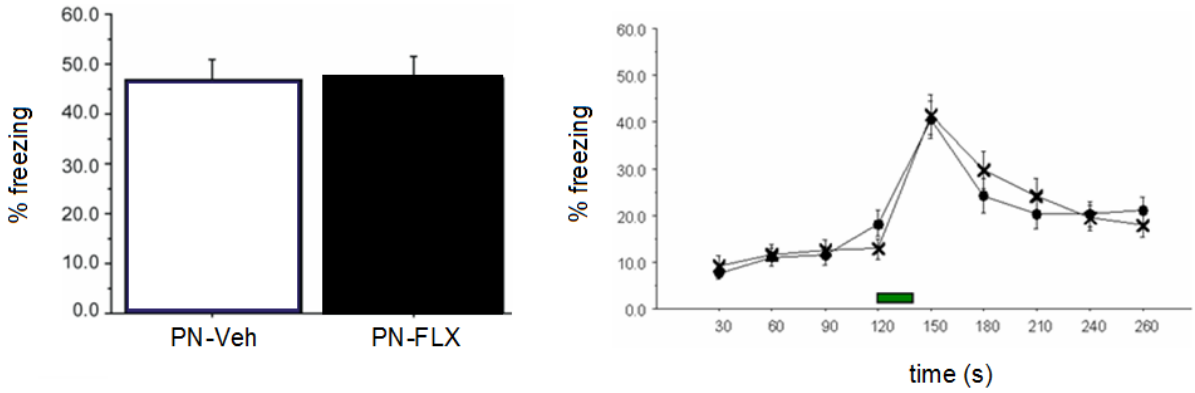

Fig C.1. No differences in % freezing to cue between PN-Veh and PN-FLX mice in a separate cohort tested. Green bar represents the tone ("cue" or "conditioned stimulus").

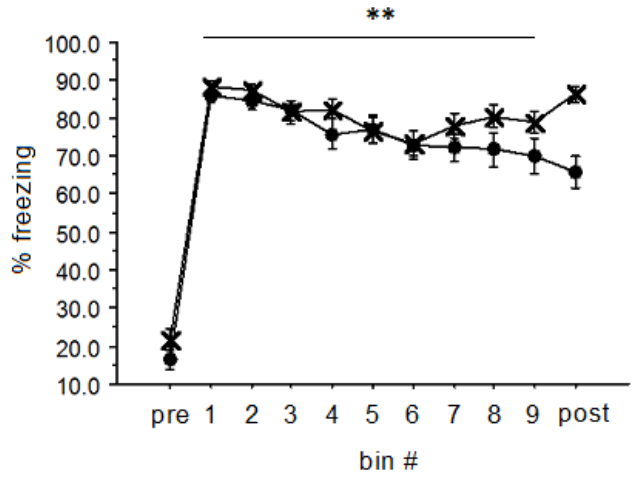

Fig C.2. Pronounced deficits in fear extinction learning displayed by PN-FLX mice (treatment x time interaction = 0.0051) in cohort 2.