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# The effects of early life adversity on the development of circuits that support flexible decision-making

By

Alaina Wren Thomas

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

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of the

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Committee in charge:

Professor Linda Wilbrecht, Chair
Professor Silvia Bunge
Professor Daniela Kaufer
Professor Ronald Dahl

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Abstract

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University of California, Berkeley

Professor Linda Wilbrecht, Chair

A large proportion of children in the United States experience childhood adversity, with over 50% reporting at least one adverse event. Early life adversity is strongly associated with increased risk for developing a number of physical and mental health disorders, yet the pathways linking childhood adversity to disease are not well understood.

This dissertation explores how the early environment can influence developing neural circuits, with a particular focus on neural circuits that support flexible goal-directed decision-making. Cognitive development plays a significant role in mental illness and prospects for recovery. Flexible decision-making is a major cognitive function that is impaired across multiple neuropsychiatric disorders including bipolar disorder and schizophrenia, that is also critical in supporting behavioral change.

In Chapter 2, I review my own collaborative work as well as work from others to describe the distinct developmental trajectories of sub-circuits that support flexible decision-making. I focus on frontal cortex dendritic spines and axonal boutons on afferents and efferents of the frontal cortex. These studies are motivated by the idea that identifying when these circuits grow and/or mature at the synaptic level will inform us when connections may be more vulnerable to adverse experiences and/or when interventions may have the greatest impact. I present *in vivo* imaging data that support previous classic findings that dendritic spines on frontal pyramidal neurons show loss of linear spine density, or in other words “prune,” across the adolescent period. However, I also present evidence that some long range frontal afferent and efferent circuits continue to grow and add synapses during the adolescent period, while others are pruning and stabilizing. These data refute the simple assumption that frontal circuits globally prune during adolescence, and raise questions about why some circuits show delayed growth.

Next, in Chapter 3, I explore how maternal separation, a mouse model of early life adversity, affects flexible decision-making across the lifespan. I find that this early life manipulation leads to changes in decision-making specifically at a juvenile, but not adult

life stage. I hypothesize that changes in decision-making strategies at the juvenile time point, a point of first independence, may serve as a cognitive adaptation to signals that indicate a harsh environment.

Finally, in Chapter 4, I investigate how maternal separation and more specifically variations in maternal care, impact the development of four long range axons (two afferents and two efferents of the frontal cortex) that each play a role in flexible decision-making. I focus on changes at the adolescent life stage, based on differences in flexible decision-making at this age discussed in Chapter 3. I find that dorsomedial prefrontal cortex (dmPFC) axons that descend to target the basolateral amygdala (BLA) are specifically sensitive to the maternal separation manipulation and variations in maternal care, while the other three axons investigated show no relationship with early life care. Specifically, I found higher bouton density and smaller bouton size on dmPFC-BLA axons in maternally separated mice compared to controls. Additionally, bouton density on this projection correlated with maternal care measures, suggesting early maternal care (P1-10) may scale the later growth of this pathway (at P35). Variations in maternal care may serve as an indicator about the type of environment offspring are growing up in. These data support the idea that the brain can sample the statistics from the early environment and tune specific circuits to adapt to that environment.

Taken together, this thesis provides new insights about the development of circuits that support flexible decision-making. Importantly, I demonstrate how early life adversity impacts specific circuits at the synaptic level. These experiments provide high-resolution data that aims to help inform intervention and treatment strategies to promote healthy child development.

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Chapter 1

# Introduction: Early life adversity and neural circuits for flexible decision-making

## Early life adversity

Childhood adversity is experienced by a large proportion of children in the US, with over 58% of children reporting at least one adverse event and almost 60% of this sub population reporting multiple events (McLaughlin et al., 2012). Early life adversity is strongly associated with risk for developing a number of physical and mental health disorders (Cohen et al., 2001; Dube et al., 2001; Edwards et al., 2003; Felitti et al., 1998; Green et al., 2010; Kessler et al., 1997).

Adverse early life experiences encompass a range of negative factors that a child may encounter from infancy through childhood. Adversity includes, but is not limited to, abuse, neglect, parental loss, parental mental health disorder, family violence and poverty (Felitti et al., 1998). These encounters may vary by type (physical to emotional), duration (a single instance to chronic exposure), timing (infancy to childhood), severity (mild to severe), as well as predictability (consistent to unpredictable). As mentioned above, most children who experience one type of adversity also experience multiple others and this may scale future risk (Green et al., 2010; McLauglin et al., 2012). The adverse childhood experience (ACE) study found a relationship between the number of adverse experiences and mental and physical health outcomes. Compared to children with no experience of adverse events, children that experienced 4 or more forms of adversity had a 4-12 fold increased risk for developing a number of psychiatric conditions including depression and substance abuse (Felitti et al., 1998). The ACE study was critical for drawing a link between adverse childhood experiences and negative health outcomes. However, lumping adversities together makes it difficult to disentangle how different types of childhood exposures uniquely impact brain development and lead to different outcomes. Recently, researchers have started to isolate how different factors, such as the timing of adverse experiences (Tottenham and Galvan, 2016) and the dimension of adversity, such as deprivation and threat (Sheridan and McLaughlin, 2014), affect brain development in unique ways. Importantly, animal models of early life adversity can aid in the independent investigation of these factors, controlling for different types of exposures, as well as genetic factors.

The pathways linking childhood adversity to disease are not well understood. Identifying how these experiences lead to changes in brain structure and function is critical for developing treatments and interventions. In this dissertation, I explore potential mechanisms by which environmental insults become biologically embedded in neural circuits. In Chapter 2, I first describe how neural circuits that support flexible decision-making mature in typically developing rodents. Then, in Chapter 3, I test how maternal separation affects flexible decision-making and anxiety-like behavior. Finally, in Chapter 4, I investigate how a mouse model of early life adversity, as well as

variations in maternal care impacts the neural circuits that support flexible decision-making.

*Maternal care as a critical aspect of the early environment*

One of the most important interactions in early life is the infant-caregiver relationship (Bowlby, 1982; Levine, 1962). Altricial species, like humans and rodents, are completely dependent on their primary caregiver (typically the mother) for survival during the postnatal period. Specifically, altricial infants receive warmth, nourishment and protection from their caregiver. Clinical descriptions of institutionalized children as well as studies in primates and rodents provided some of the first evidence that depriving altricial organisms of this species expected input has long-lasting effects on the offspring (Spitz, 1945; Denenberg; 1963; Harlow, 1958; Harlow et al., 1965; Levine and Lewis, 1959). Furthermore, rodent studies have provided evidence that specific maternal cues (e.g. licking, grooming and nursing), are able to regulate the physiology and behavior of rodent pups (Hofer, 1994). The caregiver also most likely serves as a signal about the type of environment that offspring are growing up in. Variation in maternal signals may act to shape the development of neural circuits in unique ways (Baram et al., 2012).

*Human studies of maternal deprivation*

Extreme examples of maternal deprivation in humans include institutionally reared children. These impoverished settings are characterized by low caregiver to child ratios, poor cognitive, emotional and sensory stimulation and non-individualized routines for basic needs (all children eat, sleep and use the bathroom at the same time) (Nelson et al., 2007). Children raised in these environments display a number of differences in cognitive abilities compared to typically-developing age-matched individuals, including lower IQ (Fox et al., 2011; Nelson et al., 2007), language impairments (Windsor et al., 2007), executive function deficits (Bos et al., 2009; Hostinar et al., 2012), problems with emotion regulation (Tottenham et al., 2010) and attachment (O'Conner et al., 2003), as well as increases in psychiatric disorders (Zeanah et al., 2009). For certain domains (such as IQ), children that were placed into foster families before the age of 2, showed better recovery to age-matched control levels (Nelson et al., 2007; Hostinar and Gunnar, 2013). In general earlier adoption placement is associated with better outcomes (Tottenham et al., 2010).

*Rodent models of maternal deprivation*

In order to investigate a more causal relationship between early life adversity and developmental outcomes, rodent models have been developed, which allow for greater control of both the environment and genetic background. These models have been used to examine the effects of early adversity on affective behavior, typically anxiety and depression related behaviors and also cognitive development.

A large subset of rodent studies have compared offspring that experienced variations in maternal care, comparing litters from dams that provided the lowest versus highest amounts of maternal care (Caldji et al., 2000; Liu et al., 1997). Other models

manipulate the amount of nesting material provided to the dam (Gilles et al., 1996; Ivy et al., 2008; Rice et al., 2008), which produces erratic behavior in dams. Importantly, this manipulation does not have an effect on the amount of care provided to the pups, but rather affects the quality of care, leading to sorties from the nest and greater fragmentation of maternal care (Rice et al., 2008). Extreme deprivation studies have also been used, in which rodents are artificially reared and receive no dam care at all (Lovic and Fleming, 2004). Finally, maternal separation (MS) is a common model that involves removing rodent pups from the dam daily for brief periods of time (typically 1-3 hours) during the early postnatal period (Francis et al., 2002; Plotsky and Meaney, 1993; Romeo et al., 2003).

*Effects of maternal separation on the HPA axis and anxiety-like behavior*

Studies investigating the impact of maternal separation on the hypothalamic-pituitary-axis (HPA) were extremely important in first drawing connections between infant-caregiver manipulations and long-lasting changes on brain and behavior. I highlight these studies here as they were important in inspiring my thesis work.

The HPA axis is made up of a set of connected structures that are important for responding to stressful conditions. When activated, corticotropin-releasing hormone (CRH; also referred to as corticotropin releasing factor or CRF is released from the paraventricular nucleus (PVN) of the hypothalamus (Vale et al., 1981). CRH then activates the secretion of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary gland (Rivier and Vale, 1983), which then stimulates glucocorticoid release (cortisol in humans and corticosterone in rodents or CORT) from the adrenal gland. Glucocorticoid receptors in the hippocampus play a part in mediating feedback inhibition of the hypothalamus and inhibiting CRH synthesis and release (Jacobson and Sapolsky, 1991).

A number of studies have shown that maternal separation in rodents leads to both immediate and long-term effects on the hypothalamic-pituitary-adrenal axis. Specifically, rat pups that underwent 3 hours of maternal separation from P2-14 displayed elevated plasma corticosterone levels at P7 compared to rats that were handled for 15 minutes each day (Huot et al., 2002). Furthermore, early life maternal separation in rats led to HPA axis hyper-reactivity in adulthood (Ladd et al., 1996; Plotsky and Meaney, 1993; Nishi et al., 2013; Wigger and Neumann, 1999), characterized by increased CRF mRNA in the hypothalamus (Plotsky and Meaney, 1993), elevated basal levels of plasma ACTH and corticosterone (Ladd et al., 1996) and in response to an acute stressor (Huot et al., 2001; Lippmann et al., 2007). Adult male mice that experienced 3 hours of maternal separation from P1-10 displayed a prolonged corticosterone response to an acute stressor (Parfitt et al., 2004). This observed hyper-reactivity may be mediated by dysregulated glucocorticoid negative feedback as multiple studies indicate that maternal separation led to decreased glucocorticoid receptor expression in the hypothalamus, hippocampus and frontal cortex of rats (Ladd et al., 2004; Meaney et al., 1996). Importantly, providing a foster litter to rodent dams during separation was able to mitigate some of the effects on the HPA axis, suggesting that the effects of maternal separation may in part be mediated by maternal care changes rather than from the separation itself (Huot et al., 2004).

Along with changes in the HPA axis, maternal separation also leads to changes in anxiety and depression-like behavior in adult rodents. Rats that underwent 3 hours of maternal separation from P2-14, spent significantly less time in the open arms of an elevated plus maze in adulthood (Huot et al., 2001) and in adolescence (Li et al., 2013). However, studies in mice are inconsistent. One study found adult male C57BL/6J mice that experienced 3 hours of MS from P1-14 displayed elevated anxiety-like behavior in the open field test and elevated plus maze compared to controls, while females showed reduced anxiety-like behavior in the open field test, specifically when in the diestrous phase of the estrous cycle (Romeo et al., 2003). In contrast, other studies which assessed the effects of 3 hours of MS on different mouse strains found no effect on anxiety and depressive-like behaviors in the elevated plus maze, open-field and forced swim tests in a number of different strains, including C57BL/6J mice (reviewed in Millstein and Holmes, 2007). One study investigated the effects of MS in adolescence and found that MS led to increased anxiety-like behavior, measured by increased % time in closed arms, in adolescent C57BL/6 male mice (P35-55) (Shin et al., 2016).

*Effects of maternal separation on frontal cortex and cognition*

Compared to the HPA axis, frontal cortex and cognitive changes in response to MS have been less studied. Studies have found that maternal separation leads to frontal cortex changes, but like the HPA axis studies, findings are inconsistent. One study found that maternal separation in rats for 4 hours from (P) 1-21 led to increased spine density on layer 2/3 neurons in OFC and mPFC in adulthood (Muhammad et al., 2011; 2012), while three other studies found reduced spine density on layer 2/3 neurons of the mPFC in adult rats following MS (Chocyk et al., 2013; Monroy et al., 2010; Pascual and Zamora-León, 2007). Another study found that the timing of the MS protocol was important. Specifically, it tested rats that underwent MS either for 1 hour a day before (P1-3), during (P5-7) or after (P14-16) the stress hyporesponsive period (SHRP), and found that MS rats that experienced MS before the SHRP, displayed reduced dendritic spine density on layer 2/3 pyramidal neurons of the mPFC at P21, while separation after the SHRP increased spine density on these same neurons (Bock et al., 2005). Early work with rodents suggests that the first two weeks of life are characterized by the SHRP, a period in which the HPA axis is less responsive (Rosenfeld et al., 1992). However as previously discussed, stressors that are more ethologically relevant and age-appropriate, such as maternal separation or cold stress are able to mount an increase in plasma corticosterone (Pihoker et al., 1993; Plotsky and Meaney, 1993). Maternal presence also plays a role in maintaining the SHRP (Moriceau and Sullivan, 2006). This suggests that the developing rodent and HPA axis may be particularly sensitive to stress that is relevant to the early-life stage. A recent study found that male and female rats that experienced MS for 3 hours a day during the first 3 weeks of life, had less myelination in the mPFC at P21 and this effect lasted into adulthood at P60 (Yang et al., 2017).

Male rats that were maternally separated for 4 hours a day from P2-20 displayed working memory deficits on the radial arm maze when tested at P40, but not at P25 (Brenhouse and Andersen, 2011). Rats that experienced 3 hours of maternal separation during the first two weeks of life were less flexible on a 2-choice reversal learning task in

adulthood (Baudin et al., 2012). However, studies in mice have inconsistent findings. One study found strain-dependent effects; Balb/c MS mice displayed impairments in spatial working memory and extradimensional shifts in attentional set-shifting compared to controls, while C57BL/6 MS mice did not differ from controls (Mehta and Schmauss, 2011). In Chapter 3, I explore how maternal separation affects flexible decision-making at juvenile and adult life stages. I aimed to clarify the inconsistencies across studies by looking at the effects of maternal separation on maternal care behaviors. In Chapter 4 I discuss how the development of cortico-amygdala projections are specifically sensitive to variations in maternal care.

## Goal-directed behavior and flexible decision-making

Executive function is an umbrella term used to describe a number of processes necessary for purposeful, goal-directed behavior. Goal-directed actions are controlled by their outcomes, in contrast to habits that are reflexively driven by antecedent stimuli (Yin and Knowlton, 2006). A key aspect of goal-directed behavior is cognitive flexibility, as animals must adapt their behavior in response to changing environmental contingencies (Anderson, 2002). For rodents, digging tasks that train animals to dig in pots filled with scented bedding for rewards are effective at assessing flexible decision-making strategies using ethologically relevant paradigms (Birrell and Brown, 2000). Additionally, digging tasks are quickly learned in rodents, making them useful tasks for assessing the development of cognitive flexibility in rodents that grow up quickly. Specifically, reversal learning tasks require rodents to first discriminate among various cues and the associated outcomes in order to receive a reward. In the reversal phase, a previously unrewarded cue predicts the reward and animals must stop responding to the previously rewarded odor and learn the new association (Kim and Ragozzino, 2005).

## Circuits supporting flexible goal-directed behavior and decision-making

The rodent dorsomedial prefrontal cortex (dmPFC), which includes anterior cingulate cortex (ACC) and supplementary motor area (M2), integrates information from a number of diverse regions including contralateral dmPFC, sensory and motor areas, orbitofrontal cortex (OFC), basolateral amygdala (BLA), ventral hippocampus (vHPC) and mediodorsal thalamus (DeNardo et al., 2015; Hoover and Vertes, 2007). The dmPFC also sends projections to many different regions including motor areas, the striatum, amygdala, brainstem and spinal cord (Gabbott et al., 2005; Little and Carter, 2013). Additionally, the dmPFC is reciprocally connected with neuromodulatory centers, including dopamine from the ventral tegmental area, serotonin from the raphe nuclei, noradrenaline from the locus coeruleus and acetylcholine from the basal forebrain (Dembrow and Johnston, 2014).

Previous studies have shown that regions of the frontal cortex play different roles in cognitive flexibility. There is some debate about the existence of a PFC in rodents, but a number of studies have identified different functions of anatomically distinct sub-

regions of rodent frontal cortex. Here, I consider the PFC and frontal cortex as overlapping regions in the mouse. Studies in rodents indicate that orbitofrontal lesions impair 2-choice reversal learning while medial prefrontal cortex lesions impair performance on extra-dimensional set-shifting tasks (Birrell and Brown, 2000; Bissonette et al., 2008; Kim and Ragozzino, 2005; McAlonan and Brown, 2003). However, studies using a 4-choice reversal learning task found that lesions and inactivations of the dorsomedial frontal cortex impair performance (Johnson and Wilbrecht, 2011; Ragozzino and Rozman, 2007), suggesting that the dmPFC is potentially recruited when there is more cognitive load, such as during a 4-choice compared to 2-choice reversal task.

In order to successfully navigate an unpredictable environment, an animal must use prior experience to estimate the value of cues and actions to optimize decision-making strategies. In order to do this, neurons must harbor signals related to past choices, outcomes and the values of those outcomes. Previous work indicates that prefrontal regions including the orbitofrontal (OFC) and anterior cingulate cortex (ACC), as well as the basolateral amygdala (BLA) and components of the striatum play critical roles in evaluating and deciding between different choices when the outcomes of these options are unknown.

Studies in rodents and primates indicate that the OFC plays an important role in signaling cue-based outcome expectancies (Schoenbaum et al., 2009). OFC neurons encode the sensory properties of rewards (Rolls, 2010) and also fire in response to the cue, in anticipation of a reward, as well as the outcome (Tremblay and Schultz, 2000) and thus have been implicated in processing reward information including outcome values. Neurons in the OFC have been shown to adjust their firing based on the relative preference for a reward (Tremblay and Schultz, 1999).

In contrast to the OFC, the anterior cingulate cortex has been implicated in using past action-outcome histories to guide behavior (Kennerley et al., 2006). Single neuron studies in primates and rodents have found signals related to the animal's prior choice and its outcome in the ACC (Seo and Lee, 2007; Sul et al., 2010). In primates, ACC neuron activity changes in response to reward presence (Kennerley and Wallis, 2009). Interestingly, ACC neuron firing rate in primates increases both to the presence and absence of a reward, indicating that ACC may be encoding the value of an outcome as well as the action that led to that specific outcome (Hayden and Platt, 2010; Kennerley et al., 2009) ACC neurons also show surprise signals to unexpected outcomes (Hayden et al., 2011). Anatomical studies also support a role for the ACC in integrating actions and outcomes as it is heavily connected with premotor areas (Hoover and Vertes, 2007).

Limbic and subcortical regions also play roles in goal-directed behavior and decision-making, including the BLA and DMS. BLA lesions disrupt the formation of cue-outcome associations in the OFC (Schoenbaum et al., 2003). However, bilateral lesions of the BLA have also been shown to enhance reversal learning performance compared to OFC lesioned rats (although notably, not compared to sham lesioned rats). Importantly, when looking at individual trials, BLA lesioned rats were more sensitive to negative feedback (Izquierdo et al., 2013). While goal-directed processes were long thought to be solely dependent on regions of the prefrontal cortex, recent research has implicated the dorsal striatum in reward related decision-making. Specifically, the

dorsomedial striatum (DMS) plays a role in flexible, goal-directed behavior, particularly in integrating reward-related processes with action selection (Balleine et al., 2007; Yin and Knowlton, 2006; Tai et al., 2012). Muscimol inactivations or lesions of the dorsomedial striatum all disrupt goal-directed learning, rendering choice selection insensitive to reward devaluation and degradation of the action-outcome contingency (Yin et al., 2005; 2006).

In sum, the primary aim of this dissertation is to understand how early life adversity affects the maturation of circuits that support flexible goal directed decision-making. In Chapter 2, I first describe the development of circuits that underlie flexible goal directed decision-making under normal conditions and also explore how pubertal hormones affect circuit maturation processes. In Chapter 3, I investigate how MS affects flexible decision-making, and anxiety related behavior during the late juvenile/early adolescent period and flexibility and ethanol consumption in adulthood. Finally, in Chapter 4, I explore how MS and variations in maternal care affect the development of specific long range circuits implicated in flexible decision-making during the early adolescent period.

Chapter 2

# The development of circuits that support flexible decision-making

## Introduction

During the adolescent transition from juvenile to adult, the frontal cortex undergoes complex structural changes. In the human neocortex longitudinal imaging studies have clearly shown that cortical grey matter thins in a rostral to caudal pattern with the frontal associative regions thinning last, in the second to even third decade of human life (Gogtay et al., 2004). Neocortical thinning is thought to be driven in part by synaptic pruning, meaning a loss in synaptic density (Chen and Zuo, 2014; Drzewiecki et al., 2016; Pentanjek et al., 2011), and likely also includes changes in astrocytes and vasculature, which I will not discuss here.

The major point to be made in this chapter is that not all circuits undergo pruning of arbors or synapse density during adolescence, but instead can follow independent trajectories. Here, I will describe the different developmental trajectories of individual circuits of the frontal cortex of animal models, focusing on dendritic spines on pyramidal neurons of the frontal cortices and long range afferent and efferent projection axons.

I will review the broader literature and highlight work I joint-published in the course of my thesis on the maturation of the frontal cortices in mice[1]. I will start by reviewing the maturation of dendritic spines on pyramidal neurons in the rodent PFC, as well as other cortical regions, exploring what is known about the role of puberty onset. Then I will examine what is known about adolescent maturation of long range afferents of the rodent PFC focusing on OFC and BLA inputs to mPFC as well as hippocampal, dopaminergic, serotonergic and noradrenergic afferents. I will also review what is known about the adolescent maturation of efferents from the PFC; covering maturation of PFC projections to the BLA and striatum. Finally, I will discuss what is known about experience-dependent synapse pruning and stabilization.

Taken together, these data show that the developmental pattern of maturation is not shared amongst all frontal cortex synapses, but rather diverse in both timing and pattern. Identifying when different circuits mature at the synaptic level will likely have important implications for understanding when these connections may be most vulnerable to adverse effects and when targeted interventions may be most effective.

## Synaptic structures in the neocortex: Dendritic spines and axonal boutons

The majority of excitatory synapses in neocortical circuits are formed by the contact of an axonal bouton onto a dendritic spines, the pre and postsynaptic

[1] Data from two previously published manuscripts are included in this chapter. One manuscript describes how dendritic spines and long range axons develop in the frontal cortex (Johnson et al., 2016a) and the other describes the role of gonadal hormones on dendritic spine development in the frontal cortex (Boivin et al., 2018).

components respectively. Cortical boutons are ~1-3 micron diameter varicosities on axons that are typically composed of an active zone with a collection of synaptic vesicles (Shepherd and Harris, 1998). Dendritic spines are small protrusions that extend from the dendritic shaft and can vary in volume from 0.0001 to 1 micron and in shape from thin to mushroom-like spines (Holtmaat and Svoboda, 2009) As spines mature, they tend to grow larger heads and shorter necks (Berry and Nedivi, 2017).

**Maturation of dendritic spines in the rodent PFC**

A large number of postmortem studies have reported age-related reductions in dendritic spine density in sensory and associative cortical regions (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997; Bourgeois, Goldman-Rakic and Rakic, 1994; Petanjek et al., 2011; Kolb and Whishaw, 1998; Anderson et al., 1995). Similar to grey matter thinning, spine pruning occurs across the cortical sheet in a rostral to caudal pattern with sensory areas showing earlier pruning of spine density than prefrontal regions (Elston et al., 2009; Huttenlocher and Dabholkar 1997; Kolb, 2012). In the rodent, maturation is compressed into a shorter time frame and a decrease in dendritic spine density (or synapse density) in the PFC can be observed from ~P30 to ~P60 (Johnson et al., 2016a; Zuo et al., 2005a), which corresponds to the juvenile to young adult period (Spear, 2000), a time when the sensory cortices are also maturing (Hensch et al., 2005).

Since the early 2000's, it has been possible to use two-photon microscopy and fluorescent proteins to image the maturation of dendritic spines and other structures in the living brain. Thy1 BAC transgenic lines (Feng et al., 2000), that label a subset of layer 5 cells, have been the most popular method employed in these studies because the cells are brightly labeled and the background is sparse enough to clearly see individual dendrites and spines. *In vivo* imaging experiments typically make a cranial window to image the apical dendrites of layer 5 neurons and follow the same dendritic spines across multiple days of imaging in order to study the dynamics of these structures (**Fig. 1**) (Holtmaat et al., 2009). Imaging over hours to 24 hours or longer, it can be seen that while a majority of spines are persistent, new spines can be gained and lost. Collectively this gain and loss has been referred to as "turnover."

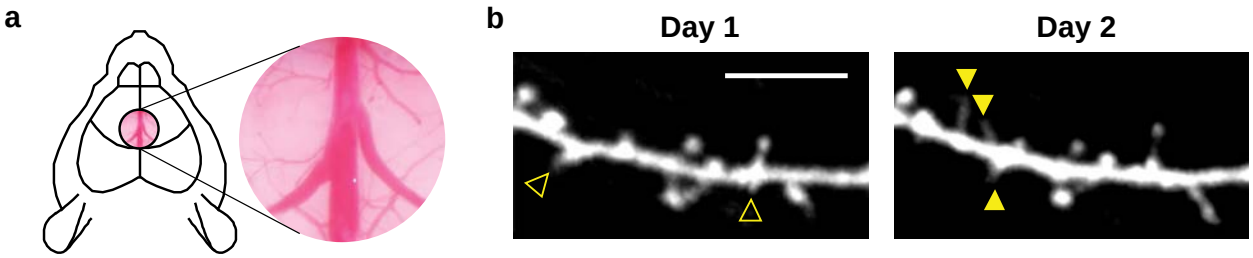



Figure 1. ***In vivo* imaging methods** (a) Schematic of a cranial window in mouse dmPFC (b) Longitudinal imaging of the same layer 5 apical dendrite across two days in Thy1-YFP-H line. Open arrows indicate spines that were lost from Day 1 to Day 2. Closed arrows indicate new spines that were gained from Day 1 to 2. Scale bar = 5 microns.

Using *in vivo* two-photon imaging it has been shown that the somatosensory, visual, and frontal cortices all undergo a loss in spine density and a decrease in daily spine gain and spine loss though the juvenile (~P20-30) to young adult (~P60-180) period of development in mice (Grutzendler et al., 2002; Zuo et al., 2005a, Holtmaat et al., 2005; Johnson et al., 2016a; Boivin et al. 2018) (**Fig. 2**). More focused sampling suggests the timing of maturation of frontal structures within this period may vary by location. Pattwell et al., (2016) found that while dendritic spine gain was reduced between P30 and P45 in the mouse mPFC, there was no difference in turnover in the adjacent frontal association cortex (FrA) in this late adolescent period.

In general, two-photon in vivo imaging studies agree that dendritic spines are quite plastic in early life, with high levels of turnover, but they become extraordinarily stable in adulthood, with a large population of them estimated to last the lifetime of the animal (Bloss et al., 2011; Holtmaat and Svoboda, 2009; Zuo et al., 2005a). It is hypothesized spine turnover allows for the sampling of new synaptic partners and reorganization of connectivity (Holtmaat et al., 2005; Trachtenberg et al., 2002; Stepanyants and Chklovskii, 2005), processes that are important during stages of learning. Increasingly lower levels of gain and loss with development would lead to stabilization of connectivity, and reduced potential for rewiring. However, the upside of stabilization in adulthood is thought to be critical for maintaining established connections, consolidating learning and promoting efficiency (Blakemore and Choudhury, 2006).

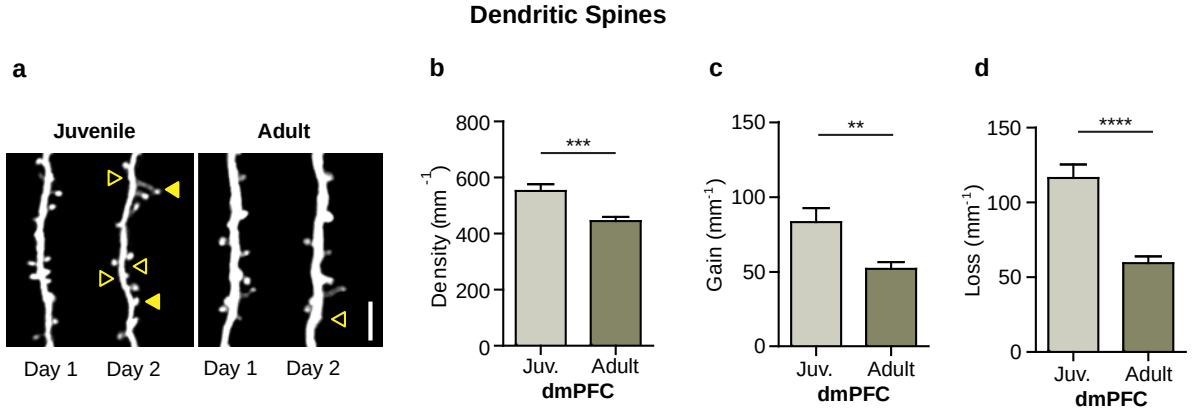



Figure 2. **Juvenile mice have higher spine density and turnover on layer 5 pyramidal cells in dmPFC compared to adults** (a) Representative dendrites from Juvenile and Adult mice followed across two days of imaging. Open arrows represent spines that were lost between day 1 and day 2 and closed arrows represent spines gained between day 1 and day 2. Scale bar = 5 microns. (b) Juvenile mice have higher spine density compared to adult mice [Juvenile: 552 ± 23 spines $mm^{-1}$, Adult: 444 ± 15 spines $mm^{-1}$; $U(77) = 413$, $P = 0.0009$] (c) Juvenile mice show greater spine gains compared to adult mice [Juvenile: 83 ± 9, Adult: 52 ± 5; U(77) = 467, $P = 0.006$]. (d) Juvenile mice show greater spine loss compared to adults [Juvenile: 117 ± 9, Adult: 59 ± 4, U(77) = 257, $P < 0.0001$]. Bars represent mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data collected as part of this thesis. From Johnson et al., 2016a.

## The role of puberty onset

The mechanisms contributing to dendritic spine remodeling in the frontal cortex during adolescence are not well understood. Multiple lines of evidence support a potential role for the increase of gonadal hormones at puberty in driving spine pruning. The initial pubertal milestones coincide with the period that spine pruning begins in human frontal cortex (Petanjek et al., 2011; Piekarski et al., 2017a). Changes in cortical thickness and surface area are related to both pubertal hormones and stage of puberty in human studies (Herting et al., 2014; 2015; Nguyen et al., 2012; 2013; Peper et al., 2009; Raznahan et al., 2010). Specifically, higher levels of testosterone were associated with thinner cortex in posterior cingulate and dorsal lateral prefrontal cortex in post-pubertal boys, while an inverted U shaped relationship was found in girls. Pre-pubertal girls showed a positive relationship, with higher levels of testosterone predicting thicker posterior cingulate cortex, while post-pubertal girls showed the opposite pattern (Nguyen et al., 2012). Work in rodents also supports a role for pubertal hormones in spine pruning. Koss et al., (2013) find that in both male and female rats there is a significant reduction in dendritic spine density on layer 5 pyramidal neurons in mPFC between P35 and P90.

Most of these studies are correlational. Few studies have experimentally manipulated gonadal hormones and looked at cortical spine density and pruning, and here the data are mixed. In adult rodents, hormone administration alters spine growth and stability in adult mouse sensory cortex (Tan et al., 2012; Wang et al., 2018). However, one study of male macaques found that gonadectomy did not affect dendritic spine pruning in a small sample (Anderson et al., 1995).

In order to test if pubertal hormones played a causal role in spine maturation, we administered ovarian hormones at P24 and P26 in order to advance puberty onset and test if this affected dmPFC spine density and turnover (**Fig. 3**) (Boivin et al., 2018). We injected 17-beta estradiol at P24 and progesterone at P26, a treatment that advances first peripubertal exposure to gonadal steroids and drives endogenous puberty (Ramirez and Sawyer, 1965; Smith and Davidson, 1968). We used two-photon imaging and the Thy1-YFP-H line to track dendritic spines from P27-P28 in female mice following hormone administration or vehicle injection. We found that vehicle and hormone treated mice, with different pubertal status, did not differ in dendritic spine density (**Fig. 3b**). There was no difference in fraction of spines gained (**Fig. 3c**) or fraction of spines lost over the 24 hour period (**Fig. 3d**) between vehicle and hormone treated mice. However, we did find morphological differences between groups. The spine head brightness to length ratio, a measure of spines' morphological maturity (Berry and Nedivi, 2017) was higher in hormone compared to vehicle treated mice, suggesting that gonadal hormones may play a role in spine morphological maturation (data in Boivin et al., 2018). These data suggest that dendritic spine pruning and stabilization are hormone independent mechanisms, while morphology maturation may be dependent on hormones. These data are consistent with null effects of gonadectomy in macaque PFC (Anderson et al 1995), but they are not consistent with data that showed decreased mPFC synapses in female rats that were post pubertal compared to prepubertal, controlling for age (Drzewiecki et al., 2016). In this latter study, the synapses were sampled globally using a non-cell specific mechanism. In the *in vivo* two-photon imaging study we only

investigated dendritic spine dynamics in a subset of Thy1 YFP+ layer 5 cells in the dmPFC. Therefore, it remains unknown if other classes of cells or other regions are sensitive to hormone manipulations. Alternate pyramidal cell types have been shown to mature later than the layer 5 Thy1 YFP+ cell type, in metrics of inhibitory neurotransmission (Vandenberg et al., 2015; Piekarski et al 2017ab). Further studies will be needed to determine if pubertal hormones drive spine pruning or stabilization in other cell types or other regions in the frontal cortex.

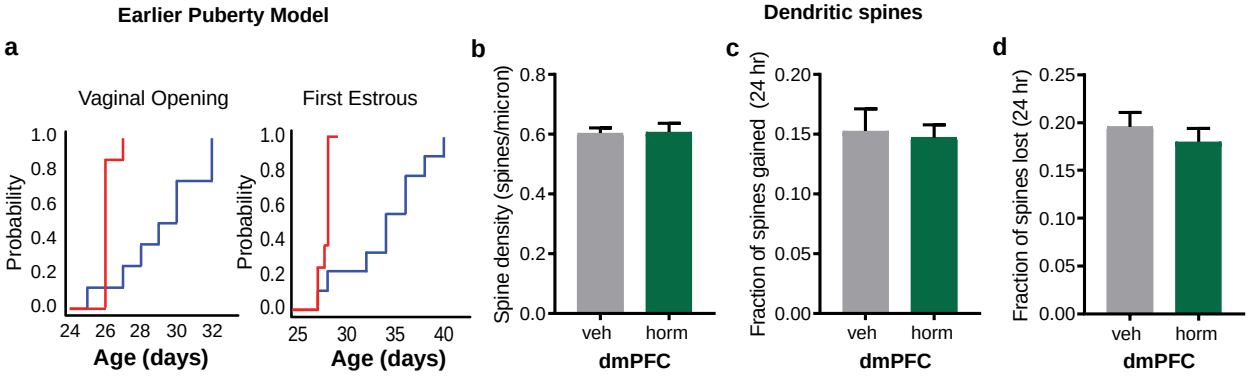



Figure 3. **Pre-pubertal hormone treatment does not accelerate spine pruning and stabilization** (a) The earlier puberty model advances vaginal opening and first estrous in hormone compared to vehicle treated mice (b) Dendritic spine density did not differ between vehicle and hormone treated mice [Vehicle: Mean = 0.6028 ± 0.01751, n=12; Hormone: Mean = 0.6057 ± 0.03051, n=12; t=0.08209 df=22, *P* = 0.9353]. (c) There was no difference in the fraction of spines gained [Vehicle: Mean = 0.1524 ± 0.01873, n=12; Hormone: Mean = 0.1471 ± 0.01071, n=12; t=0.247 df=22, *P* = 0.8072] (d) and no difference in fraction of spines lost [Vehicle: Mdn = 0.206, n=12; Hormone: Mdn= 0.1828, n=12, U= 59, *P* = 0.4680]. Bars represent mean ± SEM. Data collected as part of this thesis. From Boivin et al., 2018.

## Maturation of long range glutamatergic afferents to the PFC

The pyramidal neurons of the PFC integrate inputs from a number of different cortical and limbic regions, including the contralateral PFC, orbitofrontal cortex (OFC), basolateral amygdala (BLA) ventral hippocampus (vHPC) and also the the thalamus (Gabbott et al., 2005; Hoover and Vertes, 2007). These inputs impinge on pyramidal cell dendritic spines and are largely glutamatergic, but they may also drive feed forward inhibition through synapses on local inhibitory neurons as well (Delevich et al., 2015). The connectivity patterns of these projections undergo changes during the adolescent period, but they do not all match the average changes in spine density, pruning over development.

Reciprocal connections between the frontal cortex and amygdala are thought to underlie emotion regulation (Kim et al., 2003; Phelps et al., 2004; Cho et al., 2013; Janak and Tye, 2015; Lai et al., 2012; Pattwell et al., 2016), with greater recruitment of the mPFC and enhanced negative functional connectivity between PFC and amygdala accompanying better emotion regulation with age (McRae et al., 2012; Silvers et al., 2014). Studies in both humans and rodents suggest that these connections undergo protracted maturation into adolescence (Arruda-Carvalho et al., 2017; Bouwmeester et al., 2002; Gabard-Durnam et al., 2014; Gee et al., 2013; Johnson et al., 2016a). Rodent studies demonstrate that BLA axons first reach the PFC during the first postnatal week, but increasingly innervate the PFC during adolescence (Cunningham et al., 2002; Pattwell et al., 2016) increasing synapse density on both excitatory and inhibitory neurons (Cunningham et al., 2002; 2008). This is particularly notable at a time when spine density on pyramidal neurons is pruning.

To our knowledge, the developmental trajectory of vHPC synaptic inputs to the frontal cortex has not been studied. However, earlier studies have attributed increases in temporal lobe volume to increases in myelination in the hippocampus during adolescence (Benes, 1989; Benes et al., 1994). Further studies demonstrated that hippocampal axons have major outgrowth by P9-10, however half of these axonal branches are pruned by adulthood (Swann et al.,1999).

In rodents, mediodorsal (MD) thalamic fibers reach the PFC before birth. The majority of MD fibers innervate layer 3. Notably, these inputs reach this layer before it has fully developed, suggesting that thalamic innervation may play a role in shaping dendritic development (Ferguson and Gao, 2014). One retrograde tracing study in mice found that MD fiber innervation increases up to P10, however they found a large reduction in innervation between P10-P13, which remained stable up to P60 (Rios and Villalobos, 2004).

In order to investigate the maturation of inputs to dmPFC, we used a viral approach to label projections from the BLA and OFC with green fluorescent protein (GFP) (**Fig. 4**) (Johnson et al., 2016a). This projection from the OFC was of interest as a prefrontal region important in learning and decision-making (particularly in tasks that show change over development). We used two-photon microscopy to image long range axons from these respective regions that target the dmPFC in juvenile (P24-28) and adult (P64-69) male mice.

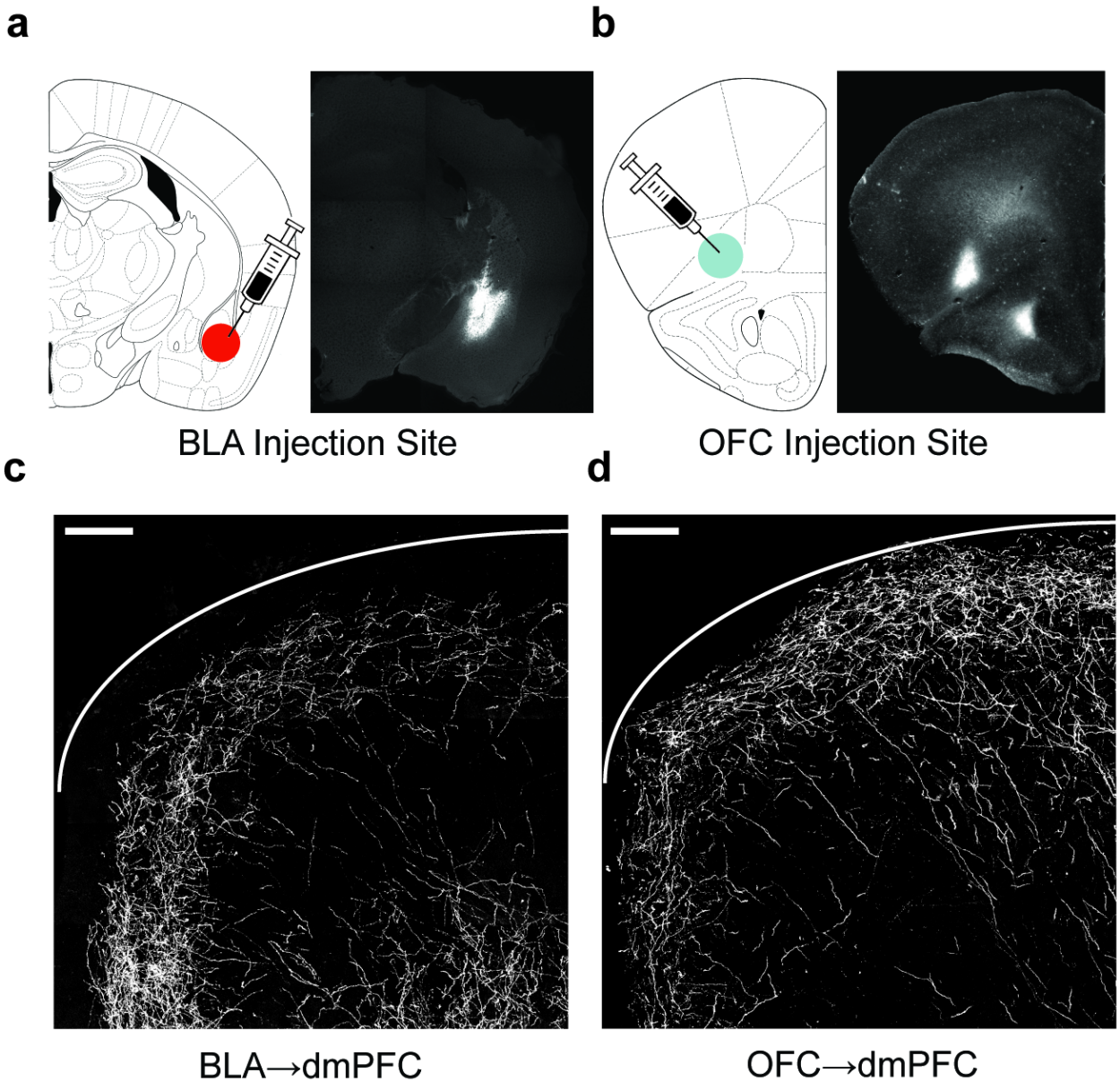



Figure 4. **Methods for viral labeling of long-range axons** (a) BLA injection site (b) OFC injection site (c) BLA axons in dmPFC (d) OFC axons in dmPFC. Scale bars = 100 microns. From Johnson et al., 2016a.

We confirmed previous findings from Cunningham et al., (2002) that BLA axons that target the dmPFC show increases in bouton density between the juvenile and adult period. While we found that OFC axons showed a different pattern; OFC axonal bouton density remained stable between the juvenile and adult period (**Fig. 5a**). Axonal bouton turnover (gain and loss dynamics) also differed between the two inputs. BLA axonal boutons showed greater gains in adults compared to the juveniles, while OFC axons showed the opposite pattern, with adults displaying fewer gains than juveniles (**Fig. 5b**) (following a stabilization pattern more in line with dmPFC dendritic spines). Losses also displayed different developmental trajectories. BLA inputs did not display changes in bouton losses between the juvenile and adult period, while OFC axons showed fewer losses in adults compared to juvenile mice (**Fig. 5c**). These data provide evidence that sub-circuits of the frontal cortex mature at different times and do not all uniformly "prune" over the adolescent transition. We replicate evidence for late growth of afferents of the frontal cortex, in particular, axons projecting from the BLA. In Chapter 4 we investigate how early life adversity affects these circuits.

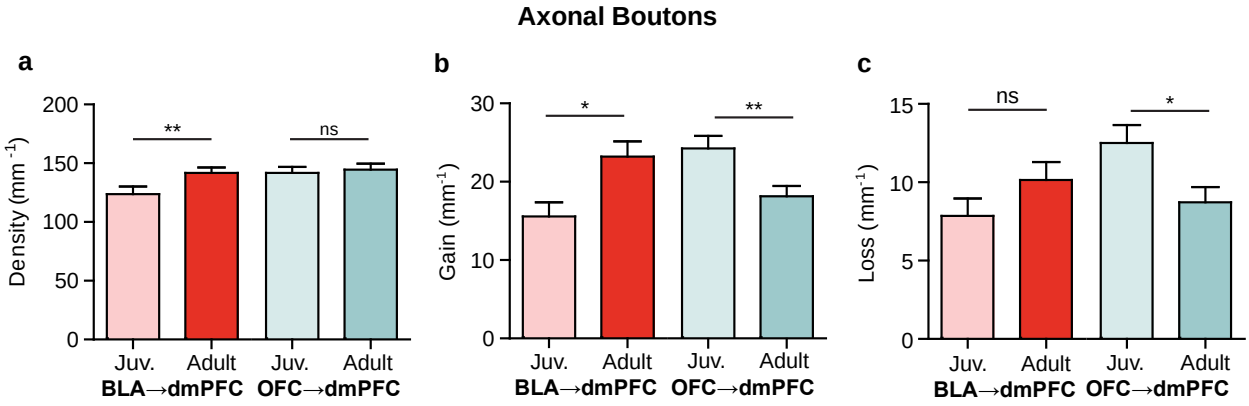



Figure 5. **BLA and OFC inputs to the dmPFC display divergent developmental trajectories** (a) BLA→dmPFC bouton density was higher in adults compared to juveniles [Juvenile: 124 ± 6 boutons mm−1, Adult: 142 ± 5 boutons mm−1, U(124) = 1,392, *P* = 0.008]. There was no difference in bouton density on OFC→dmPFC axons [Juvenile: 142 ± 5 boutons $mm^{-1}$, Adult: 145 ± 5 boutons $mm^{-1}$,U(154) = 2,817, *P* = 0.44] (b) Adult mice showed greater bouton gains compared to juvenile mice on BLA→dmPFC axons [Juvenile: 16 ± 2, Adult: 23 ± 2;U(124) = 842.5, *P* = 0.02] and adult mice showed fewer gains compared to juveniles on OFC axons [Juvenile: 24 ± 2, Adult: 18 ± 1; U(154) = 2,289, *P* = 0.008] (c) There was no difference in bouton loss between adults and juveniles on BLA→dmPFC axons [Juvenile: 8 ± 1, Adult: 10 ± 1; U(124) = 1,039, *P* = 0.32]. Juveniles showed greater bouton loss compared to adults on OFC→dmPFC axons [Juvenile: 13 ± 1, Adult: 9 ± 1;U(154) = 2,363, *P* = 0.02. Bars represent mean ± SEM. *$P < 0.05$; **$P < 0.01$. Data collected as part of this thesis. From Johnson et al., 2016a.

## Maturation of neuromodulatory inputs to the PFC

The PFC also receives input from neuromodulatory regions including dopamine from the ventral tegmental area (VTA), serotonin (5-HT) from the raphe nucleus (RN) and noradrenaline (NA) from the locus coereleus (LC) (Dembrow and Johnston, 2014).

In both rodents and primate, dopaminergic connections continue to ramify in the frontal cortex and add synapses throughout adolescence and into early adulthood (Benes et al., 2000; Hoops et al., 2018; Kalsbeek et al., 1988; Rosenberg and Lewis, 1995; Willing et al., 2017). Interestingly, dopaminergic axons continue to grow from targets in the striatum extending into the PFC (Reynolds et al., 2018). Serotonergic fiber density in the mPFC also increases up to the first month in rats and mice, and further morphological changes continue into adulthood (Lidov and Molliver, 1982; Maddaloni et al., 2017). Noradrenergic innervation of the PFC reaches adult levels within the first week in the rat, however studies suggest that these axons continue to mature in terms of their biochemical machinery even though anatomically they reach maturity much earlier (Foote and Morrison, 1987). Additionally, norepinephrine transporter density declines from P40 to P60 in the prelimibic and lateral orbitofrontal cortex of rats (Bradshaw et al., 2016)

## Competition for target innervation of the PFC

During postnatal development, long-range inputs to the frontal cortex may compete for synaptic space in an activity dependent manner, similar to inputs in sensory cortices (Trachtenberg, 2015). Indeed, a recent study found that following neonatal lesions to the vHPC, there was increased bouton density on BLA axons targeting the mPFC in adulthood (Guirado et al., 2015). Additionally, these axons both terminate in similar regions of the mPFC (Bacon et al., 1996). Neuromodulatory afferents also show evidence for activity dependent competition. Following dorsal raphe 5-HT lesions, dopamine fibers show increased innervation of the mPFC (Taylor et al., 1998). Taken together, this evidence supports the idea that similar to sensory cortices, afferents of associative regions may also compete for cortical territory. In Chapter 4, I test if early life adversity acts on the competition of BLA and OFC inputs.

## Maturation of efferents from the PFC

Efferents from the PFC also undergo late growth in adolescence, at the time when dendritic spine density is pruning. One study using retrograde labeling in rats found that mPFC begins to innervate the BLA between the second and third postnatal week (Bouwmeester et al., 2002). This is consistent with findings in humans, in which mPFC-amygdala connectivity (positive coupling) first appears in preadolescence, around age 10 (Gabard-Durnam et al., 2014). A recent study in mice provided a more detailed description of the development of mPFC axons that target the BLA. Using anterograde labeling they found that mPFC fibers show late innervation of the BLA compared to other target regions (including the striatum, thalamus and claustrum), first reaching the amygdala at P15 and increasing until P30. They also determined that this connection undergoes synaptic strengthening during adolescence, reaching peak levels

at P30 (Arruda-Carvalho et al., 2017), with a transient increase in feed-forward inhibition at P30 as well. Notably, another study suggests that mPFC inputs to the BLA in rats undergo late pruning between P45 and P60 (Cressman et al., 2010).

Corticostriatal projections from the cingulate cortex (a region in the dmPFC) are present in the ipsilateral dorsomedial striatum at P1 in rats and increase innervation up to P7 (Christensen et al., 1999). Corticostriatal projections have been shown to be functional as early as P6 and undergo rapid synaptic changes between P10-18 in mice (Peixoto et al., 2016). In unpublished work, we recently investigated how corticostriatal afferents mature from the juvenile to adulthood period in mice at the synaptic level. We virally labeled axons projecting from the dmPFC to the dorsomedial striatum with GFP and compared bouton density between juvenile (P28), adolescent (P35) and adult mice (P67-87). We found that axonal bouton density development followed an inverted U shaped curve, with adolescents displaying the highest density compared to juveniles and adults (**Fig. 6**). These data, comparable at either end of the inverted U shape, suggest even more intensive sampling of development may yet reveal further complex differences in the trajectory of development of specific circuits during adolescence.

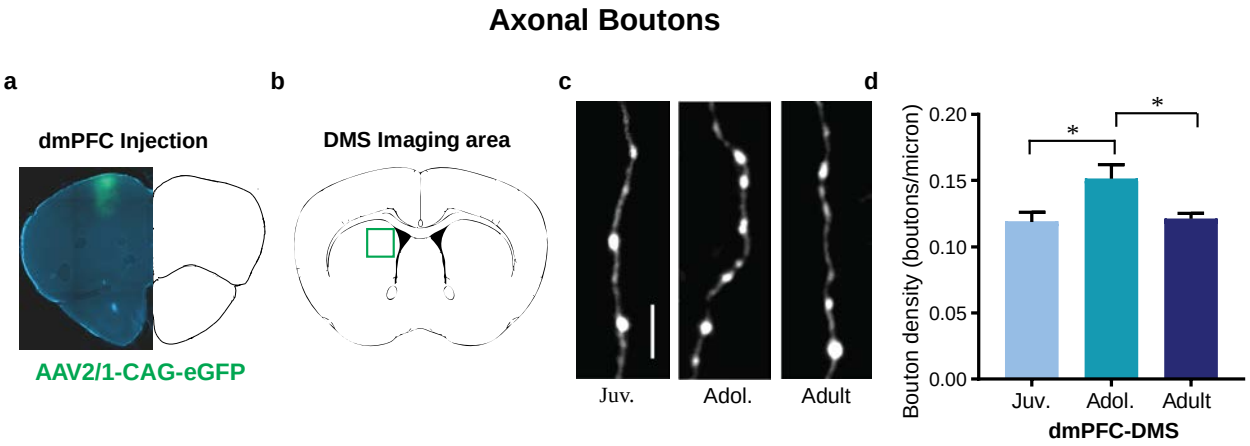



Figure 6. **dmPFC→DMS bouton density development follows an inverted U-shaped pattern.** (a) dmPFC injection site (b) DMS imaging area (c) Representative dmPFC→DMS axons for juvenile (juv.), adolescent (adol.) and adult mice (d) Adolescent mice show higher bouton density on dmPFC→DMS axons compared to juvenile and adult mice. [Juvenile: Mean = 0.1183, n = 11; Adolescent: Mean = 0.1512, n = 6; Adult: Mean = 0.09575, n = 15; one-way ANOVA, $F$ (2, 29) = 5.4, $P$ = 0.0102. Bouton density compared with that for Adolescent by Tukey's test: Juvenile, $P$ = 0.0135; Adult, $P$ =0.0152]. Bars represent mean ± SEM. *P < 0.05.

## Why does pruning and stabilization occur in development?

Overall analyses of prefrontal synapses during adolescent development show pruning and/or stabilization is eventually the norm, even if there are late bursts of growth. Structural growth and retraction of synaptic structures are both thought to

underlie learning and memory in the adult brain (Bailey and Kandel, 1993; Johnson et al., 2016b; Lai et al., 2012; Munoz-Cuevas et al., 2013; Roberts et al., 2010; Xu et al., 2009). In development it is less clear if changes are due to experience and learning or more automatic developmental processes. Deprivation studies in the somatosensory cortex suggest spine pruning is blocked by whisker plucking in mice (Zuo et al., 2005b). However, in the visual cortex deprivation is consistently associated with greater spine pruning in rats (Riccio and Matthews, 1985; Wallace and Bear, 2004; Valverde, 1967; 1971; O'Kusky, 1985), while housing in continuous illumination leads to increased spine density in rats (Parnavelas et al., 1973). Enrichment of the environment during development is associated with greater dendritic branching, dendritic length and spine density (Faherty et al., 2003; Leggio et al., 2005; Volkmar and Greenough, 1972; Globus et al., 1973; Turner and Greenough, 1985; Greenough and Volkmar, 1973; Greenough et al., 1973; Nithianantharajah and Hannan, 2006; Jung and Herms, 2014).

Stress and excessive glucocorticoid exposure can also alter dendritic spines in diverse regions of cortex. Chronic exposure to stress in adult rodents or repeated administration of glucocorticoids leads to decreased dendrite length and reduced spine density in medial prefrontal pyramidal cells (Wellman, 2001; Cook and Wellman, 2004; Radley et al. 2004; 2006). In addition, these structural changes have been associated with learning impairments (Liston et al. 2006). Importantly, one study found a circuit specific effect of stress dependent remodeling of mPFC neurons. Randomly filled neurons in the IL region of mPFC showed dendritic retraction and reduced spine density following chronic immobilization stress in rats, while BLA projecting IL neurons did not show dendritic remodeling in response to stress (Shansky et al., 2009). A recent study found that stress induced dendritic spine loss in layer 5 pyramidal cells in mouse barrel cortex was rescued by parvalbumin expressing interneuron activation or environmental enrichment (Chen et al., 2017). Interestingly, studies investigating dendritic spines in the basolateral amygdala have shown dendritic enhancement as well as an increase in spine density following chronic stress in adult rats (Vyas et al., 2002; 2006), while acute stress leads to a gradual increase in dendritic spine density, only observed 10 days, but not 1 day post stressor (Mitra et al., 2005). More recently, studies have focused on the effects of stress-related mechanisms in developing animals and found that chronic glucocorticoid exposure enhanced spine turnover, specifically increasing elimination in the barrel cortex of adolescent mice (Liston and Gan, 2011). A recent study found that maternal separation in rats led to increased dendritic length, branching and spine density in the BLA in adulthood (Koe et al., 2016). Notably, environmental enrichment in adulthood was able to rescue these effects. Studies assessing the effects of early life adversity on long-range afferents are lacking. Future studies will be needed to disentangle critical variables for each cell type and region.

## Conclusions

Adolescent development is frequently characterized as a period dominated by synapse pruning. However, some circuits show surprising late growth in frontal afferents and efferents of the frontal cortex at a time when dendritic spines are heavily pruned.

*In vivo* imaging of living cells further reveals a developmental process of stabilization, characterized by a decrease in the daily gains and losses of spines and or axonal boutons. This stabilization process contributes to the sampling of potential partners and thus likely regulates the capacity for rewiring and learning. Stabilization of the rate of new spine and bouton formation can be observed to occur across a period when density is invariant and therefore is dissociable from pruning.

Identifying when different PFC afferents and efferents mature has important implications for understanding when these circuits may be sensitive to experience, either vulnerable to adverse experiences or amenable for intervention. In Chapter 3, I discuss how a mouse model of early life adversity impacts the development of bouton density on afferents and efferents of the dmPFC that were described in this chapter.

Chapter 3

# Early maternal separation impacts flexible decision-making at the age of first independence in mice

## Introduction

Early life experiences are known to have a profound impact on brain development and behavior. Epidemiological data and clinical studies suggest a strong link between childhood maltreatment and the development of substance use disorders, mental health disorders, obesity, and other physical health problems (Heim and Nemeroff, 2001; Sanchez et al., 2001; Fishbein et al., 2009; Felitti et al., 1998; Dube et al., 2001; Jaffee et al., 2002; Gilbert et al., 2009). Changes in flexible decision-making caused by early neglect or maltreatment could cause and/or exacerbate mental and physical health conditions. For example, flexible decision-making deficits may contribute to the development and management of substance use disorders (Goldstein and Volkow, 2011).

Our goal here was to use a mouse model of early life adversity to investigate effects on flexible decision-making[2]. Additionally, we sought to investigate how early life stress might contribute to the development of addiction-related behaviors by assessing ethanol consumption using an intermittent access "drinking in the dark" paradigm that leads to binge drinking episodes in mice (Rhodes et al., 2005; Thiele and Navarro, 2014). We choose to focus on mice to enable use of the wealth of tools for the study of neural circuits that are currently most developed in this species. Neural circuit data will be covered in the following chapter.

There is a large body of work that has used rats as a model system to study the effects of early adverse experience on anxiety and fear behavior and also, to a lesser extent, cognitive function. Many of these models involve disruptions of the infant-mother relationship, which is thought to be one of the most important relationships in early life (Levine, 1962; Bowlby, 1982). Some studies have focused on comparing the offspring of mothers that provide low versus high levels of maternal care (Liu et al., 1997), while others have manipulated the amount of bedding to induce maternal stress and erratic behavior (Giles et al., 1996; Ivy et al., 2008) or employed more invasive separation paradigms which remove pups from their mother during the early postnatal period (Plotsky and Meaney, 1993; Francis et al., 2002). The most invasive separation studies have used artificial rearing with no dam care at all (Lovic and Fleming, 2004). Rat pups that have experienced low care levels or maternal separation (MS) have been shown by a wealth of studies to exhibit altered stress reactivity and anxiety behavior (Liu et al., 1997; Huot et al., 2002; Ladd et al., 2004; Lippmann et al., 2007; Aisa et al., 2007; Pryce and Feldon, 2003; Gilles et al., 1996; Dalle Molle et al., 2012).

[2] This chapter consists of a previously published manuscript (Thomas et al., 2016).

A small but growing body of work has found evidence of cognitive changes in rats following early adverse experience. Adult rats that were artificially-reared (with no dam contact) have been shown to exhibit impairments in tests of cognitive flexibility in a 2-choice attentional set shifting paradigm (ASST) in which rodents learn to dig for cereal reward in scented or textured material and the rewarded contingency is reversed or the rewarded dimension is shifted (Lovic and Fleming, 2004). Impairments in 2-choice reversal in this same digging based task have also been found in adult rats that underwent 3 hours of maternal separation during the first two weeks of life (Baudin et al., 2012). Working memory and flexibility in spatial tasks has also been found to be altered in adolescent and adult rats following early maternal separation (Brenhouse and Andersen, 2011; but see Wang et al., 2015).

Notably, there is less evidence of a maternal separation effect on flexible decision-making in mice, a species in which we have greater access to the study of specific circuits. Furthermore, past studies in mice have found inconsistent results which call into question the reliability of the rodent model. One study found the effects of maternal separation on cognitive function was strain-dependent (Mehta and Schmauss, 2011). The Balb/c strain showed spatial working memory and set-shifting deficits in adulthood following maternal separation, while the adult C57Bl/6 strain showed no impairments across multiple cognitive domains. Importantly, this mouse study found no deficit in reversal learning in either strain. In contrast, other studies have found adult spatial learning impairments and working memory impairments, including deficits in spatial reversal in both C57Bl/6J (Fabricius et al., 2008) and Balb/cJ strain (Wang et al., 2011a), and Y maze spontaneous alternation and temporal order memory in the C57Bl6 strain (Yang et al., 2015). Two of these positive findings however focus on spatial tasks dependent on the hippocampus rather than odor or texture based digging tasks that have been found to be dependent on the integrity of the frontal cortex of rodents (Birrell and Brown, 2000; Garner et al., 2006; Bissonette et al., 2008; Kim and Ragozzino, 2005; McAlonan and Brown, 2003).

We have previously reported developmental changes in reversal learning in mice using a 4-choice, odor-based digging task (similar to the ASST but with 4 choices) and we established is dependent on the integrity of the dorsomedial frontal cortex (dmPFC) (Johnson and Wilbrecht, 2011). Designing this current study, we hypothesized that maternal separation may alter the developmental trajectory of flexible decision-making, and that the 4-choice task may be more sensitive to detecting this effect in mice. Four-choice tasks likely produce greater cognitive load and ambiguity or uncertainty than 2-choice tasks and can be argued to be more naturalistic in their resemblance to real world foraging environments (Ragozzino and Rozman, 2007).

As mentioned above, MS treatment can have significant effects on anxiety-related behavior, particularly in rats. Studies in mice present inconsistent findings in anxiety-like behavior outcomes, with some reporting increased anxiety in MS mice, while others find no change (Romeo et al., 2003; Millstein and Holmes, 2007). Here we also tested anxiety behavior in case this may also play a role in cognitive or substance use related measures.

Exposure to early life stressful events has been postulated to be associated with increased vulnerability to develop substance use disorders. Human subject studies have found an association between early life adversity and increased incidence of

alcohol use disorders (MacMillan., et al. 2001; Green et al., 2010; Enoch, 2011). Maternal separation in rats (Huot et al., 2001) and mice (Cruz et al., 2008) has been shown to lead to increased ethanol consumption in adulthood, but the ethanol consumption paradigms used have differed and one study suggests handling protocols may impact later drinking (Ploj et al., 2003; Moffett et al., 2007). We sought to confirm if maternal separation experience in mice leads to greater consumption of ethanol using a more recent "drinking in the dark" model (Rhodes et al., 2005) that promotes binge-levels of drinking.

Here, we report that a fairly low dose of 60 minutes per day of maternal separation in mice for the first ten days of life can impact reversal performance measured using a 4-choice odor based task in juvenile male mice tested at postnatal day 26. Maternal separation did not lead to changes in the elevated-plus maze in male mice tested at P32-33, suggesting enhanced anxiety was not the cause of the cognitive difference P26. Effects on cognitive performance were developmental, such that when testing in adulthood (~P60), early maternal separation experience did not lead to any difference in performance in the 4-choice reversal task, even after the daily separation time was raised to 180 minutes. We discuss how a change in the developmental trajectory of cognitive function may be interpreted as adaptive earlier maturation in adverse circumstances or stress induced impairment. Our data also strengthen links between early adversity and substance use disorders. When adult MS and littermate control mice were allowed intermittent access to 20% ethanol, we found a significant interaction between MS experience x time on cumulative ethanol consumption.

## Methods

### *Animals*

Male and female C57Bl/6 *Mus musculus* (lines originally obtained from Charles River) were used for this study. Dams and sires were housed in pairs throughout the breeding and rearing period. Post-weaning, experimental mice were group housed in single sex cages, 2-5 per cage. All cages were on a 12/12 reverse light cycle (lights off at 10 AM). Testing took place during the dark period. All animals received nesting material and plastic hut in their home cage. All procedures were approved by the Ernest Gallo Clinic and Research Center and UC Berkeley Animal Care and Use Committees.

### *Maternal Separation (MS)*

*(MS):* From postnatal (P) day 1 to P11, with a one day break on the weekend for some litters, half of each litter of pups was removed daily from their cage for either 60 or 180 minutes (MS group), while half of the litter remained with the dam (littermate, LM group). (Group sizes: Cohort 1 Juvenile 60 minutes: MS=14, LM=15; Cohort 2 Adult 60 minutes: MS=12, LM=8; Cohort 3 Adult 180 minutes: MS=19, LM=11). This particular paradigm was selected as a combination of elements from a broad variety of MS protocols used in other rodent studies (Bock et al., 2005; Monroy et al., 2010; Macrí et al., 2008). During separation, MS pups were kept in a clean cage warmed by an electric pad. MS pups were able to hear and smell each other, but not touch during seperation due to a

cardboard divider. The control group were littermates (LM group), forming the other half of the litter who were initially handled and marked the same as the MS half, but who were placed back in the home cage with the dam during the period of separation. All mice were identified by marker and then ear clipping. After P11 mice were not handled until weaning at P21, with the exception of one weekly cage cleaning. At weaning, the average weight of maternally separated mice was not distinguishable from littermates (MS = 8.99 ± 0.34, n=14, LM = 8.83 ± 0.35 n=15; $t$ (27) =0.3258, $P$ = 0.75, cohort 1).

*Four-choice odor discrimination and reversal task*

Extensive methods for the four-choice reversal were published previously (Johnson and Wilbrecht, 2011). Briefly, testing of MS offspring and littermate controls took place over 5 days with 2 days of food deprivation, followed by habituation, shaping and one final day of discrimination and reversal testing. Juvenile mice started food deprivation at P22 and were tested at P26 (**Fig. 7a**), while adult mice started food deprivation at P56 and were tested at P60 (**Fig. 7b and c**). The same cohorts of mice that underwent the maternal separation or littermate procedure were food deprived (to 90% of *ad libitum* of adult weight or 90% of peer weights if testing before P50). On habituation day, mice were placed in the behavioral testing box and consumed a novel cereal reward (Cheerio fragments, General Mills) for 30 minutes. On shaping day, mice learned to retrieve cereal pieces from the bottom of a single bowl that was increasingly filled with unscented shavings. On the main testing day, mice were presented with 4 bowls (all sham baited with a screen over a cheerio), each containing shavings of different scents (odors 1-4: anise, clove, litsea, thyme, respectively). Only one bowl contained an accessible reward (**Fig. 7d**). This was Odor 1 during the discrimination phase and Odor 2 during the reversal phase. During each trial mice were allowed 3 minutes to explore the arena and indicate a single choice by purposively digging in a bowl with both paws. Bowl locations were changed each trial, so mice were forced to use odor to reliably retrieve the cereal reward. An absence of a choice during the 3 minutes was scored as an omission, and mice were returned to a start cylinder and a new trial was initiated. Upon reaching criterion of 8/10 sequential trials correct during the discrimination phase, the reversal phase began on the next trial in the same session. During this phase, Odor 2 was now rewarded and a novel odor replaced Odor 4 (eucalyptus). Perseverative errors were scored as choices to dig in the bowl with the previously rewarded odor (Odor 1). Irrelevant errors were choices to dig in the bowl with odor 3, which was present during both phases of the behavior. Novel errors were choices to dig in newly introduced $4^{th}$ odor, which were not rewarded.

*Intermittent-Access Ethanol Consumption (Single bottle paradigm) procedure*

The intermittent access “drinking in the dark” ethanol consumption model was chosen since it produces binge drinking behavior in which rodents will often attain blood alcohol levels comparable to the legal limits for driving after ethanol consumption in humans (Rhodes et al., 2005; Thiele and Navarro, 2014). To measure ethanol consumption, mice that had undergone 180 minutes of maternal separation or littermate experience and then were tested on 4-choice reversal task (MS=16; LM=9; The initiation of the ethanol experiment was delayed and so 3 MS mice and 2 LM mice from cohort 3 were

not tested on ethanol consumption.), were moved to single housing at ~P70 (**Fig. 7**). Mice had ad lib access to water except during testing when the water bottle was replaced with 20% ethanol solution during a 4 hour test session conducted from 1 PM to 5 PM in the reverse dark light cycle (which started at 10 AM). Ad lib water was available until 1 PM and then after 5 PM. Testing was complete after three weeks with a total of 9 sessions conducted on Monday, Wednesday and Friday (**Fig. 11a**). Bottles and mice were weighed after each session to calculate ethanol consumption (ethanol per kg mouse weight). Blood was sampled from a tail vein after the 9$^{th}$ session for a subset of the mice (n=4-6 per group) to measure blood alcohol content (BAC).

*Elevated-Plus Maze (EPM)*

In a separate cohort of MS and control mice, we used an elevated plus maze to quantify anxiety and exploration related behavior at P32-33. The elevated plus maze was made of opaque white acrylic and consisted of 2 open arms (30 cm long x 6 cm wide) and 2 closed arms (30 cm long x 6 cm wide x 20.5 cm high walls) and a center square (6 cm x 6 cm). The maze was raised by a platform 66 cm from the floor. On the day of testing, the homecage was brought into the EPM room 30 minutes prior to testing to allow time to acclimate. Mice were then placed at the center of the maze and allowed to freely explore for 10 minutes. The maze was cleaned with 70% ethanol in between testing sessions. The number of times animals entered each zone of the maze (i.e. open, closed and center), total time spent in each zone and distance traveled in each zone were videotaped and analyzed using EthoVision tracking software (Noldus: Sacramento, CA).

*Maternal care quantification (for EPM cohort)*

Maternal care quantification was performed only for cohort of mice tested in anxiety behavior. This was initiated after the other experiments on cohorts for reversal learning and alcohol consumption were completed. Maternal behavior was monitored in both control and MS cages every other day from P1-P10, for a total of 5 monitoring days. Cages were observed 3 times a day during the dark phase (11 AM, 2:30 PM and 5 PM) under infrared light and each monitoring session lasted for 30 minutes, in which dam behavior was scored every other minute (15 mins total of observation per session). The amount of time the dam spent on the nest and the number of instances the dam left the nest (sorties) were scored. Nests were built up into dome-like structures, which have been described in detail in other studies assessing the C57Bl/6 strain (Millstein and Holmes, 2003; Rice et al., 2008). These domes typically occluded behavior in the nest, thus more detailed maternal behaviors such as arched-back nursing and licking and grooming were not scored.

*Statistical Analysis*

Values are reported as mean (*M*) ± SEM or median (*Mdn*) ± IQR. Data were tested for normal distribution. Data that were normally distributed were analyzed using two-tailed *t* tests or ANOVAS, followed by post-hoc analysis. Pearson's correlations were used to test the linear relationship between variables. Data that were not normally distributed

were analyzed using the Mann-Whitney *U* test for group comparisons. Statistical significance was set at $P < 0.05$; analysis and graphing were performed with GraphPad Prism v6

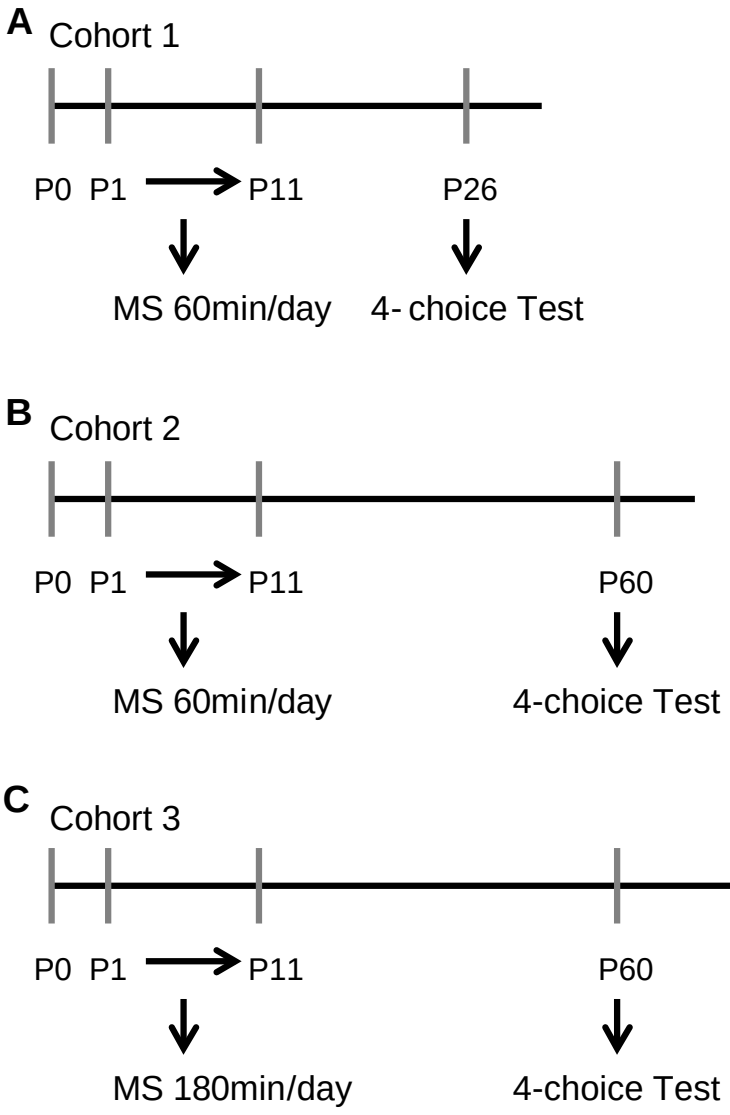



Figure 7. **Experimental timeline and set-up.** (A) Timeline of maternal separation paradigm and 4-choice testing for juvenile 60 minute (B) adult 60 minute maternal separation and (C) adult 180 minute maternal separation and ethanol drinking paradigm. (D) Schematic of arena in 4-choice task. Mice learned to discriminate among four odors to learn which odor contained a buried cheerio reward. In the reversal phase, a previously incorrect odor predicted the reward location. All odor locations were shuffled each trial.

## Results

4-choice odor discrimination and reversal: Juveniles (60 minute Separation)-Cohort 1

To explore how maternal separation influences flexible decision-makingduring development we compared performance of mice that had undergone 60 minutes of maternal separation (MS60) to littermate controls (LM60) on a 4-choice odor discrimination and reversal task tested at a juvenile age (P25-P26). During the discrimination phase, animals learned to dig for a buried cheerio reward in bowls with differently scented shavings. Only one bowl was rewarded. Maternally separated ($N$=14) mice did not differ from littermates ($N$=15) in the discrimination phase of the task [littermate: $M$ = 30.47 ± 3.95; maternal separation: $M$ = 26.00 ± 1.27; $t$ (27) = 1.05, $P$ = 0.30] (**Fig. 8a)**. However, in the reversal phase, in which a previously incorrect odor predicted the location of the reward, maternally separated mice required significantly more trials to reach criterion compared to littermates [$t$(27) = 3.02, $P$ < 0.01] (**Fig. 8a**). Analysis of error type using a two-way ANOVA revealed a significant main effect of MS experience [$F$(1,135) = 25.38, $P$ < 0.0001] and error-type [$F$(4,135) = 75.04, $P$ < 0.0001] and a significant interaction of maternal experience and error type [$F$(4,135) = 3.82, $P$ = 0.0057] (**Fig. 8b**). Bonferroni post-hoc tests found that the MS mice made more total errors ($P$ < 0.0001) and odor 1 errors ($P$ < 0.01) compared to littermates (**Fig. 8b**). Analyzing errors made before or after the first reward revealed that MS mice made significantly more errors before the first reward compared to LM mice [$t$(27) = 2.38, $P$ < 0.05], but there was no difference in the number of errors made after the first reward [$t$(27) = 1.62, $P$ = 0.12] (**Fig. 8c**). Maternally separated mice did not differ from littermates in latency to dig in incorrect [$t$(27) = 0.32, $P$ = 0.75) or correct trials [$t$(27) = 0.92, $P$ = 0.37) (**Fig. 8d**).

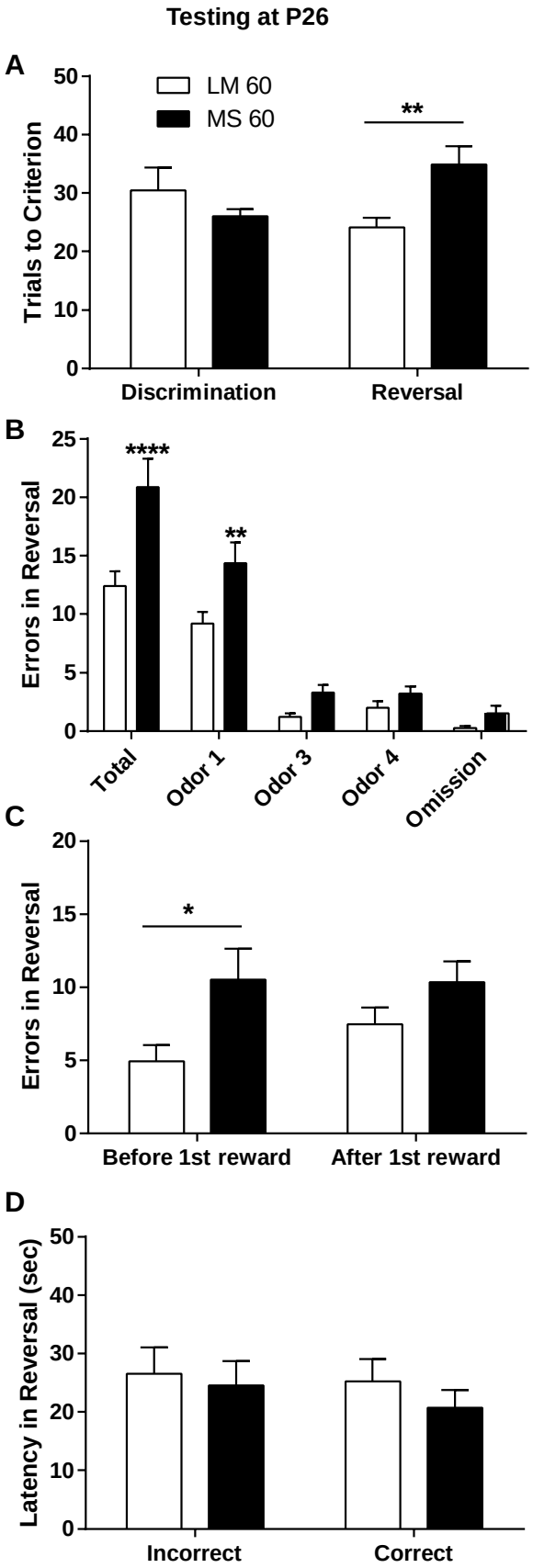



Figure 8. **Maternally separated (MS60) mice tested at a juvenile stage show less flexibility in a 4-choice odor discrimination and reversal task compared to littermate (LM60) controls**. (A) Juvenile MS60 (*N*=14) and LM60 mice (*N*=15) did not differ in the total trials to reach criterion in the discrimination phase ($P = 0.30$). In the reversal phase, MS60 mice required significantly more trials to reach criterion compared to littermates ($P < 0.01$). (B) Analysis of error type revealed that MS60 mice made more total errors ($P < 0.0001$) and odor 1 errors ($P < 0.01$) compared to littermates. (C) MS60 mice made more errors prior to the first reward than littermates ($P < 0.05$). (D) MS60 mice did not differ from littermates in the latency to dig in incorrect ($P = 0.75$) or correct trials ($P = 0.37$). Bars represent mean ± SEM *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

4-choice odor discrimination and reversal: Adults (60 Minute Separation)-Cohort 2

To test the impact of maternal separation on adult reversal performance, a second cohort of mice underwent the same 60 minute maternal separation (*N*=12) and littermate (*N*=8) procedure from P1-11 as above, and then were tested on the 4-choice task in adulthood (P59-P60). Adult maternal separation mice did not differ from littermates in the total trials to criterion in the discrimination phase [$t(18) = 1.49$, $P = 0.15$) (**Fig. 9a**). In contrast to maternally separated mice tested as juveniles, there was no difference between adult maternally separated mice and littermates in the number of trials it took to reach criterion in the reversal phase [$t(18) = 0.06$, $P = 0.95$) (**Fig. 9a**). Analysis of error type using a two-way ANOVA revealed a main effect of error type [$F(4, 90) = 41.32$, $P < 0.0001$], but no main effect of MS experience [$F(1, 90) = 0.08$, $P = 0.78$] or interaction [$F(4, 90) = 0.15$, $P = 0.96$] (**Fig. 9b**). There was no difference between MS and LM mice in the number of errors made before the first reward [$t(18) = 0.01$, $P = 0.99$] or after first reward [$t(18) = 0.14$, $P = 0.89$] (**Fig. 9c**). Additionally, latency to dig did not differ between groups in incorrect trials [$t(18) = 0.76$, $P = 0.46$] or correct trials [$t(18) = 0.93$, $P = 0.36$] (**Fig. 9d**).

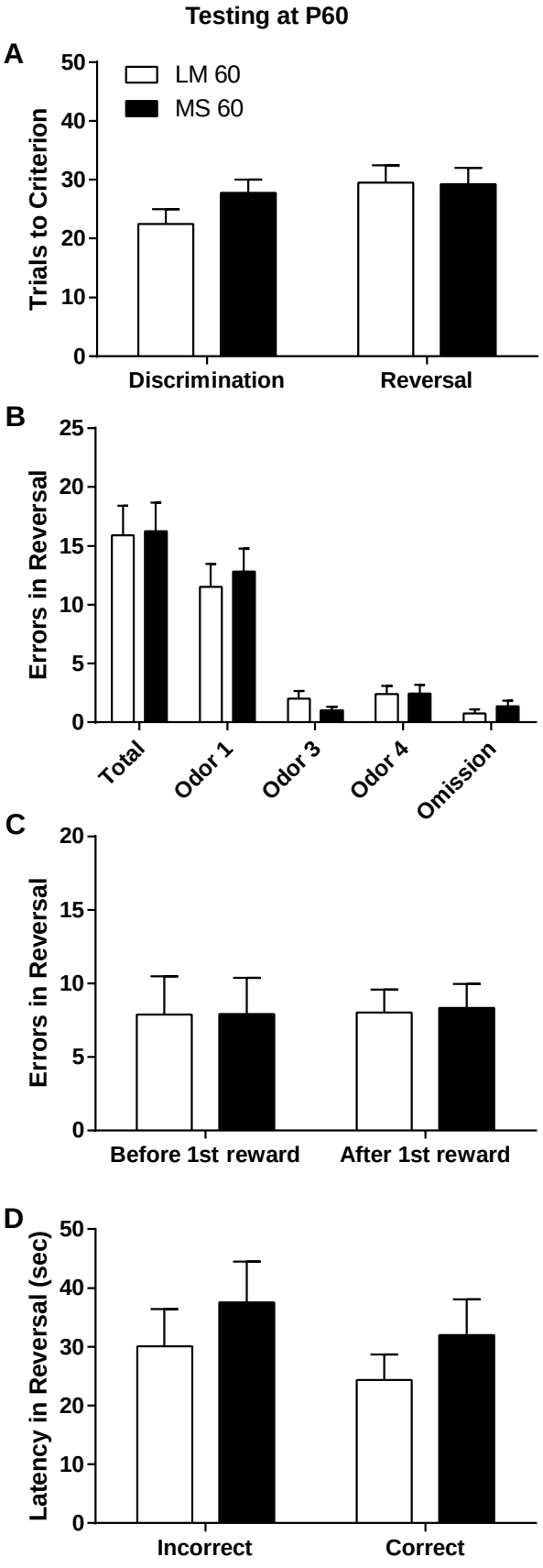



Figure 9. **Maternally separated mice (MS 60) tested in adulthood do not differ from littermates (LM 60) in the 4-choice task** (A) Maternally separated (*N*=12) adult mice did not differ from littermates (*N*=8) in the total trials to criterion in the discrimination phase ($P = 0.15$), or reversal phase ($P = 0.95$). (B) Analysis of error type using a two-way ANOVA revealed a main effect of error type ($P < 0.0001$) but no main effect of maternal experience ($P = 0.78$) or interaction ($P = 0.96$). (C) There was no difference between MS and LM mice in the number of errors made before the first reward ($P = 0.99$) or after first reward ($P = 0.89$). (D) Latency to dig did not differ between groups in incorrect trials ($P = 0.46$) or correct trials ($P = 0.36$). Bars represent mean ± SEM.

Four-choice odor discrimination and reversal: Adults (180 Minute Separation)-Cohort 3

We next ran a third cohort of animals in the maternal separation (*N*=19) and littermate (*N*=11) paradigm, increasing the separation period to 180 minutes a day from P1-11 (MS180). Again, mice were tested using the 4-choice task in adulthood (P59-P60). Similar to the adults that had undergone maternal separation for 60 minutes, we found no difference in the total trials to criterion in the discrimination phase [$t(28) = 0.28$, $P = 0.78$], and in the reversal phase [$t(28) = 0.66$, $P = 0.51$] between the 180 minute maternal separation group and littermates tested as adults (**Fig. 10a**). Analysis of error type using a two-way ANOVA revealed a main effect of error type [$F(4, 140) = 40.75$, $P < 0.0001$], but no main effect of MS180 experience [$F(1, 140) = 1.58$, $P = 0.21$] or interaction [$F(4, 140) = 0.32$, $P = 0.86$] (**Fig. 10b**). There was no difference between MS180 and LM groups in the number errors made prior to the first reward [$t(28) = 0.15$, $P = 0.88$] or after first reward [$t(28) = 1.00$, $P = 0.33$] (**Fig. 10c**). There was no difference in latency to dig between maternally separated and littermate adult mice in incorrect [$t(28) = 0.99$, $P = 0.33$] and correct trials [$t(28) = 0.45$, $P = 0.66$] (**Fig. 10d**).

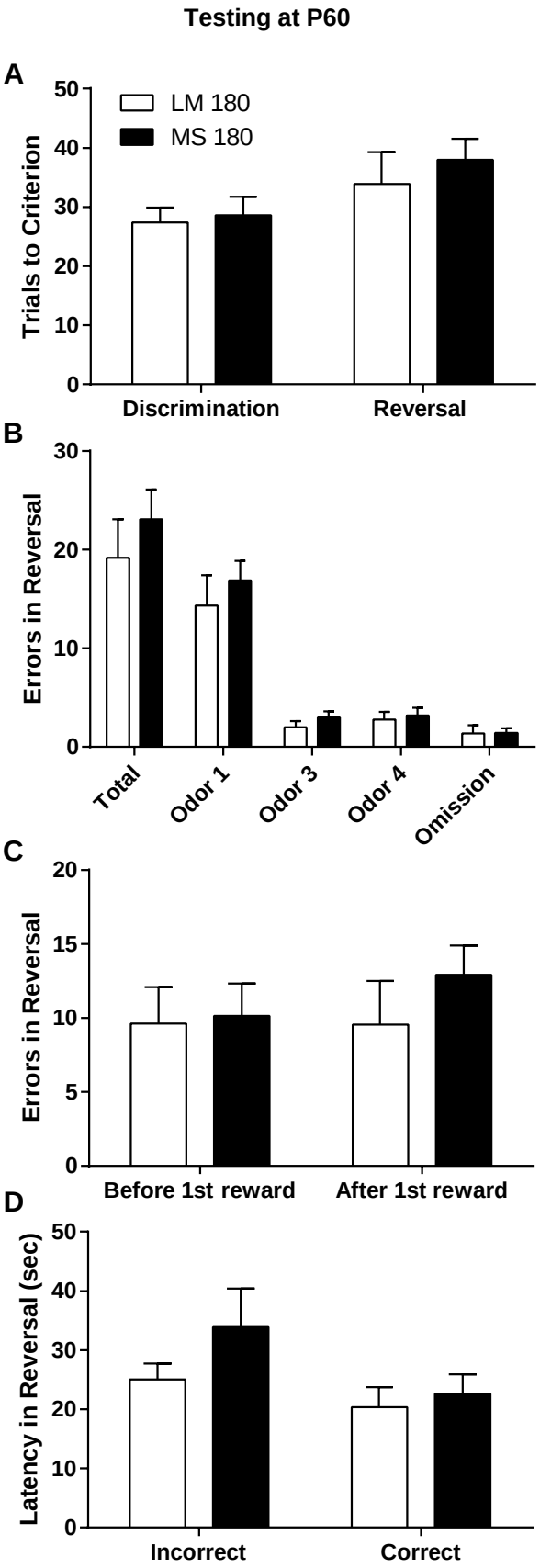



Figure 10. **A longer daily separation dose does not result in differences between maternally separated and littermates tested on the 4-choice task in adulthood.** (A) Adult MS180 did not differ from littermates in the discrimination phase ($P = 0.78$) and reversal phase of the 4-choice task ($P = 0.51$). (B) MS180 and LM180 mice did not differ in number of errors. (C) There was no difference between MS180 and LM180 groups in the number errors made prior to the first reward ($P = 0.88$) or after first reward ($P = 0.33$). (D) There was no difference in latency to dig between MS180 and LM180 mice in incorrect ($P = 0.33$) and correct trials ($P = 0.66$). Bars represent mean ± SEM.

*Intermittent-Access 20% Ethanol Consumption: Adults (180 Minute Separation)- Cohort 3*

The same cohort of animals that underwent maternal separation or littermate experience for 180 minutes and were tested on the 4-choice task in adulthood (P59-P60), were next tested on ethanol consumption using an intermittent-access ethanol drinking paradigm starting at ~ P70 (MS=16; LM=9) (**Fig. 11a**). A two-way repeated measures ANOVA revealed a main effect of session [$F(8, 184) = 156.3$, $P < 0.0001$] and a significant interaction of MS180 experience and session [$F(8, 184) = 2.17$; $P < 0.05$] with MS180 treated mice drinking more than LM180 controls over time (**Fig. 11b**). No correlation was found between reversal performance and cumulative ethanol intake [$r = -0.032$, $P = .88$] (**Fig. 11c**). Blood ethanol concentration taken at the end of the final 3 hour drinking period revealed mice with ranges from 0.004-0.149% with a median of 0.039% and group average of .045 ± .027%, for the LM group (n= 4) and .056 ± 0.26% for the MS group (n=6). For reference, the legal driving limit in humans is set at 0.08% in the United States. As mice may have reached higher levels earlier in the 3 hour period when blood was not sampled, these blood values should be interpreted with caution.

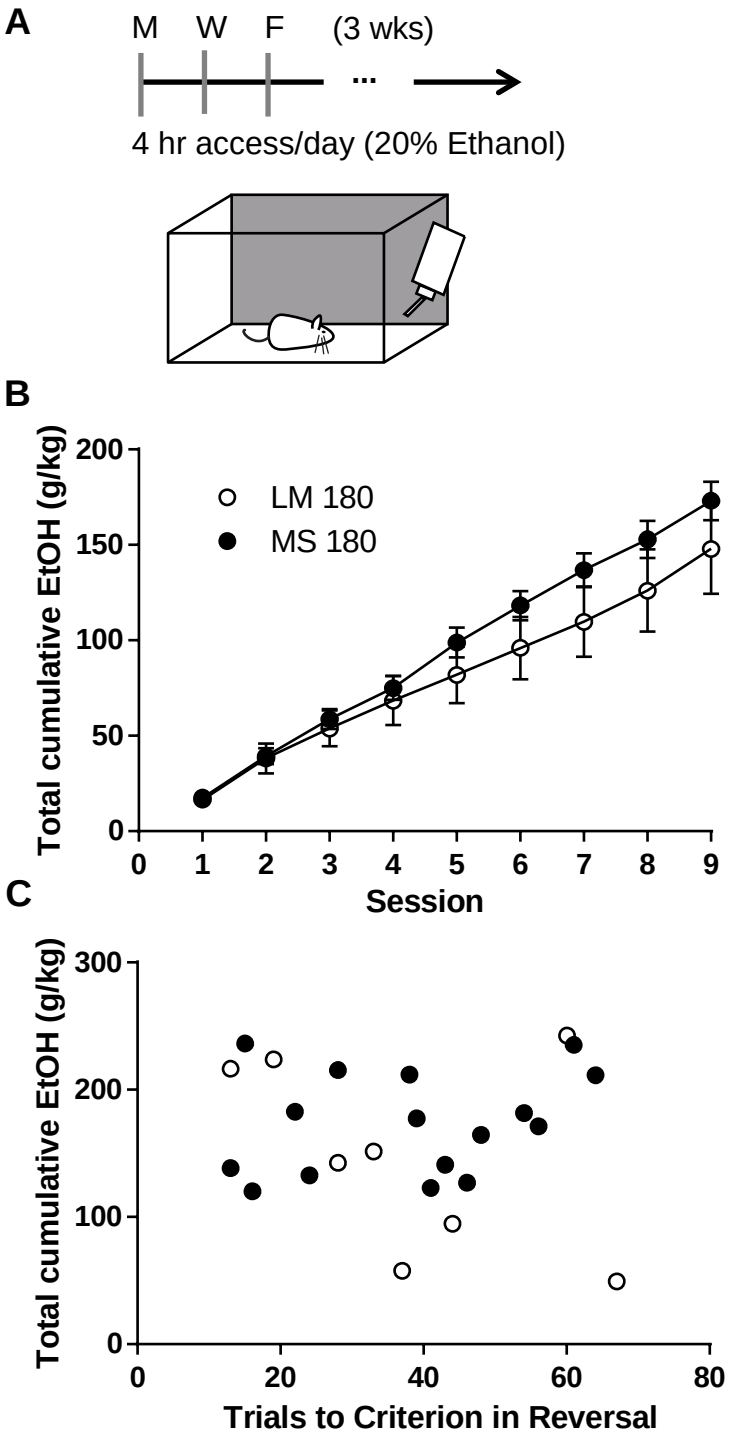



Figure 11. **Maternal separation (MS180) enhanced cumulative alcohol consumption compared to littermate controls** (A) Schematic of ethanol paradigm (B) Cumulative ethanol consumption across 9 sessions. A two-way repeated measures ANOVA revealed a significant interaction of MS180 experience and session ($P < 0.05$) (C) No correlation was found between 4-choice reversal performance (trials to criterion) and cumulative ethanol consumption on Day 9 ($r = -0.032$, $P = .88$). Bars represent mean ± SEM.

Elevated-Plus Maze: Adolescents (3 hour separation) –Cohort 4

To put our above results in context of the broader literature, we added a final cohort in which we quantified maternal care behavior after MS P1-10 (3 hours daily) and then measured anxiety behavior in male offspring at P32-33. MS and control mice did not differ in anxiety-like behavior in the elevated plus maze. Control and MS mice did not differ in % time spent in open arms [Control: *Mdn* = 0.1063 (0.05129 to 0.1574), n=27, MS: *Mdn* = 0.09925 (0.05708 to 0.1511), n=22, U= 289, *P* = 0.8813] (**Fig. 12a**) or in % time spent in closed arms (**Fig. 12b**) [Control: *M* = 0.7933 ± 0.01884, n=27, MS: *M* = 0.7972 ± 0.02064, n=22, t=0.1376 df=47, *P* = 0.8912]. There was no relationship between % time in open arms and time over nest [r = -0.1238, *P* = 0.3968] (**Fig. 12c**) and no relationship between % time in open arms and sorties [r = 0.2084, *P* = 0.1508] (**Fig. 12d**). Additionally, there was no relationship between % time in closed arms and time over nest [r = 0.05305, *P* = 0.7173] (**Fig. 12e**) and no relationship between % time in closed arms and sorties [r = -0.1292, *P* = 0.3763] (**Fig. 12f**).

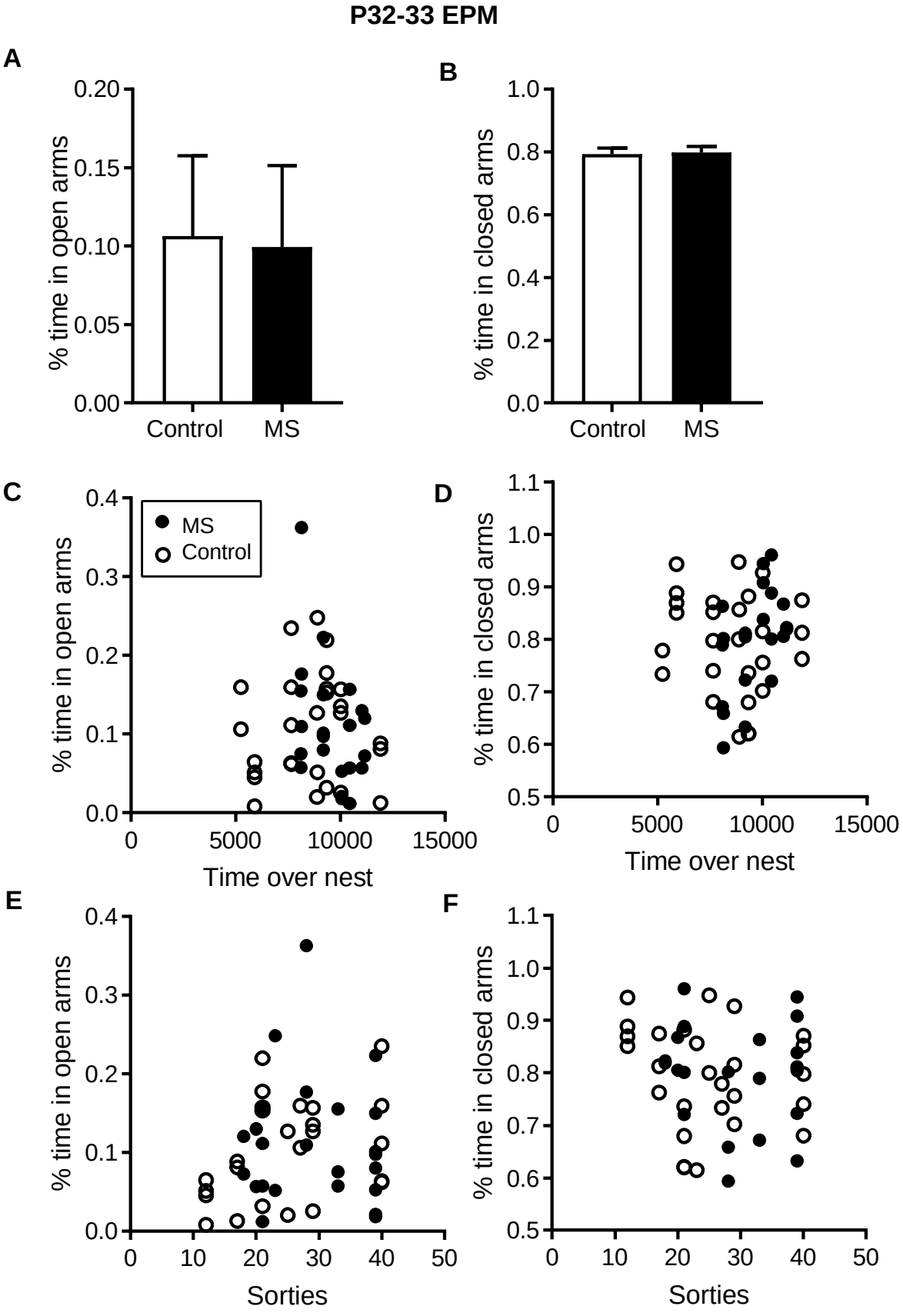

P32-33 EPM
A
% time in open arms
0.20
0.15
0.10
0.05
0.00
Control
MS
B
% time in closed arms
1.0
0.8
0.6
0.4
0.2
0.0
Control
MS
C
MS
Control
% time in open arms
0.4
0.3
0.2
0.1
0.0
0
5000
10000
15000
Time over nest
D
% time in closed arms
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
5000
10000
15000
Time over nest
E
% time in open arms
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
Sorties
F
% time in closed arms
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
10
20
30
40
50
Sorties

Figure 12. **Maternal separation does not alter anxiety-like behavior in the elevated-plus maze in adolescence.** (A) MS and control mice did not differ in % time spent in open arms Control: *Mdn* = 0.1063 (0.05129 to 0.1574), n=27, MS: *Mdn* = 0.09925 (0.05708 to 0.1511), n=22, U= 289, *P* = 0.8813]. Bars represent median ± IQR. (B) MS and control mice do not differ in % time spent in closed arms [Control: *M* = 0.7933 ± 0.01884, n=27, MS: *M* = 0.7972 ± 0.02064, n=22; t=0.1376 df=47; *P* = 0.8912]. Bars represent mean ± SEM. (C) There was no association between % time in open arms and time over nest [r = -0.1238, *P* = 0.3968] (D) There was no association between % time in closed arms and time over nest [r = 0.05305, P = 0.7173] (E) There was no association between % time in open arms and sorties [r = 0.2084, *P* = 0.1508] (F) There was no relationship between % time in closed arms and sorties [r= -0.1292, *P* = 0.3763].

## Discussion

Our goal was to use a maternal separation paradigm to determine how an early life insult impacts the development of flexible decision-making in mice. We found that maternally separated mice tested at a juvenile age (P25-P26) showed less flexibility in learning to reverse a previously learned association compared to littermates. Maternally separated mice required more trials to reach criterion (**Fig. 8a**) and made more errors than littermates, specifically during the reversal phase of the task (**Fig. 8b**). These data indicate that maternal separation induces changes in flexible decision-making early in life when mice have just been weaned and must forage for their own food. Notably, we found that juvenile MS mice made more errors prior to the first reward compared to littermates (**Fig. 8c**). This increase in perseveration compared to littermates could result from insensitivity to disappointing outcomes leading to lack of updating or difficulty inhibiting their responses to the previously rewarded odor. However, once the first reward in the new odor was experienced, maternally separated and littermate juvenile mice did not differ in the number of errors made, suggesting that MS mice and littermate controls updated behavior at a similar rate once positive feedback was encountered.

We did not detect any significant effects of MS on reversal performance in the 4-choice task when mice were tested in adulthood (**Fig. 9**), even if the daily separation period was extended from 60 minutes to 180 minutes (**Fig. 10**). This could suggest that early maternal separation has no lasting consequences on decision-making and underlying neural circuits in mice despite extensive evidence of changes in executive function related circuitry in rats (Pascual and Zamora-Leon, 2007; Chocyk et al., 2010; Monroy et al., 2010; Uchida et al., 2010; Muhammad and Kolb, 2011, Brenhouse et al 2013; Anier et al 2014) and degus (Helmeke et al., 2008). This species difference seems unlikely. Yang et al. (2015) recently reported that early maternal stress driven by inadequate supply of bedding produces lasting effects on neuronal morphology in the anterior cingulate cortex in P75 mice and this stress led to impairments in tests of

working memory. Additionally, it is possible that the effects of maternal separation on adults could be observed with a more difficult task.

We also find that in adulthood, mice that had experienced early postnatal maternal separation had higher cumulative ethanol consumption compared to littermates in an intermittent access 20% ethanol single bottle choice "drinking in the dark" paradigm (**Fig. 11**), suggesting that at least some differences in reward and decision-making circuits remain in mice into adulthood. Our ethanol data are consistent with a previous study that found that a 180 minute maternal separation paradigm increases 10% ethanol intake in a daily three bottle choice paradigm and also increases 6% and 10% ethanol consumption in a daily operant paradigm in adult mice (Cruz et al., 2008). Our ethanol consumption data, acquired using a different paradigm which is able to promote binge-like levels of ethanol drinking in short periods of time, strengthen these previous findings, which together strongly suggest that early maternal separation enhances voluntary ethanol consumption in adulthood. This adds to growing evidence that early adversity may enhance risk for the development of substance use disorders. Although many studies in rats find increased anxiety-like behavior following maternal separation, we did not find that our maternal separation manipulation led to changes in anxiety-like behavior (**Fig. 12**), and therefore anxiety is not likely playing a driving role in cognitive differences or ethanol consumption.

Our results may also explain why previous studies found no effect of early MS on reversal learning in mice. If, as we observe, the MS effect on flexible decision-making is limited to juvenile development in mice, then effects may have been missed by previous researchers that only tested adults. Task design may also be a factor. Prior studies of MS in mice that found no effect on reversal used the 2-choice attentional set shifting task (Mehta and Schmauss, 2011) while our current study used a 4-choice paradigm. Prior work in rats has shown that the 4-choice reversal task is more difficult to learn than the 2-choice task (Kim and Ragozzino, 2005; Ragozzino et al., 2003; Ragozzino and Rozman, 2007) placing greater cognitive load on the animal to sort out the best of the multiple options. Testing mice with this 4-choice task may have made MS effects easier to detect in mice even when using a supposedly more "stress resistant" C57Bl/6 strain.

Although rats and mice both show similar cognitive differences as a result of early MS, it is notable the age at which MS effects become apparent differs by species. Here we show effects in mice on flexible decision-making are limited to the juvenile period, while in the literature rats have shown MS effects that emerge after the juvenile period (post P30). For example, working memory deficits assessed using the radial arm maze were observed in maternally separated rats tested at P40, but not at P25 (Brenhouse and Andersen, 2011). Differences in MS rats and controls also emerged after P30 in the Morris water maze (Wang et al., 2015). In this latter study, spatial reversal notably became more flexible in adolescent and adult rats compared to controls. This variability in MS effects on cognition suggests there are complex interrelationships between species, age and the specific cognitive domain tested by a task (e.g. spatial vs. non-spatial).

It may help to frame this variability in the context of two models that are used to discuss how the brain and body respond to stress (Hostinar and Gunnar, 2013). In one, the allostatic load model (McEwen and Stellar, 1993), repeated stress has negative impacts on the brain. In another, the adaptive calibration model (Del Giudice, Ellis and

Shirtcliff, 2011), stress calibrates or tunes developmental processes to allow an animal to match its behavior to the environment. The allostatic load model attempts to explain how organisms respond to insults, positing that adaptations, in the short-term, can provide benefits, however if experienced for longer periods can lead to negative consequences (Hostinar and Gunnar, 2013). The adaptive calibration model attempts to answer why systems act the way they do in the context of life history strategies instead of viewing certain outcomes as dysfunctional or pathological.

Under the umbrella of adaptive calibration model, there is a growing body of literature suggesting that early social deprivation may accelerate the maturation of threat related behavior. A series of studies focused on extinction and recovery of fear conditioning memory have noted that rats exposed to MS show early adult-like fear extinction and recovery behavior (Callaghan and Richardson, 2011). Stress in the early homecage has also been shown to alter the developmental trajectory of attachment and avoidance learning (Moriceau et al., 2009). This has been echoed by discovery of adult-like functional connectivity in the amygdala-ventromedial PFC of children who were institutionalized during early life (Gee et al., 2013).

Consistent with the earlier maturation hypothesis, the juvenile MS mice tested in our 4-choice reversal paradigm performed in a manner indistinguishable from littermate adults (reversal trials to criterion: MS juvenile $M = 34.86 \pm 3.20$, LM adults (LM 60) $M = 29.50 \pm 2.98$, $t(20) = 1.11$, $P = .3$) or untreated adults from previously published data (Control adults $M = 36.18 \pm 4.78$, $t(23) = 0.24$, $P = .8$; data from Johnson and Wilbrecht, 2011). The age at which we observe a significant effect of MS (P25-26) is likely a transitional phase of life in which the animal is moving from parental dependence to independence and in which the animal must quickly learn about its changing environment and make adjustments based on those experiences (Spear, 2000). In our prior study which described a developmental decrease in flexibility in a 4-choice reversal task (Johnson and Wilbrecht, 2011), we hypothesized that this heightened flexibility observed in juveniles may be particularly important for navigating the ambiguous and/or uncertain environment that an animal faces at this unique life stage. Periods of enhanced flexibility have been observed in other developmental studies in rats (Simon and Moghaddam, 2015) and humans (van der Schaff et al., 2011). Interpreted in the context of the adaptive calibration model and the earlier maturation hypothesis our current MS results could suggest that in the face of adversity it might be adaptive for mice to use more perseverative, adult-like strategies during the juvenile period.

Our results can also be interpreted in the context of the chronic stress and allostatic load model. We can compare our MS data to studies of chronic restraint stress, that have found repeated stress in adulthood can lead to alteration in frontal circuit neural morphology (Radley et al., 2004; 2009) and disrupt flexible decision-making in the 2-choice ASST task (Liston et al., 2006). Although chronic restraint stress in adulthood selectively affected attentional set shifting (changing a rule from odor to texture) and not reversal learning (switching from odor 1 to odor 2) (Liston et al., 2006), it is possible that early MS might simply impair circuit function underlying reversal learning in an analogous fashion. Loss of function could thus be quite different than acceleration of maturation. In summary, both the allostatic load and adaptive calibration model could be used to explain our current data. We speculate that different strategies adopted at the

time of dispersal (whether evolutionarily adaptive or maladaptive) could potentially influence the criteria and timing of territory and reproductive decisions that could influence the whole life trajectory of a wild living rodent. This idea could also be applied to human society and decision-making in its greater complexity. Future work on the biological changes following MS in rodents should shed light on the appropriateness of the adaptive calibration model versus the allostatic load model and help guide translational efforts to ameliorate the impact of early life adversity on human health.

In the next chapter we explore how maternal separation effects the development of long-range axons that support flexible goal-directed decision-making. Given that we found that maternal separation impacted flexible decision-making at P26 but not P60, we focus on brain development during the late juvenile/early adolescent period.

Chapter 4

# Variation in early life maternal care predicts adolescent prefrontal cortex to amygdala synapse development in mice

## Introduction

The adolescent transitional period between childhood and adulthood is a time marked by striking changes in neural circuits and behavior. The frontal cortices, as well as its connections with other brain regions, undergo a protracted period of maturation. However, sub-circuits of the frontal cortex display different developmental trajectories. While dendritic spine density decreases (Huttenlocher and Dabhokhar, 1997; Petanjek et al., 2011; Anderson et 1995; Bourgeois, Goldman-Rakic and Rakic, 1994) and the neocortex is thins (Gogtay et al., 2004), a subset of connections exhibit growth during adolescence. In rodent models, we can observe projections from the medial prefrontal cortex (mPFC) to the basolateral amygdala (BLA) and from the BLA to the mPFC ramify and undergo new synapse formation during the adolescent to young adult period (Cunningham et al., 2002; Landers and Sullivan, 2012; Arruda-Carvalho et al., 2017; Johnson et al., 2016a, but see Cressman et al., 2010). Dopaminergic axons also increasingly innervate the mPFC and orbitofrontal cortex (OFC) into young adulthood (Benes et al., 2000; Cunningham et al., 2002; Hoops et al., 2018; Reynolds et al., 2018) and inhibitory synapses onto subtypes of prefrontal cortical neurons are also remodeled (Tseng and O'Donnell; 2007; Vandenberg et al., 2015; Piekarski et al., 2017ab). The corticostriatal pathway undergoes rapid synaptic changes in P10-18 mice (Peixoto et al., 2016) and corticostriatal functional connectivity is strengthened in humans when quantified by MRI (Larsen et al., 2017; van den Bos et al., 2012).

Theoretical work on the evolution of plasticity suggests circuits with extended or late growth may evolve to support changes appropriate to the life stage or adapt to an individual's specific environment (Nettle, Frankenhuis and Rikard, 2013; Mabry and Stamps, 2008). We were particularly interested in learning if these late developing prefrontal cortex-BLA connections would show changes in response to early life adversity. Functional connectivity between these structures has been shown to be affected by early institutional care in children (Gee et al., 2013). More broadly, early-life adversity is associated with increased risk for the development of a number of physical and mental health conditions including depression and anxiety disorders (Felitti et al., 1998; McLaughlin et al., 2012). However, the specific neurodevelopmental pathways linking adversity to disease are not well understood. By comparing development of these PFC-BLA pathways with OFC and cortical striatal axons at the synaptic level, we hope to gain new insights into the specificity and potential mechanisms at play in the neurodevelopmental response to a specific form of adversity.

In humans, multiple variables often contribute to early life adversity. These variables can vary in type (physical vs psychological), severity, developmental age and duration, which make it difficult to discern which factors are most important in sculpting circuits in early life (Sheridan and McLaughlin, 2014). In mice, we can model adversity

in a more controlled fashion. Altricial species, like humans and rodents, rely on their caregiver for warmth, nourishment and protection (Bowlby, 1982). Caregivers also most likely serve as a critical signal about the type of environment in which offspring grow up. Variation in maternal care may convey information about environmental conditions and influence the developmental trajectory of neural circuits in an adaptive manner (Baram et al., 2012). In this way, maternal care may serve as an important signal that enables the juvenile brain to anticipate the environment it will encounter when it reaches independence and adjust its developmental trajectory accordingly.

Here, we used maternal separation (MS), three hours per day P1-10, as a rodent model of early life adversity and added quantification of maternal care to capture variation in care in the MS and control groups. We then used a viral injection approach to label long range axons in adolescence that show extended growth in development. We focused on two divergent frontal efferents projecting from the same dorsomedial prefrontal cortex (dmPFC) region out to the basolateral amygdala (BLA) and dorsomedial striatum (DMS), as well as two convergent frontal afferents whose axons emerge from cell bodies in the BLA and OFC and ramify in the same target region in the dmPFC. We quantified axonal bouton density on each projection at a mid-adolescent timepoint, compared density between MS and controls and looked for relationships between variation in maternal care and bouton density.

Our study was motivated by a previous study from our lab in which we found that mice that experienced MS P1-10 are less flexible in a reversal learning task when tested at P26 (Thomas et al., 2016). Flexibility in reversal has been shown to be dependent on the integrity of brain regions investigated in the current study, as lesions to dmPFC, BLA, OFC and DMS all impair reversal learning (Johnson and Wilbrecht, 2011; Bissonette et al., 2008; Kim and Ragozzino, 2005; Rudebeck et al., 2013; Schoenbaum et al., 2003; Yin et al., 2005; 2006).

We also hoped to clarify the meaning of results from previous studies that demonstrated that maternal separation leads to changes in layer 2/3 pyramidal cells in the mPFC, an input layer of both OFC and BLA long-range axons. Multiple studies show that MS in rats leads to reduced mPFC spine density in adults (Chocyk et al., 2013; Monroy et al., 2010; Pascual and Zamora-León, 2007), however one study found increased spine density specifically on the apical dendrites of these same mPFC neurons (Muhammad et al., 2012). Importantly, another study found that the timing of the MS protocol was important (Bock et al., 2005). These mixed data suggest specific sub-circuits may be remodeled within the PFC in a manner that is sensitive to the manipulation.

In the current study, we found that MS treatment and variation in maternal care have specific effects on the projection from the dmPFC to the BLA, but not the three other projections under study. MS led to greater total dam time on nest and more dam sorties. In the MS treated offspring at P35, a mid-adolescent time point, we found dmPFC boutons were more numerous but smaller in size. These synaptic differences could alter connectivity and plasticity in this projection, potentially contributing to differences in flexible decision-making in development.

## Methods

*Animals*

Male C57Bl/6 *Mus musculus* (lines originally obtained from Charles River) were used for this study. Dams and sires were housed in pairs throughout the breeding and rearing period. At P21, experimental mice were weaned and group housed in same-sex cages, 2-5 per cage. All cages were kept on a 12/12 reverse light dark cycle (lights off at 10 AM). All animals received nesting material and paper huts in their home cage. All procedures were approved by the UC Berkeley Animal Care and Use Committees.

*Maternal Separation (MS)*

From postnatal (P) day 1 to 10, pups from the MS group were removed daily from their home cage for 3 hours from 11:30 AM to 2:30 PM. During the 3 hour separation, pups were kept in a clean cage placed on an electric heating pad. MS pups were separated from each other by dividers and thus could hear and smell each other, but not touch during the separation. The control group stayed in the homecage with the dam and the litter was untouched.

To better understand our MS manipulation and enhance potential for comparison to an alternate model of early-life adversity that provides rodent dams with limited nesting material (LNM) (Rice et al., 2008), we also quantified maternal care in MS and control dams. It has been shown that the LNM manipulation leads to greater fragmentation of care, perhaps best indicated by an increase in the number of sorties, instances when the dams leave the nest (Rice et al., 2008).

*Maternal care quantification*

*Maternal care monitoring 1 (P1-P10):* Maternal behavior was monitored in both control (n = 18) and MS (n= 15) cages every other day from P1-P10, for a total of 5 monitoring days. Cages were observed 3 times a day during the dark phase (11 AM, 2:30 PM and 5 PM) under infrared light and each monitoring session lasted for 30 minutes, in which dam behavior was scored every other minute (15 mins total of observation per session). The amount of time the dam spent on the nest and the number of instances the dam left the nest (sorties) were scored. Nests were built up into dome-like structures, which have been described in detail in other studies assessing the C57Bl/6 strain (Millstein and Holmes, 2007; Rice et al., 2008). These domes typically occluded behavior in the nest, thus more detailed maternal behaviors such as arched-back nursing and licking and grooming were not scored.

*Maternal care monitoring 2 (P16-P20):* Maternal and pup behavior was also monitored just before weaning from P16-P20 in both control (n= 16) and MS (n= 14) cages. The number of litters monitored during this period differed from the earlier monitoring period due to errors in data collection. Cages were observed at 11 AM, 2:30 PM and 5 PM over 3 days. Here we marked each observation minute as simply positive or negative for dam sortie, pup sortie, all pups in nest and instances of dam mounting pups (aggressive behavior sometimes observed near weaning age; Franks et al., 2015).

*Viral injections*

Stereotaxic viral injections were performed under isoflurane anesthesia at P21. In the first cohort of mice, a Nanoject II injector (Drummond Scientific Company, Broomall, PA) was used to deliver 50 nl of AAV2/1-CAG-eGFP (UNC Vector Core) to the left dmPFC (AP +2.6 mm, ML +1.0 mm, DV 0.5 mm relative to bregma). In the second cohort, 50 nl of AAV2/1-CAG-eGFP or AAV2/1-CAG-Tdtomato were delivered to both the left OFC (AP +2.7 mm, ML + 1.65 mm, DV 2.1 mm) and bilateral BLA (AP -1.0 mm, ML +/- 3.2, DV 4.25) (reporter virus was counterbalanced between brain regions). Before surgery mice were given 10 mg/kg of meloxicam. Dosing was repeated twice post-surgery at 24 hour intervals and mice were monitored for health and weight gain.

*Histology*

Mice were transcardially perfused at a mid-adolescent time point with 4% paraformaldehyde in PB (0.1 M, pH 7.4). Perfusion age was P35 for all data in Fig. 2 and Fig. 3 and P28 for data in Fig. 4. Brains were extracted and post-fixed in 4% paraformaldehyde overnight and then placed in PB. Coronal sections (200 µm) were cut on a vibratome and mounted on slides with Fluoromont-G (Southern Biotech). Sections that included the injection sites were stained with DAPI and checked for accuracy.

*Microscopy*

To image dmPFC axons, we used an Ultima IV laser scanning microscope (Bruker, Middleton, WI) and a 40 x 0.8 NA water immersion objective (Olympus, Center Valley, PA). A Mai Tai HP laser (Spectra physics, Santa Clara, CA) was tuned to 910 nm in order to excite GFP. We imaged axon segments (~40 microns in length) and obtained image stacks with a 1 µm z-step. To image BLA and OFC axons that target the same region of the dmPFC (labeled with GFP and td-tomato in the same mouse), we used a Zeiss LSM 710 laser scanning confocal microscope using 40 x 0.8 NA oil immersion objective.

*Image processing and analysis*

Images were median-filtered 3-dimensional z stacks. All images were analyzed blind to experimental group. Axonal boutons were scored based on established criteria (Holtmaat et al., 2009) using custom Matlab software (Mathworks). Briefly, axonal boutons were scored if the intensity was more than 3 times as bright as the adjacent axon shaft, a conservative criterion established using imaging and electron microscopy (Holtmaat et al., 2009). Bouton density was calculated as the number of boutons, divided by the length of analyzed axon. We also looked at bouton volume based on brightness measures, motivated by work that shows a strong relationship between bouton size and synaptic strength (Cheetham et al., 2014; Murthy et al., 2001). We measured the average intensity of the adjacent axon backbone and the bouton of interest and a nearby region of the background. Background subtracted bouton intensity

was then divided by the background subtracted backbone intensity to get a background subtracted normalized intensity for the bouton of interest.

*Statistics*

All statistical comparisons were performed using GraphPad Prism 7 (GraphPad, San Diego, CA). Data were tested for normality using D'Agostino normality tests. Data that were normally distributed were analyzed using unpaired student's t-tests for group comparisons. Pearson's correlations were used to test the linear relationship between variables. Data that were not normally distributed were analyzed using the Mann-Whitney *U* test for group comparisons. Kolmogorov-Smirnov tests were used to measure differences in cumulative frequency distributions. All normally distributed data are presented as means ± SEM. Data that were not normally distributed are presented as medians ± IQR. Statistical significance was **P* < 0.05; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

## Results

### Maternal separation leads to fragmented early maternal care

To measure how early life maternal separation impacts maternal care, we quantified the behavior of dams from both control and MS cages from P1-P10 (**Fig. 13a**). We recorded the amount of time dams spent over the nest and the number of sorties (instances when the dam left the nest) during each monitoring session. We found that dams experiencing the MS manipulation spent significantly more time over the nest compared to control dams during observations from P1-P10 [Control: *M* = 8865 ± 346.4, n=18; MS: *M* =10128 ± 143.5, n=15; t=3.139 df=31, *P* = 0.0037] (**Fig. 13b**). Additionally, dams experiencing the MS manipulation made significantly more sorties than control dams [Control: *M* =16.89 ± 1.193, n=18; MS: *M* = 22.93 ± 2.345, n=15; t=2.414 df=31, *P* = 0.0219] (**Fig. 13c**). There was no difference between treatment groups in offspring weight at weaning (P21) [Control: *M* = 10.92 ± 0.2954, n=18; MS: *M* = 10.6 ± 0.3242, n=19, t=0.7396 df=35, *P* = 0.4645] (**Fig. 13d**).

P16-20 maternal care measures did not differ between control and MS mice [Dam out of nest: Control: *M* = 59.88 ± 5.491, n=16; MS: *M* = 56.93 ± 6.328, n=14, t=0.3535 df=28, *P* = 0.7264; Pups out of nest: Control: *M* = 64.94 ± 5.915, n=16; MS: *M* = 60.5 ± 5.073, n=14; t=0.561 df=28, *P* = 0.5793; All in nest: Control: *M* = 69.63 ± 6.353, n=16; MS: *M* = 77.36 ± 4.008, n=14; t=0.9956 df=28, *P* = 0.3280; Mounting behavior: Control: *Mdn* = 0 (0-2.5), n =16; MS *Mdn* = 0 (0-2.25), n =14, U = 106, *P* = 0.7847].

These observations indicate that maternal separation impacts maternal care during early life (P1-10), but not in the later pre-weaning period (P16-20). Specifically, maternal separation alters both the quantity and quality of early care. We speculate that the greater amount of time MS dams spend over nest is a compensatory response, making up for missed maternal care bouts. Increases in maternal care after reunion with pups have been noted in other studies as well (Millstein and Holmes, 2007). The

increase in sorties suggests MS has parallels to the limited nesting material model of adversity (**Fig. 13e**) (Rice et al., 2008).

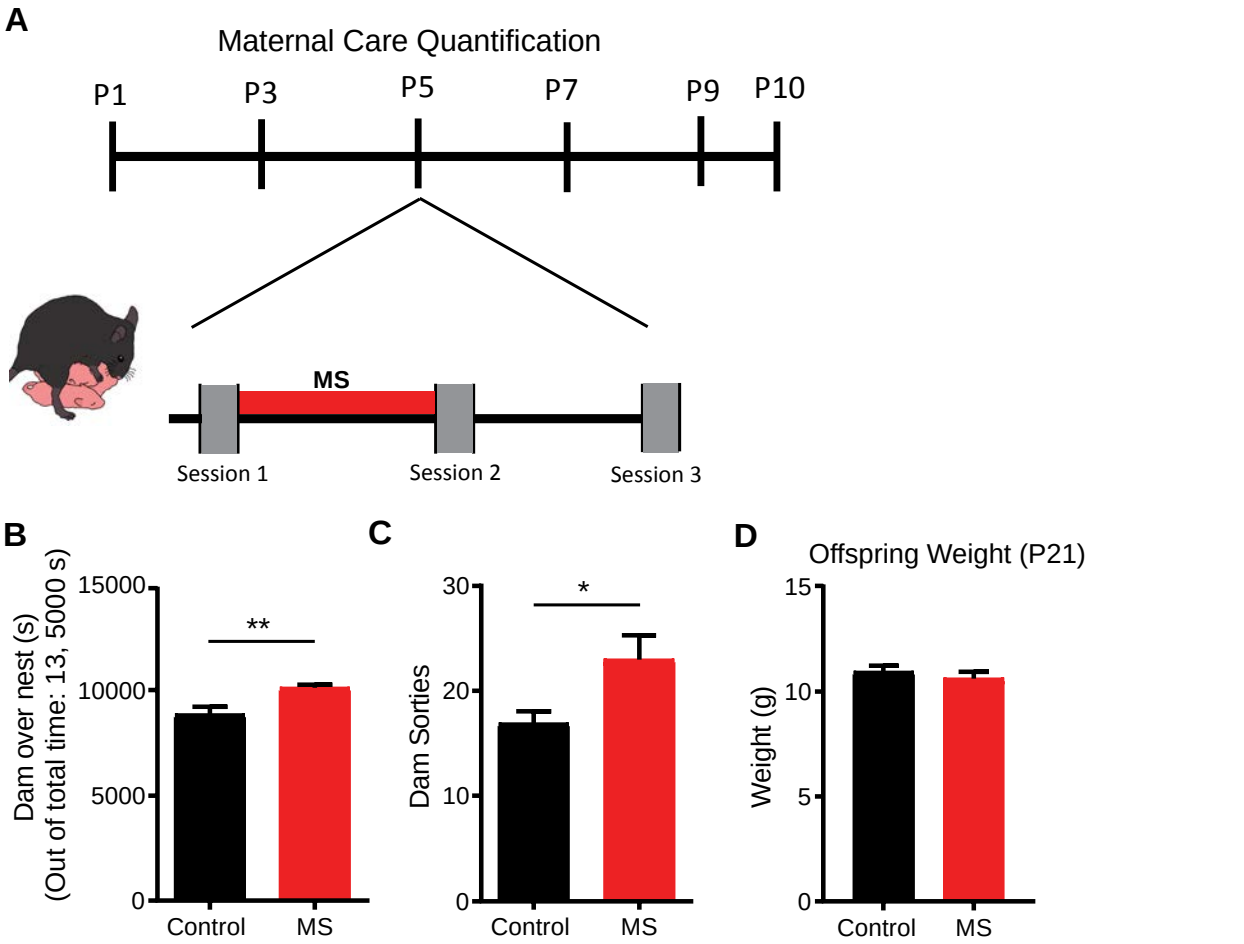

A
Maternal Care Quantification
P1
P3
P5
P7
P9
P10
MS
Session 1
Session 2
Session 3
B
Dam over nest (s)
(Out of total time: 13, 5000 s)
15000
10000
5000
0
**
Control
MS
C
Dam Sorties
30
20
10
0
*
Control
MS
D
Offspring Weight (P21)
Weight (g)
15
10
5
0
Control
MS
E
Activity Grids
Control Dam
MS Dam
Red = Dam off nest entire observation minute
Blue = Dam on nest entire observation minute
Yellow = Dam on and off nest during observation minute

Figure 13. **Maternal separation dams display more fragmented care compared to control dams**
(A) Timeline of maternal care quantification and maternal separation. Cages were monitored for 5 days during the P1-P10 period. Each day of monitoring cages were monitored 3 times a day (sessions shown in grey bars). MS offspring were separated from the homecage for 3 hours per day (red bar). (B) MS dams spend more time over the nest compared to control dams [Control: *M* = 8865 ± 346.4, n=18; MS: *M* = 10128 ± 143.5, n=15; t=3.139 df=31, *P* = 0.0037]. (C) MS dams display significantly more sorties compared to control dams [Control: *M* =16.89 ± 1.193, n=18; MS: *M* = 22.93 ± 2.345, n=15; t=2.414 df=31, *P* = 0.0219] (D) MS and Control offspring do not differ in weight at P21 [Control: *M* = 10.92 ± 0.2954, n=18; MS: *M* = 10.6 ± 0.3242, n=19, t=0.7396 df=35, *P* = 0.4645]. (E) Activity grids representing dam behavior. Red squares = dam off nest entire observation minute, blue squares = dam on nest entire observation minute, yellow = mixed minute (dam on and off nest during observation minute). Bars represent mean ± SEM. *P < 0.05; **P < 0.01.

## dmPFC afferents that target the BLA are specifically sensitive to early life maternal care

We next used a viral strategy to label dmPFC axons with GFP in the MS and control group offspring (**Fig. 14a,b**) and examined innervation of two target regions, the BLA (**Fig. 14c,d**) and the dorsomedial striatum (DMS) (**Fig. 14h, i**). When comparing linear bouton density, we found that dmPFC-BLA axons exhibit higher bouton density in MS mice compared to control mice at P35 [Control: *M* = 0.118 ± 0.005706, n=59; MS: *M* = 0.1637 ± 0.006604, n=61, t=5.224 df=118, *P* <0.0001] (**Fig. 14e**). Furthermore, dmPFC-BLA bouton density correlated with early maternal care measures. There was a positive correlation between dmPFC-BLA bouton density and P1-10 time over nest (r = 0.6981, *P* = 0.0116) (**Fig. 14f**) and dam sorties (r = 0.6197, *P* = 0.0316) (**Fig. 14g**).

In contrast to dmPFC-BLA axons, the density of boutons on dmPFC-DMS axons (labeled by the same viral injection) did not differ between controls and MS mice [Control: *M* = 0.1528 ± 0.006769 n=60; MS: *M* = 0.1406 ± 0.0058, n=61, t=1.371 df=119, *P* = 0.1729] (**Fig. 14j**). Also, dmPFC-DMS axonal bouton density did not show any relationship with amount of time dam spent over nest (r = -0.3198, *P* = 0.3110) (**Fig. 14k**) or with dam sorties (r = -0.1286, *P* = 0.6904) (**Fig. 14l**).

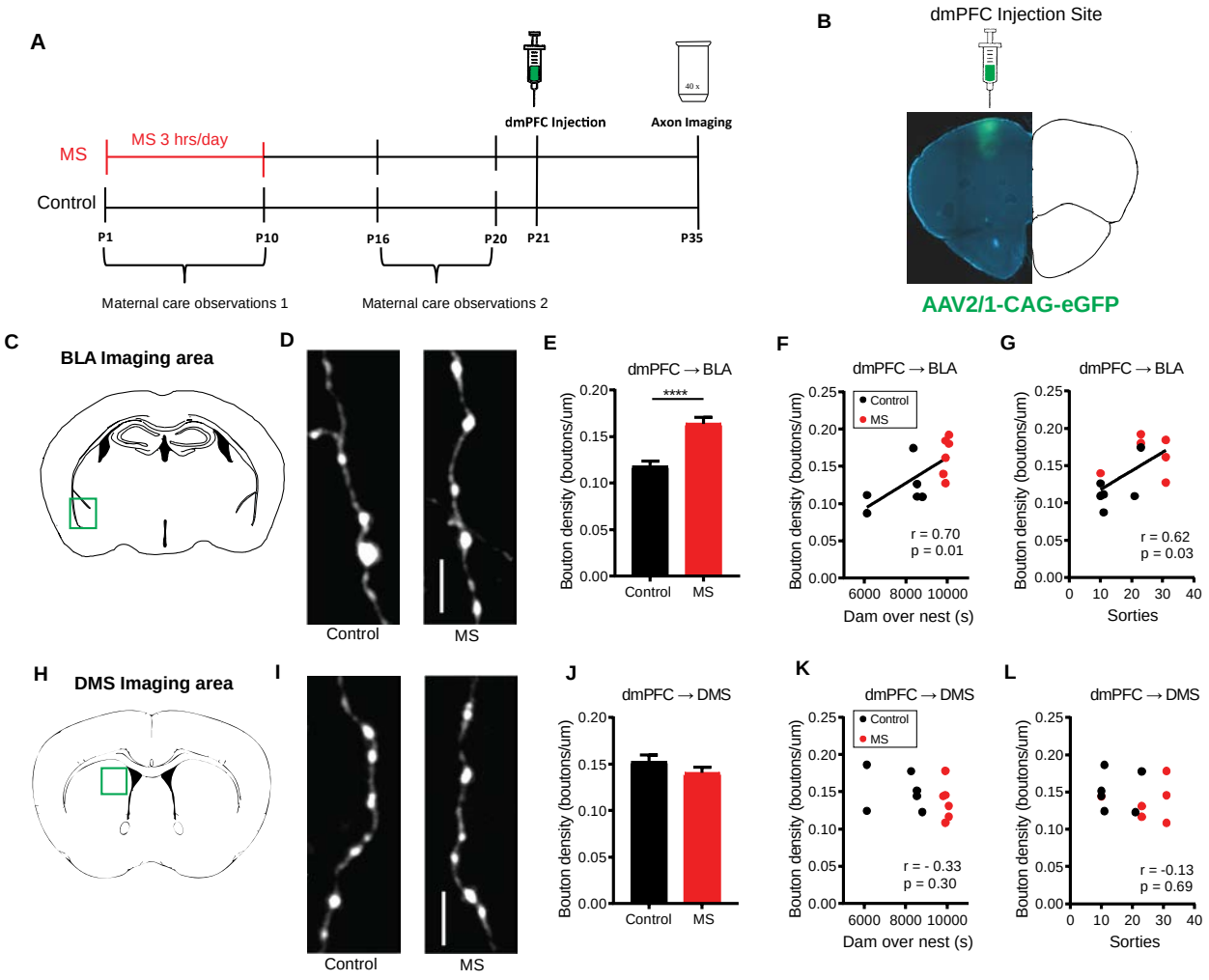



Figure 14. **Maternal care affects dmPFC→BLA bouton development**
(A) Experimental timeline (B) Injection site of dmPFC with AAV2/1-CAG-eGFP (C) Schematic of dmPFC→BLA imaging area (D) dmPFC→BLA example axons (scale bar = 5 microns) (E) MS mice have higher dmPFC→BLA bouton density compared to controls at P35 [Control: *M* = 0.118 ± 0.005706, n=59; Maternal separation: *M* = 0.1637 ± 0.006604, n=61, t=5.224 df=118, *P* <0.0001]. (F) The amount of time dams spend over the nest in early life correlates with dmPFC→BLA bouton density in adolescent offspring (r = 0.6985, *P* = 0.0115), with higher time over nest associated with higher bouton density. (G) Early life dam sorties are correlated with dmPFC→BLA bouton density in adolescent offspring (r = 0.6197, *P* = 0.0316), with more sorties associated with higher bouton density. (H) Schematic of dmPFC→DMS imaging area (I) dmPFC→DMS example axons (scale bar = 5 microns) (J) MS mice did not differ in dmPFC →DMS bouton density compared to controls at P35 [Control: *M* = 0.1528 ± 0.006769 n=60; Maternal separation: *M* = 0.1406 ± 0.0058, n=61, t=1.371 df=119, *P* = 0.1729]. (K) The amount of time dams spend over the nest did not correlate with dmPFC→DMS bouton density in the adolescent mice (r = -0.3262, *P* = 0.3008). (L) The number of dam sorties did not correlate with dmPFC→DMS bouton density in adolescent offspring (r = -0.1286, *P* = 0.6904). Bars represent mean ± SEM. ****$P <$ 0.0001.

**MS leads to smaller bouton volume on dmPFC-BLA afferents**

To further explore the nature of the differences in bouton density in the dmPFC projection to the BLA we performed additional analyses of bouton volume using normalized and background subtracted fluorescent intensity as a proxy measure (Holtmaat et al., 2009). We found that the median bouton volume estimate in the MS mice was smaller on dmPFC-BLA axons than in control mice (Control: *Mdn* = 941.7 (647.9 to 1447), n = 326; MS: *Mdn* = 855.3 (557.1 to 1307), n = 452; U = 65909, *P* = 0.012) (**Fig. 15a**). Cumulative distribution of bouton volume estimates showed a trend level leftward shift in the MS group compared to controls (KS test 0.09573, *P* = 0.0621) (**Fig. 15b**).

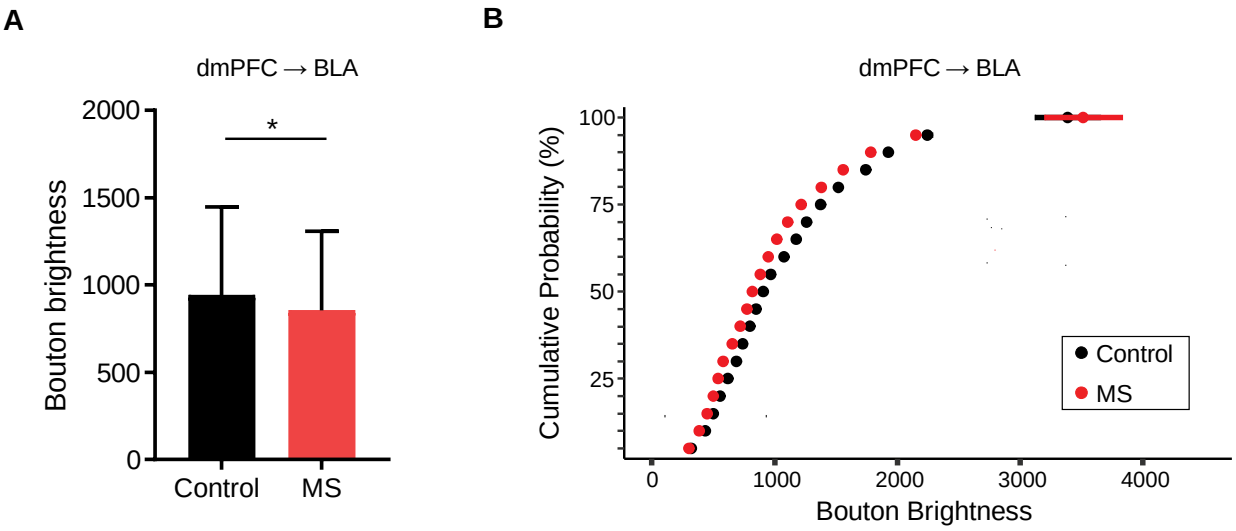


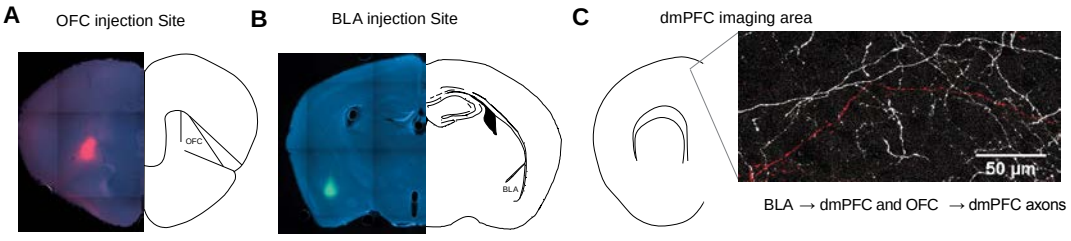

Figure 15. **Maternal separation mice have smaller bouton volumes on dmPFC → BLA axons**
(A) MS mice have less bright boutons compared to control mice on dmPFC → BLA axons [Control: *Mdn* = 941.7, n = 326; MS: *Mdn* = 855.3, n = 452; U = 65909, P = 0.0120 (B) Cumulative probability plots showed MS mice had a trend level leftward shift in bouton brightness on dmPFC → BLA axons (P = 0.06, KS test). Bars represent median ± IQR. **P* < 0.05

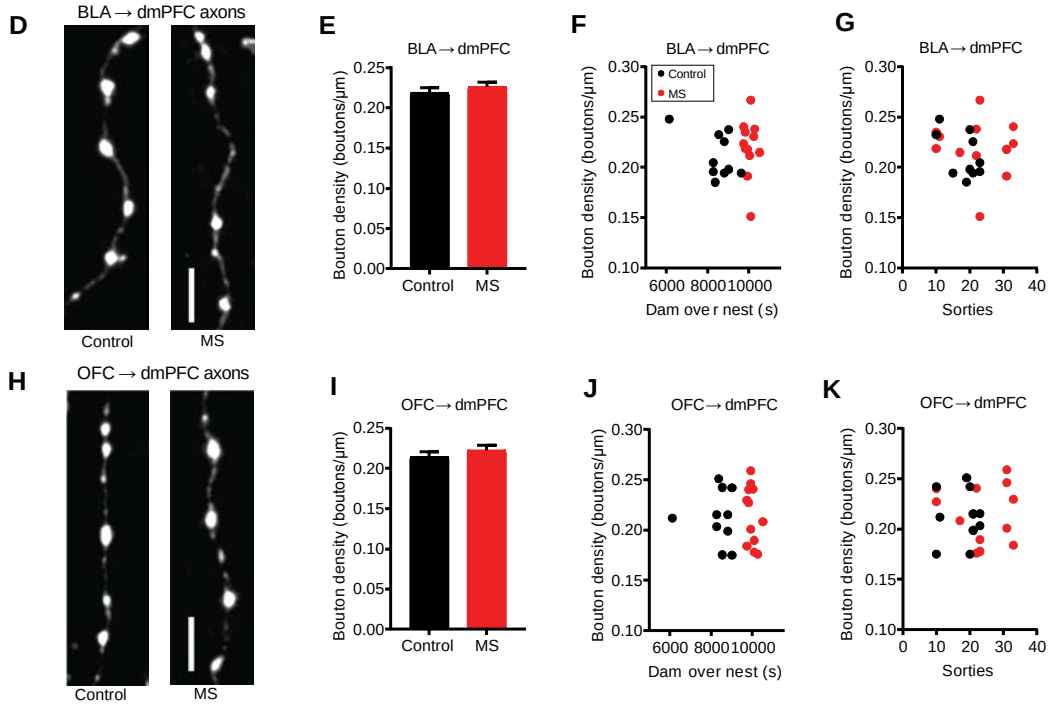
**OFC and BLA axons that target the dmPFC were not sensitive to MS and changes in maternal care**

The prefrontal cortex integrates inputs from a number of different regions, including the basolateral amygdala (BLA) and orbitofrontal cortex (OFC) among others (Hoover and Vertes, 2007; Johnson et al., 2016ab). During postnatal development, these long-range axons could potentially compete for synaptic space in an activity dependent manner, similar to inputs in sensory cortices (Antonini and Stryker, 1993; Taylor et al., 1998; Guirado et al., 2016; Trachtenberg, 2015). We hypothesized that BLA and OFC axons may compete for synaptic partners in the superficial dmPFC and that maternal separation may enhance BLA activity and trophic growth. This could tip the balance in a putative BLA vs. OFC afferent competition, allowing one input dominance over the other. In order to test these hypotheses, we quantified the density of BLA and OFC axonal boutons at sites where they overlap in layer 2 of the dmPFC (**Fig. 16a,b,c**).

We found that BLA axons that targeted the dmPFC did not differ in bouton density between control and MS mice (Control: $M$ = 0.2191 ± 0.005813, n = 70; MS: $M$ = 0.226 ± 0.005793, n = 81, t=0.8429 df=149, $P$ = 0.4007) (**Fig. 16e**). Additionally, there was no relationship between BLA-dmPFC bouton density and dam time over nest (r = -0.04871, $P$ = 0.8296) (**Fig. 16f**), as well as no relationship between bouton density and dam sorties (r = -0.1548, $P$ = 0.4917) (**Fig. 16g**). OFC axons also did not differ in bouton density between control and MS mice (Control: $M$ = 0.2146 ± 0.005942, n=67; MS: $M$ = 0.2228 ± 0.00613, n=76, t=0.9477 df=141, $P$ = 0.3449) (**Fig. 16i**). There was no relationship between OFC-dmPFC bouton density and time over nest (r = -0.1155, $P$ = 0.6086) (**Fig. 16j**), or dam sorties (r = -0.005116, $P$ = 0.9820) (**Fig. 16k**).

In order to determine if MS alters the balance of OFC/BLA inputs, we calculated the ratio between OFC and BLA bouton density (BLA density – OFC density/BLA density + OFC density), however there was no difference in BLA:OFC ratio between MS and controls (Control: $M$ = 0.007103 ± 0.03086, n=10; MS: $M$ = 0.01546 ± 0.02947, n=11, t=0.1958 df=19, $P$ = 0.8469) (**Fig. 16l**) and no relationship between the ratio and time over nest (r = 0.051, $P$ = 0.8309) (**Fig. 16m**) or sorties (r= 0.03589, $P$ = 0.8806) (**Fig. 16n**). These data do not support the hypothesis that MS treatment would have a trophic effect on BLA afferents in dmPFC at the cost of other afferents.


A
OFC injection Site
B
BLA injection Site
C
dmPFC imaging area
50 μm
BLA → dmPFC and OFC → dmPFC axons
AAV2/1-CAG-Tdtomato/AAV2/1-CAG-eGFP


D
BLA → dmPFC axons
Control
MS
E
BLA → dmPFC
Bouton density (boutons/μm)
Control
MS
F
BLA → dmPFC
Control
MS
Bouton density (boutons/μm)
Dam over nest (s)
G
BLA → dmPFC
Bouton density (boutons/μm)
Sorties
H
OFC → dmPFC axons
Control
MS
I
OFC → dmPFC
Bouton density (boutons/μm)
Control
MS
J
OFC → dmPFC
Bouton density (boutons/μm)
Dam over nest (s)
K
OFC → dmPFC
Bouton density (boutons/μm)
Sorties

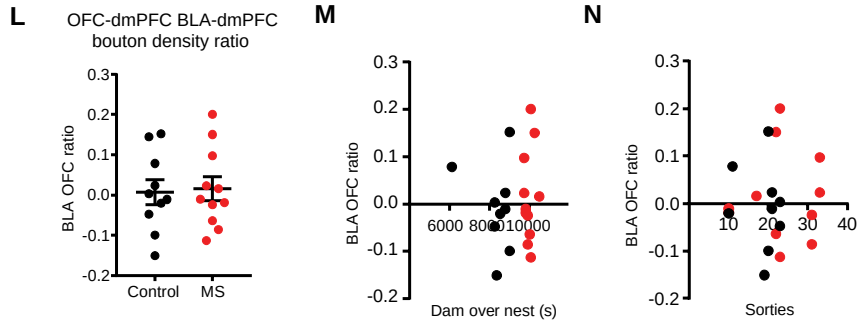

L
OFC-dmPFC BLA-dmPFC
bouton density ratio
BLA OFC ratio
Control
MS
M
BLA OFC ratio
Dam over nest (s)
N
BLA OFC ratio
Sorties

Figure 16. **BLA → dmPFC and OFC → dmPFC axons are not sensitive to variations in maternal care**
(A) Example OFC injection (B) Example BLA injection (C) Image of both axons converging in dmPFC (D) BLA → dmPFC example axons (scale bar = 5 microns) (E) MS mice do not differ from controls in BLA → dmPFC bouton density (Control: $M = 0.2191 \pm 0.005813$, n=70; MS: $M = 0.226 \pm 0.005793$, n=81, t=0.8429 df=149, $P = 0.4007$) (F) There is no relationship between BLA→ dmPFC bouton density and dam time over nest (r = -0.04871, $P = 0.8296$) (G) There is no relationship between bouton density and dam sorties (r = -0.1548, $P = 0.4917$) (H) OFC→ dmPFC example axons (scale bar= 5 microns) (I) MS mice do not differ from controls in OFC→ dmPFC bouton density (Control: $M = 0.2146 \pm 0.005942$, n=67; MS: $M = 0.2228 \pm 0.00613$, n=76, t=0.9477 df=141, $P = 0.3449$) (J) There is no relationship between OFC→dmPFC bouton density and dam time over nest (r = -0.1155, $P = 0.6086$) (K) There is no relationship between OFC→dmPFC bouton density and dam sorties (r = -0.005116, $P = 0.9820$) (L) BLA and OFC bouton density ratio (BLA density-OFC density/BLA density +OFC density) did not differ between controls and MS mice (Control: $M = 0.007103 \pm 0.03086$, n=10; MS: $M = 0.01546 \pm 0.02947$, n=11, t=0.1958 df=19, $P = 0.8469$) (M) There was no relationship between the ratio and dam time over nest (r = 0.051, $P = 0.8309$) (N) There was no relationship between the ratio and dam sorties (r = 0.03589, $P = 0.8806$). Bars represent mean ± SEM.

## Discussion

Here, we find that early life maternal care predicts later axonal bouton density on dmPFC-BLA neurons at an adolescent time-point and has no effect on dmPFC-DMS, BLA-dmPFC or OFC-dmPFC axonal bouton density at this time. These data suggest that early life maternal care has highly specific effects on the adolescent growth and maturation of the dorsomedial PFC axons targeting the amygdala, but not the ascending amygdala projection to the PFC, or other projections with an overlapping origin or target zone.

### Data are consistent with and inform studies of human neglect

Our findings in mice support parallels to research in human subjects who experienced childhood neglect and maltreatment. Previous brain imaging work in children who experienced early life institutional care followed by adoption, found abnormal functional connectivity between PFC and BLA while viewing fearful faces, compared to age-matched controls (Gee et al., 2013). Our data are consistent with these human data and suggest differences in development of descending connectivity from the PFC into the BLA, rather than ascending connectivity, may be responsible for these findings.

### What is the function of the change in dmPFC to BLA synapses?

Rodent and human studies have identified the dorsomedial and or medial PFC-BLA circuit as playing a key role in fear expression and extinction as well as emotion regulation (Arruda-Carvalho and Clem, 2015; Cho et al., 2013; Corcoran and Quirk,

2007; Lai et al 2012; Likhtik et al., 2005;) but also appetitive associative learning and flexible goal directed behavior (Costa et al., 2016; Stuber et al., 2011; Haber and Behrens, 2014). Dysfunction of PFC-BLA connectivity has been implicated in various psychiatric disorders (Maren et al., 2013). The PFC is typically thought to exert top-down dampening control over BLA in order to regulate emotional behavior (Sotres-Bayon and Quirk, 2010), but this projection could also invigorate associative learning and affect flexible decision-making.

Here, we see an upregulation of boutons on dmPFC-BLA axons, accompanied by a decrease in bouton volume. How this change in bouton density impacts the function of this circuit is unclear and could be involved in pathological processes, adaptation to the environment, or resilience. A recent study found that maternal separation in rats led to higher dendritic spine density in the BLA in adulthood, as well as increased anxiety-like behavior (Koe et al., 2016). Together with our data, greater spine density may allow for more synaptic sites in the BLA for dmPFC axons to target. Another study found that observing a distressed conspecific generated 'silent synapses', glutamatergic synapses lacking functional AMPAR, specifically in the dmPFC-BLA pathway. This same stress model also augmented passive avoidance learning (Ito et al. 2015). This enhanced learning is potentially due to these newly generated glutamatergic synapses, which likely strengthen circuit transmission between the dmPFC-BLA pathway (Hanse et al., 2013). A similar mechanism may occur in maternally separated mice, in which we see a greater number of boutons, with smaller volumes, on the dmPFC-BLA circuit in MS compared to control mice. Further research is needed to test if these additional boutons form silent synapses or are indeed critical to changes in function.

In a previous study, we have found that P26 mice that experienced MS show inflexibility in an appetitive odor based reversal task (Thomas et al., 2016), that is known to depend on the integrity of the dmPFC (Johnson and Wilbrecht, 2011) the BLA (Loucks, 2014), as well as OFC (Bissonette et al., 2008; McAlonan and Brown, 2003). Based on the specificity of the MS effect in these circuits at this age, we speculate that changes in the dmPFC-to BLA play an important role in sculpting performance in this flexible decision-making task.

Other relevant mouse studies have focused on connectivity between the mPFC and the BLA. Li et al., (2018) found that maternal immune activation led to enhanced excitatory synaptic transmission in the mPFC–BLA pathway, while postnatal immune activation led to a reduction in feed-forward inhibition in this same pathway, suggesting that the timing of these insults is important for developmental outcomes. Increased connectivity of the mPFC and BLA pathway has also been implicated in a mouse model of autism spectrum disorder. Pten mice displayed increased axonal branching and bouton density of mPFC-BLA axons and in turn increased activity in the BLA (Huang et al., 2016). Importantly, reducing BLA activity was able to rescue social behavioral deficits.

The mechanisms that might be underlying the changes in bouton density and bouton volume are unclear. Previous studies have shown that reductions in hippocampal dendritic spine density following adult chronic stress are dependent on corticotropin releasing factor (CRF) and CRF receptors; blocking CRF binding rescued dendritic spine loss in the hippocampus and memory deficits due to adult acute stress

(Chen et al., 2010). Additionally, mice lacking the CRF1 receptor were resistant to the cognitive effects of early life stress (Wang et al., 2011b) and adult chronic stress (Wang et al., 2011c). CRF gene expression has also been shown to be sensitive to early life maternal care as adult rats that experienced handling (which leads to increased maternal care upon reunion with the pups) showed reduced CRF expression in hypothalamic neurons (Plotsky and Meaney, 1993; Liu et al., 1997). How this might come into play in dmPFC-BLA axons is unclear. Studies have found an upregulation of dendritic spines in BLA following both adult stress as well as maternal separation (Vyas et al., 2002; 2006; Koe et al., 2017). Importantly, CRF also acts on the amygdala (Roozendaal et al., 2002; Gallagher et al., 2008; Regev et al., 2012) and CRF is released within layer 5 of the PFC (Yan et al., 1998), the same layer that the cell bodies that project to the BLA reside. CRF may specifically act on BLA projecting cells to upregulate axonal boutons on these projections.

There is evidence from human studies that greater amygdala reactivity may serve as a mechanism for both risk and resilience in maltreated children. Studies indicate that childhood maltreatment is associated with more severe and chronic cases of depression (Wiersma et al., 2009) as well as poor treatment outcomes (Nanni et al., 2012). However, amygdala activity in individuals who have experienced early life adversity may also be protective. Goldstein-Piekarski et al., (2016) found that depressed people with high levels of early life adversity who show higher amygdala reactivity to happy faces are more likely to experience remission from depression following antidepressant exposure, whereas the opposite is true for depressed individuals with low adversity experience.

In sum, it is difficult to say in laboratory mice whether the changes we see after MS are “good” or “bad” without the context of the natural environment. However, our data are consistent with life history models that predict that brain development should be sculpted by the statistics of the early environment. Often in neuroscience we think about experience dependent processes as situations in which synaptic growth is generated in an experience-independent manner and is then selectively pruned by an experience. Here early experience appears as a critical variable in scaling later growth. These data support previous speculation that the brain can sample the statistics of the environment (here through maternal care in the nest) and then set the developmental trajectory accordingly (Rickard, Frankenhuis and Nettle, 2014; Baram et al., 2012). These processes are likely in place to adapt to more harsh or uncertain environments.

**Limitations of our study**

Although we did not see differences in bouton densities in the projection from the dmPFC to the striatum or projections from the BLA and OFC to the dmPFC, our sampling was not exhaustive. Alternate time points and alternate projections may have revealed MS effects or correlation with variation in maternal care. Also, alternative stressors with different timing may have impact on different circuits. For example, the cortico-striatal circuit might be particularly sensitive to stress experienced during adolescence, when strengthening of this connectivity is observed (Larsen et al., 2017). These data were also collected post mortem, and cannot reveal changes in the dynamic

turnover of synaptic structures which are can be highly responsive to experience during development and adulthood (Johnson et al., 2016ab).

## Conclusion

Adolescent development is a time of flux for the prefrontal cortex and a period of vulnerability for mental health (Paus et al., 2008; Silberg et al., 1999). Here we show that variables in the early life environment show strong and highly specific influences on developmental processes during the adolescent period, enhancing the density and altering the size of the dmPFC boutons in the BLA. We speculate that these changes may support developmental differences in learning in mice that experienced differences in maternal care (Thomas et al 2016), and potentially serve to adapt the brain to environments with different statistics. These data are consistent with imaging studies of children who experienced early life institutional care (Gee et al., 2013), and suggest at a more granular level which circuits and neurons are likely to be most affected. In future, our knowledge of human brain development and health should be enriched by further rodent studies with high resolution circuit and cell-type specificity. We envision studies with increasing specificity will enhance our capacity to design and deliver more effective interventions to promote positive development (Crone and Dahl, 2012; Dahl et al., 2018).

Chapter 5

# Conclusions and Future Directions

## Sub-circuits of the frontal cortex display divergent developmental trajectories

Adolescence is a time of growth and remodeling of cortical and subcortical circuits. Changes at the level of neural circuits likely underlie behavioral transitions that characterize adolescence including gains in social, emotional and cognitive skills and increased exploration and risk taking. While many studies point to large scale modifications in brain volume and grey and white matter during adolescence, cell and circuit specific data at the synaptic level are lacking. Here, we have focused on the development of sub-circuits of the frontal cortex that support flexible decision-making and emotion regulation. The motivating factor behind this research is the idea that by understanding how and when these sub-circuits mature during normative development we will gain understanding about when and how these circuits may be vulnerable to adverse experiences or be most amenable for intervention.

In Chapter 2, I review my own collaborative work and the work of others that illustrate how local dendritic spines and axonal arbors and boutons of afferents and efferents of the frontal cortex mature with different trajectories during the adolescent period. Our more recent *in vivo* imaging data show that local layer 5 dmPFC pyramidal cells prune their dendritic spines during the adolescent period, with adult male and female mice displaying reduced spine density compared to juveniles (Johnson et al., 2016a; Boivin et al., 2018). These data are consistent with previous histological data from humans, non-human primates, rats and mice (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997; Bourgeois, Goldman-Rakic and Rakic, 1994; Petanjek et al., 2011; Kolb and Whishaw, 1998; Anderson et al., 1995). Additionally, we find dendritic spines show less turnover with age, there is less spine formation and elimination in adult compared to juvenile male mice (Johnson et al., 2016a). We also tested our hypothesis that gonadal hormones at puberty play a causal role in driving spine pruning and stabilization. Here we found that for Layer 5b neurons labeled in the Thy1-YFPH line, pre-pubertal ovarian hormone administration in females did advance puberty onset but did not enhance spine pruning or stabilization of daily gain or loss (Boivin et al., 2018). We did find more subtle effects of pubertal manipulation on dendritic spine morphology (Boivin et al., 2018). Further studies will be necessary to investigate if other sub classes of cells in the frontal cortex are sensitive or insensitive to hormone manipulations.

Compared to local dmPFC dendritic spines, long-range afferents and efferents of the PFC also display differential patterns of maturation during the adolescent time period. BLA axons that target the dmPFC undergo late maturation compared to OFC afferents that are more stable in adulthood (Johnson et al., 2016a). Additionally, corticostriatal axons display an inverted U-shaped developmental trajectory, with adolescent male mice displaying higher bouton density compared to juvenile and adult male mice.

These studies, as well as work from others, demonstrate that contrary to the popular framework that circuits prune during adolescence, some circuits undergo late growth. Inputs to associative cortices also show evidence for competition, similar to the activity-dependent battleground seen in sensory cortices. Future studies are needed to investigate how other intermingled frontal circuits mature and which if any subsets of circuits compete for synaptic space in the frontal cortex.

Many studies also indicate that dendritic spines and axonal boutons are sensitive to environmental manipulations, including both deprivation and enrichment. Some cortical regions display opposite effects in response to environmental changes, with some showing spine growth, while others show spine loss in response to similar stressors or sensory deprivation. Less focus has been on how adversity during development, including childhood and adolescence, affects synaptic maturation.

**Early life adversity induces changes in decision-making strategies at a juvenile period**

There are various theories for explaining how and why the body responds to stress. The allostatic load model has been used to describe how a certain level of stress can be adaptive (allostasis), while too much or chronic stress can lead to disease states (allostatic load) (McEwen, 2012). Indeed, multiple lines of evidence indicate that early life adversity leads to and/or contributes to negative outcomes, including mental health disorders such as anxiety and depression. While many have viewed adversity within this framework, describing outcomes as deficits, alternative theories have been used to describe why the body responds in particular ways. The adaptive calibration model (Del Giudice, Ellis and Shirtcliff, 2011) posits that stress tunes developmental processes to match an organism's behavior to the environment. A number of studies fit within this framework and have shown that early life adversity accelerates the maturation of fear learning systems in both rodents (Bath et al., 2016; Callaghan and Richardson, 2011; Moriceau) and humans (Gee et al., 2009). The impact of early life adversity on development may differ depending on dose. The most severe forms of early life adversity may simply derail 'normal' development in the direction of pathology. More mild forms may drive adaptive plasticity, such as earlier maturation, where development is not derailed but accelerated to allow an organism to grow-up 'fast' in a threating world (Hostinar and Gunnar, 2013; Gee et al., 2013).

In Chapter 3, I explored how early life maternal separation sculpts decision-making strategies across the lifespan. I found that early maternal separation reduces flexible decision-making at a juvenile stage; however I do not see lasting effects on flexibility into adulthood. Additionally, I found that maternal separation did not alter anxiety-like behavior when measured at P32-33, suggesting that anxiety is not driving the differences in flexible decision-making I observe. I hypothesized that the difference in decision-making strategies could reflect accelerated maturation. Previous studies in our lab have shown that adult mice are less flexible than juvenile mice on the same cognitive test (Johnson et al., 2011). When I compare our juvenile MS mice to adult control mice, they display similar performance levels (similar total trial to criterion), suggesting their decision-making strategies may be adult-like. However, from these

behavioral measures it is hard to rule out the possibility that stress-induced impairments have altered development and led to differences in flexibility via routes different from typical maturation.

Finally the transience of the behavioral effect after MS, may suggest it is of minor importance. However, the juvenile period is an important life stage for both rodents and humans, when individuals are first exploring outside of the nest or home. Differences in decision-making strategies at this critical point could have long term changes on the developmental trajectory of an animal. It is impossible to test in the lab, but I speculate based on our data that increased perseveration in newly independent mice dispersing into harsh conditions may prove beneficial to long term survival or reproduction. In Chapter 4, I turn to mechanism, and examine how circuits that support flexible decision-making are altered by maternal separation.

**Variations in maternal care lead to circuit specific changes in adolescent development**

In Chapter 4, I assessed how maternal separation and variations in maternal care affected the development of four long range axonal projections between regions implicated in learning and flexible decision-making. I found that offspring that experienced maternal separation have higher bouton density and smaller bouton volume on dmPFC-BLA axons. Importantly, I found that amount of time the dam spent over the nest and dam sorties (a measure of care fragmentation) was predictive of the later development of the dmPFC to BLA pathway, but showed no relationship with the other afferent and efferents I investigated. These data suggest that the dmPFC-BLA pathway is specifically sensitive to variations in maternal care. How these changes in bouton density and volume impact the function of this circuit is unclear. While many studies in rodents and humans commonly implicate the mPFC-BLA circuit as playing a key role in fear expression and emotion regulation, it is also important for appetitive associative learning. In Chapter 3, I describe how maternal separation impacts decision-making strategies in an associative learning task at the juvenile, but not adult life stage. Differences in bouton density and bouton volume on dmPFC-BLA axons at the adolescent, but not adult time point could explain these age-dependent differences in decision-making in an appetitive task. Further research will also be needed to test how these smaller more numerous boutons contribute to changes in function. Our descriptive data cannot tell us if these changes reflect pathological processes, adaptation to the adverse environment or an alternate form of resilience. A simple interpretation is that flexible decision-making, enhanced ethanol consumption and bouton density changes may simply illustrate pathological processes. In an alternate interpretation, these data may be in line with the early maturation hypothesis.

Future studies should also focus on how adversity experienced at other developmental windows and through different modalities affect these specific circuits. I speculate that stress experienced during adolescence may uniquely impact corticostriatal circuits, which show an inverted U shaped trajectory of maturation through adolescence to adulthood.

**Late growth may allow for fine tuning of circuits to adapt to an environment**

As described in Chapter 2, sub-circuits of the basolateral amygdala and prefrontal cortex undergo late maturation, continuing to grow during adolescence. Theoretical work on the evolution of plasticity suggests that circuits with extended or late growth may adapt to the environment (Nettle et al., 2013; Mabry and Stamps, 2008). I hypothesized that variations in early life maternal care would differentially impact late growing sub-circuits compared to earlier developing ones. I used maternal separation as a mouse model of early life adversity and found that this manipulation led to variations in maternal care. I also found that variations in maternal care showed a relationship with dmPFC-BLA bouton density at a late juvenile early adolescent time point. However, I did not see a relationship between our measures of maternal care and the other circuits I investigated (for example in OFC to dmPFC axons which show stable bouton density after P26). Taken together, this suggests that late developing circuits may evaluate the statistics of the early environment and then adjust their developmental trajectory accordingly. In this manner, fragmented maternal care may provide a signal about the adverse environment offspring are growing up in and upregulate dmPFC-BLA projections in response. Indeed, studies in children who experienced early life maternal care deprivation show evidence for accelerated maturation of mPFC and amygdala circuits (Gee et al., 2013), suggesting this pathway is particularly sensitive to maternal care variations. However, these changes may also reflect an adaptation that takes an alternate trajectory rather than simply accelerates development. In either pattern, these changes could enhance vulnerability and/or support some aspects of resilience. Further studies are necessary to clarify the functional significance of the structural changes I observe.

This thesis describes how prefrontal circuits develop at the synaptic level in mice in the typical laboratory rearing environment and in mice exposed to early life maternal separation and variations in maternal care. While rodent research is valuable in that it can provide a more causal relationship between early life factors and circuit development, I recognize the limitations of this research. Research in mice cannot encompass the complex social, emotional and psychological factors that may come into play in human children experiencing adversity and variations in caregiving. Additionally, I recognize that similar processes may not be in play in human children. However, our data provide important information about specific circuits that show similar developmental trajectories in humans and rodents that might be affected by early childhood adversity in a similar manner. Our high resolution data should inform future studies focused on children who have experienced adverse early life experiences.

Determining when specific circuits mature and are most sensitive to the environment, may yield insights about the most effective timing for interventions and treatments. Our data suggest the early life environment leads to changes in the circuits and behavior in the adolescent period and that early experience may have delayed effects. If similar processes are in place in humans, then there may be a second opportunity for intervention when circuits are changing and growing in humans who have experienced abuse or neglect. Interventions aimed at middle childhood may also be easier to implement as school-based programs, compared to interventions in infancy, targeted at the home environment. Together, this thesis provides data on the

development of circuits at the synaptic level. This data also points to specific circuits that may be specifically affected by early life adversity and aims to guide future studies that will aid in healthy child development.

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