Effects of early-life stress on the developing basolateral amygdala and associated long-term fear behaviors

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February 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Acknowledgements

There are many people whom I want to thank and acknowledge for helping me during the course of my PhD research. I would like to start by thanking my supervisor, Dr. Claire-Dominique Walker. I first joined your lab as an undergraduate student and was immediately drawn to your passion for science and the breadth of your knowledge. Thank you for taking a chance on me and welcoming me back into your lab with open arms. You have been an incredible joy to work with and I will deeply miss our interactions. I am forever grateful for your motivation, support and the enviable opportunities you have given me.

I want to thank our collaborator and member of my advisory committee, Dr. Tak Pan Wong. Thank you for the insightful ideas and instrumental feedback you provided during my studies and for generously guiding me through complex electrophysiological techniques. Your patience and calm instruction have always been appreciated. I am grateful for the support and time of my advisory committee member, Dr. Naguib Mechawar. Thank you for tracking my progress over the years and sharing invaluable input on my project. I would also like to acknowledge the help of our collaborators Drs. Pedro Rosa-Neto and Mallar Chakravarty. Special thanks to Dr. Joe Rochford for his valuable assistance with behavioral experiments, statistical insight and comedic relief.

I must acknowledge the organizations that financially supported me throughout my studies. I am thankful for the gracious studentships I received from the Integrated Program in Neuroscience (IPN), Dr. Alice Chan-Yip and the Faculty of Medicine, as well

as my external funding from NSERC. Thank you to Marie Giguère, the Douglas Institute, IPN and the Pan American Neuroendocrine Society for supporting my travel to enriching international conferences.

Many of the experiments presented in this thesis would not have been possible without the exceptional technical support of Hong Long and Silvanna Verlezza. You are both true lab magicians and I am equally thankful for your optimism and emotional support. I have been fortunate to work with many talented members of the Walker lab, past and present, including Mary, Alyssa, Jiamin, Lyla, Paula, Lisa… the list goes on. Thank you for celebrating the great moments with me, but also sympathizing when times were stressful. You have made this experience truly unforgettable.

I would like to thank my friends and family. I appreciate you listening to all my crazy rat stories and being incredibly understanding when I was sometimes too caught up in research to make time for you. I am immensely blessed to have so many of you that have cheered me on every step of the way.

Last, but certainly not least, I am forever in debt to my husband James, my parents, Al and Lilly, and my brother, Michael. Thank you for standing by me and always believing I could make it this far. Achieving this milestone would not have been possible, let alone imaginable, without your encouragement, unconditional love and support. I love you all and dedicate this thesis to you.

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Abstract

Exposure to early-life stress (ELS) associates with increased vulnerability to anxiety, depression and cognitive illness. The basolateral amygdala (BLA) is known to promote dysregulated fear responses and stress reactivity, particularly through its robust connections with the medial prefrontal cortex (mPFC). Although the enduring and sex-dependent effects of ELS on the BLA are well established, the proximal effects and cellular mechanisms operating during the neonatal period are still unclear. Using a rodent model of ELS, the limited bedding (LB) paradigm during the first 10 postnatal days, we investigated the morphological and functional consequences of ELS on the developing BLA. We demonstrated that LB male, but not female, preweaning rats exhibited BLA neuron hypertrophy, along with enhanced synaptic responsivity. LB decreased BLA-mPFC functional connectivity in preweaning and adult males, likely contributing to the exaggerated long-term fear responses and anxiety-related behaviors in adult males exclusively. Lateralized BLA connectivity was already present in preweaning pups and maintained until adulthood, with right hemisphere connectivity changes outnumbering those from the left. We further probed for asymmetrical effects and sex differences of ELS and revealed that right BLA synaptic plasticity and neuron excitability were exclusively enhanced in juvenile males, whereas females displayed reduced neuron excitability and NMDAR subunit expression. LB conditions also suppressed parvalbumin (PV) cell activity and increased perineuronal net density around PV cells only in the right BLA of juvenile males, possibly contributing to a reduced inhibitory tone in the right BLA after ELS. Our findings suggest novel sex and hemispheric-dependent mechanisms through which ELS disrupts the developing BLA to program lifelong impairments in emotional regulation.

Resumé

L'exposition au stress précoce (ELS) est associée à une vulnérabilité accrue à l'anxiété, la dépression et aux maladies cognitives. En particulier, l'amygdale basolatérale (BLA) est une structure du cerveau reconnue pour sa participation dans la genèse des réactions de peur et pour son implication dans les symptômes d'anxiété et de réponses dysfonctionnelles au stress. Cette structure possède de robustes connexions avec le cortex préfrontal médian (mPFC) et l'activation de ce circuit est responsable du comportement de « freezing » observé dans des situations de peur conditionnée. Bien que les effets à long terme du stress précoce sur la BLA soient relativement bien établis, les effets proximaux et les mécanismes cellulaires opérant pendant la période néonatale ne sont pas encore clairs. Ceci est important car le circuit BLA-mPFC responsable de la peur se développe en période postnatale et est donc plus vulnérable au stress environnemental durant cette période. En limitant l'accès à la litière (LB) pour construire un nid, cela crée un stress chez la mère et chez les petits ratons, un paradigme d'ELS que nous imposons entre les jours 1 et 10 de vie postnatale. Nous avons étudié les conséquences morphologiques et fonctionnelles d'ELS sur la BLA au cours du développement. Nous avons démontré que les ratons LB mâles, mais pas femelles, présentaient une hypertrophie des neurones BLA, ainsi qu'une réactivité synaptique accrue comparée au rats contrôles (NB). Dans des conditions de ELS, la connectivité fonctionnelle entre BLA et mPFC est diminuée chez les mâles avant le sevrage et le reste jusqu'à l'âge adulte. Ceci contribue probablement aux réponses de peur exagérées et aux comportements liés à l'anxiété que nous avons observées chez les rats mâles adultes. Une latéralisation dans la connectivité BLA-mPFC était déjà présente chez les

ratons avant le sevrage et maintenue jusqu'à l'âge adulte, favorisant les changements dans l'hémisphère droit par rapport à ceux de l'hémisphère gauche. Finalement, nous avons examiné les effets du sexe et de l'asymétrie hémisphérique sur les changements de plasticité synaptique induite par ELS chez les jeunes rats. Cette étude a révélé que la plasticité synaptique et l'excitabilité des neurones BLA était augmentée par ELS seulement chez les jeunes mâles et dans l'hémisphère droit. Un tel effet n'était pas observé chez les femelles suite au ELS, par contre nous avons observé une réduction de l'excitabilité neuronale de la BLA ainsi qu'une diminution de l'expression des sous-unités formant le récepteur NMDA dans cette structure et chez les femelles exclusivement. Les conditions de ELS (LB) ont également supprimé l'activité des interneurones inhibiteurs dans la BLA, en particulier ceux contenant de la parvalbumine (PV). Ces PV neurones sont stabilisés par l'ajout de « filets » périneuronaux (PNNs) qui se trouvent être augmentes par l'exposition à l'ELS chez les mâles seulement et ceci, uniquement dans la BLA droite. Il est possible que ce phénomène contribue à diminuer le tonus inhibiteur au sein de la BLA droite après l'ELS et ainsi permette une excitabilité accrue de la BLA chez les jeunes mâles. Nos résultats mettent de l'avant de nouveaux mécanismes, différemment modulés selon le sexe, par lesquels le stress au cours du développement néonatal perturbe le développement de la BLA ainsi que celui du circuit BLA-mPFC. Nous avons démontré que cela a pour conséquence de programmer à long terme d'importantes dysfonctions dans la régulation émotionnelle chez les mâles et que les femelles semblent être protégées de certains des effets du stress néonatal.

Contributions to Original Knowledge

Chapter II: In the published manuscript (Guadagno et al., 2018a) presented in this chapter, we are the first to show that LB exposure during the first 10 postnatal days induces sexually dimorphic effects on BLA morphology in neonatal and preweaning rats. We also demonstrate for the first time that increased BLA neuron spine numbers and dendritic hypertrophy in LB male pups occurs in parallel with heightened evoked synaptic responses. Lastly, we provide evidence that early morphological and functional alterations in the developing BLA associate with male-specific enhancements in fear and anxiety-related behaviors in adulthood.

Chapter III: In the published manuscript (Guadagno et al., 2018b) we show, for the first time, that LB exposure significantly disrupts resting-state functional MRI connectivity of the BLA with the mPFC, as well as with other regions, in PND18 male rats. We are also the first to demonstrate that many of these alterations are lateralized to the right BLA and persist until adulthood with almost the exact same connectivity distribution. This is also the first manuscript to show that LB alters the connectivity of the BLA with the contralateral, in addition to the ipsilateral mPFC in PND18 pups. The experiments presented in this manuscript are the first to link reduced BLA-mPFC connectivity in preweaning and adult male rats with increased long-term fear behaviors.

Chapter IV: The manuscript presented in this chapter is not published, however we anticipate that it will be submitted for publication by the end of March 2020. This manuscript will be the first to examine sex and hemispheric-dependent effects of LB on

synaptic plasticity, neuron excitability and NMDAR expression in the juvenile BLA. We will also be the first to show that enhanced synaptic plasticity and neuron excitability in the right BLA of LB juvenile males coincides with increased PNN expression, notably around PV cells, and decreased activity of PV interneurons in the right, but not the left BLA.

Contributions of Authors

Chapter I: Angela Guadagno wrote the comprehensive review of the literature under the supervision of Dr. Claire-Dominique Walker.

Chapter II: Angela Guadagno and Dr. Claire-Dominique Walker conceived the experiments. Angela Guadagno, Dr. Claire-Dominique Walker and Dr. Tak Pan Wong designed the experiments. Angela Guadagno and Dr. Claire-Dominique Walker performed the experiments. Angela Guadagno analyzed the data and wrote the manuscript. Dr. Claire-Dominique Walker and Dr. Tak Pan Wong edited the manuscript.

Chapter III: Angela Guadagno and Dr. Claire-Dominique Walker conceived and designed the experiments. Angela Guadagno, Dr. Claire-Dominique Walker and Dr. Axel Mathieu performed the experiments. Angela Guadagno, Min Su Kang and Dr. Gabriel Devenyi processed the data. Angela Guadagno analyzed the data and wrote the manuscript. Dr. Mallar Chakravarty directed the volumetric analyses. All authors edited the manuscript.

Chapter IV: Angela Guadagno, Dr. Claire-Dominique Walker and Dr. Tak Pan Wong designed the experiments. Angela Guadagno, Dr. Claire-Dominique Walker, Silvanna Verlezza and Hong Long performed the experiments. Angela Guadagno wrote the manuscript. Dr. Claire-Dominique Walker edited the manuscript.

Chapter V: Angela Guadagno wrote the general discussion under the supervision of Dr. Claire-Dominique Walker.

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List of Abbreviations

AB: Accessory basal nucleus of the amygdala

ACTH: Adrenocorticotropic hormone

ANOVA: Analysis of variance

BLA: Basolateral nucleus of the amygdala

BNST: Bed nucleus of the stria terminalis

CCK: Cholecystokinin

CeA: Central nucleus of the amygdala

CIS: Chronic immobilization stress

CORT: Corticosterone

CRF: Corticotropin-releasing factor

CRH: Corticotropin-releasing hormone

CS: Conditioned stimulus

ELS: Early-life stress

EPM: Elevated plus maze

EPSP: Excitatory postsynaptic potential

GD: Gestation day

GR: Glucocorticoid receptor

HPA: Hypothalamic-pituitary adrenal

Iba1: Ionized calcium binding adaptor molecule 1

ITC: Intercalated cell masses

ITI: Inter-trial interval

LA: Lateral nucleus of the amygdala

LB: Limited bedding

LC: Locus coeruleus

LTD: Long-term depression

LTP: Long-term potentiation

MeA: Medial nucleus of the amygdala

mEPSCs: Miniature excitatory postsynaptic currents

mPFC: Medial prefrontal cortex

- PL mPFC: Prelimbic medial prefrontal cortex
- IL mPFC: Infralimbic medial prefrontal cortex

MR : Mineralocorticoid receptor

NB: Normal bedding

NMDAR: N-methyl-d-aspartate receptor

NTS: Nucleus of the solitary tract

PFC: Prefrontal cortex

PND: Postnatal day

PNN: Perineuronal net

PTSD: Post-traumatic stress disorder

PV: Parvalbumin

PVN: Paraventricular nucleus

rs-fMRI: Resting-state functional magnetic resonance imaging

S-aCSF: Sucrose-substituted artificial cerebrospinal fluid

SEM: Standard error of the mean

US: Unconditioned stimulus

vHIPP: Ventral hippocampus

Introduction

The developing brain during fetal and early postnatal life is particularly plastic and undergoes a series of maturational processes. During these critical periods of development, genetic and environmental factors act to reorganize the connectivity of neural networks (Stiles and Jernigan, 2010; Kolb and Gibb, 2011). The heightened neuronal plasticity of the immature brain is usually advantageous and adaptive, allowing an individual to cope with and adapt to unfavorable conditions. However, this system may also become maladaptive in face of early life adversity, according to genetic differences between individuals, and through epigenetic changes of specific genes (McEwen, 2006; Buss et al., 2012; Franklin et al., 2012; Dunn et al., 2019). Numerous human epidemiological and observational studies have strongly linked adverse early life experiences, such as emotional, sexual and physical abuse, as well as emotional and physical neglect with consequences on cognitive and emotional health (Bremne and Vermetten, 2001; Pechtel and Pizzagalli, 2011; Gould et al., 2012). These forms of early adversity often involve the primary caregiver and may contribute to chronic early-life stress (ELS) in children, increasing their vulnerability to develop future psychiatric disorders, including anxiety, depression, and substance abuse (Weber et al., 2008; Green et al., 2010; Carr et al., 2013). Furthermore, the effects of ELS are often sex-specific, depending on the type, timing and duration of stress exposure (Fox et al., 2010; Gee and Casey, 2015; White and Kaffman, 2019). It is estimated that 45% of childhood-onset mental health disorders and over 30% of later-onset disorders are associated with early life adversity (Green et al., 2010). The severity of the problem is underscored by the fact

that adverse childhood experiences in many different forms impact 29% of the world population (VanTieghem and Tottenham, 2017) and display marked sexual dimorphism. For instance, girls are more likely to experience chronic sexual abuse, whereas boys are more likely to be exposed to physical violence and neglect (Maikovich-Fong and Jaffee, 2010; Gauthier-Duchesne et al., 2017; Coelho et al., 2018).

As a result, in order to better treat and understand stress-induced mental health disorders, it has been suggested that research should attempt to investigate how early life perturbations begin to shape later physiological and behavioral responses in a sex-dependent manner. Therefore, studies which examine the effects of early stress exposure on the immature, developing brain will not only be essential in identifying the potentially modified neuronal circuits that contribute to future psychopathologies, but will also assist in the design of specific interventions and therapeutic strategies (Callaghan et al., 2014; Walker et al., 2017).

The amygdala processes emotions and fear and could promote dysregulation of stress reactivity and anxiety-related disorders, particularly through its widespread connections with other limbic structures, such as the medial prefrontal cortex and ventral hippocampus (vHIPP) (Ressler, 2010; Shin and Liberzon, 2010). Projections from the amygdala and limbic or emotion regulation circuitry converge onto the hypothalamic paraventricular nucleus (PVN) to modulate activity of the hypothalamic-pituitary-adrenal (HPA) axis during periods of stress exposure (Jankord and Herman, 2008). The high density of glucocorticoid receptors present in the amygdala likely increases its sensitivity to the effects of chronic ELS (Geuze et al., 2012). In humans, ELS exposure enhances amygdala reactivity during the presentation of negative emotional stimuli and weakens

amygdala-prefrontal cortex (PFC) resting-state functional MRI (rs-fMRI) connectivity (Tottenham et al., 2011; Burghy et al., 2012; Pagliaccio et al., 2015). Importantly, dysfunction in the amygdala-prefrontal circuitry predicts behavioral outcomes and symptoms of depressive and anxiety disorders (Burghy et al., 2012; Gee et al., 2013a; Yan et al., 2017). While many studies across species have documented long-term structural and functional effects of ELS on the amygdala, very few have investigated sex-specific proximal effects and tentative mechanisms operating during the neonatal and juvenile periods. These knowledge gaps are addressed in my thesis using a naturalistic rodent model of ELS commonly known as the limited bedding (LB) paradigm (Walker et al., 2017).

Chapter I: Comprehensive review of the literature

1.1 The hypothalamus-pituitary-adrenal (HPA) axis

Homeostasis represents the delicate balance of physiological systems to maintain a constant optimal state of functioning for the individual (McEwen and Wingfield, 2010). It is essential for survival and can be disturbed by real or perceived threats, broadly defined as 'stressors' (Schneiderman et al., 2005). These challenges trigger alarm responses such as activation of the neuroendocrine HPA axis as well as the sympathetic nervous system (Szabo et al., 2012; Kumar et al., 2013). The HPA axis is a major adaptive system responsible for the initiation of neuroendocrine stress responses in all vertebrates (Herman et al., 2012). Physical and psychological stressors activate the HPA axis through brainstem areas, as well as cortical, limbic and hypothalamic inputs terminating on corticotropin-releasing hormone (CRH)-containing neurons in the hypothalamic paraventricular nucleus (PVN) (Walker et al., 2001; Jankord and Herman, 2008; Flak et al., 2009). Secretion of CRH into the hypophyseal portal circulation stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) and ultimately, the release of adrenal glucocorticoids (Walker et al., 2001). Glucocorticoid hormones (cortisol in humans, corticosterone (CORT) in rodents) are the end products of this neuroendocrine cascade and provide a negative inhibitory feedback on the HPA axis, allowing for the return of the neuroendocrine response to baseline levels. Glucocorticoids also play a major role in metabolic processes regulating homeostasis (Herman et al., 2012).

Glucocorticoids act at various sites in the brain and pituitary via glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) to regulate the activity of the HPA axis through negative inhibitory feedback (Tasker and Herman, 2011). MRs have high affinity for endogenous glucocorticoids and are bound even when glucocorticoid levels

are low. In contrast, GRs have lower affinity and are highly occupied at high circulating levels of glucocorticoids, as encountered during stress responses (Herman et al., 2012). There are two primary mechanisms of negative feedback inhibition, including non-genomic 'fast' feedback at the level of the PVN and 'delayed' feedback mediated mostly through GRs in limbic regions (amygdala, PFC, ventral hippocampus) (Tasker and Herman, 2011; Herman et al., 2012).

1.1.1 Maturation of the neuroendocrine stress response

The neuroendocrine response to stress in neonatal life is often much different than that observed in adulthood (Sapolsky and Meaney, 1986; Walker et al., 1986a; Dallman, 2000). Developmental changes in functioning of the HPA axis may therefore impact vulnerability to ELS. The stress hypo-responsive period (SHRP) represents a period of blunted pituitary-adrenal stress responsiveness in postnatal day (PND) 4-14 rats that is critically maintained by mother-infant interactions (Walker et al., 2004; Schmidt, 2019). Equivalent periods of suppressed stress responsiveness during infancy have also been identified in humans (Gunnar and Quevedo, 2007). The diminished pituitary-adrenal hormonal output during the rat SHRP partially results from enhanced adrenocortical sensitivity to CORT negative feedback and does not appear to be a consequence of low hypothalamic secretion of CRH (Walker et al., 1986b). While the SHRP was initially designated as the "stress nonresponsive period", it was later realized that neonates are capable of displaying robust CRH activity, ACTH and CORT responses, particularly in response to maternal deprivation (Levine et al., 1991; Suchecki et al., 1993; Dent et al., 2000). Thus, the SHRP is not universal and neonatal adrenocortical responsiveness likely

depends on the type of stressor encountered (Dent et al., 2000). Evidence suggests that the SHRP serves as a mechanism to protect the developing brain from damaging effects of excess glucocorticoid secretion (Sapolsky and Meaney, 1986). Indeed, high glucocorticoid levels negatively affect neuron structure and growth in multiple brain regions, especially the amygdala (McEwen et al., 2016). As development progresses, adrenocortical responses to stress steadily increase in parallel with GR expression in the amygdala (Sapolsky and Meaney, 1986; Walker et al., 1986a). Prior to adolescence around PND30, rats exhibit a window of stress hyper-reactivity, as evidenced by prolonged stress-induced ACTH and CORT release (Romeo, 2013). This enhanced stress reactivity is reported in humans as well, but at a later developmental time point in adolescence (15-17 years) (Romeo, 2013).

1.1.2 Effects of early-life stress on the HPA axis in humans and rats

While glucocorticoid secretion is beneficial in the short term for survival, temporal prolongation of exposure to glucocorticoids, encountered during some instances of chronic ELS, can cause long-term alterations in the functioning of the HPA axis and induce metabolic, immune and psychological dysfunctions (Jankord and Herman, 2008; Wilkinson and Goodyer, 2011; Flak et al., 2012). In humans, the effects of adverse childhood experiences on the reactivity of the HPA axis are mixed. For instance, adult women with a history of chronic childhood abuse (minimum of one incident per month lasting at least one year) exhibit elevated ACTH levels in response to a psychosocial stress (i.e., the Trier Social Stress Test; TSST) (Heim et al., 2000). Conversely, a recent meta-analysis found that early adversity was associated with reduced cortisol responses

to social stress, and effect sizes were greater in adults compared to children (Bunea et al., 2017). Blunted cortisol responses to the TSST are found in both childhood maltreated men and women (Carpenter et al., 2007; Carpenter et al., 2011) and are reminiscent of the situation of post-traumatic stress disorders, characterized by blunted glucocorticoid secretion (Yehuda, 2009). For resting stress hormone levels, a recent prospective longitudinal study documented that female children exposed to maternal stress during the first year of life display increased baseline cortisol levels (Burghy et al., 2012). However, retrospective studies of adults reporting exposure to early chronic stress show the opposite effect on basal cortisol levels (Trickett et al., 2010). It is postulated that hyper-activation of the HPA stress system precedes later hypo-activity (Trickett et al., 2010; Bunea et al., 2017). Therefore, the type and severity of adversity as well as the time elapsed after stress exposure might be critical factors in understanding HPA axis alterations after ELS.

In line with results reported in humans, ELS exerts significant effects on the rodent HPA axis, although the direction of these perturbations is inconsistent (Walker et al., 2017) and depends in part, on the model of early adversity that is employed. For reasons that will be described in Section 1.5, the limited bedding (LB) and nesting procedure is among the most widely used naturalistic models of ELS in rodents. The major proximal and distal outcomes elicited by this paradigm were the topics of a recent review by Walker et al (Walker et al., 2017). A study conducted in our laboratory observed blunted secretion of ACTH and CORT in PND10 rats following immobilization stress, while basal CORT levels remained unchanged, suggesting that under specific conditions, adversity might suppress HPA axis activation to conserve energy resources (McLaughlin et al., 2016)

rather than cause an increased HPA activation. Other groups have reported increases or decreases in basal plasma CORT in neonatal rats (Walker et al., 2017). At weaning age (PND21), LB rat pups display elevated basal CORT levels, with more pronounced hypercorticosteronemia in females relative to males (Moussaoui et al., 2017). However, these effects do not appear to persist into adolescence (Molet et al., 2016) or adulthood (Brunson et al., 2005; Ivy et al., 2008).

Unfortunately, there is limited understanding of the specific mechanisms by which ELS contributes to HPA axis alterations (Essex et al., 2011; Flak et al., 2012). Furthermore, HPA axis output may not always give a complete picture of underlying stress-induced consequences. Indeed, LB exposure significantly reduces volume and impairs dendrite structure in the dorsal hippocampus of late adolescent rats without significantly affecting HPA activity (Molet et al., 2016). As such, research has examined various brain structures that provide direct or indirect inputs to regulate HPA axis activity during stress exposure and the amygdala inputs might be of particular importance for modulating hypothalamic PVN neurons during emotional stressors (Beaulieu et al., 1987; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009).

Under situations of chronic stress in the adult rodent, a number of brain structures otherwise quite silent, become activated and they now modify HPA axis regulation to respond to continuous demands on homeostasis. Such structures include the bed nucleus of the stria terminalis (BNST), paraventricular thalamus and amygdala (Bhatnagar et al., 2000; Herman et al., 2016). However, it is currently unknown whether similar recruitment occurs in developing rats and if this mediates some of the long-term effects of ELS on regulation of the HPA axis and emotional responses. Work in this thesis

will attempt to clarify this issue by focusing on changes occurring in the amygdala following chronic ELS.

The effects of ELS on the developing amygdala are discussed in the remaining chapters and manuscripts of this thesis. To place these findings into context and understand how the amygdala is well positioned to modulate HPA activity and emotional behaviors, we will first review its anatomy, function, connectivity and development under normative conditions.

1.2 The amygdala

Significant progress has been made over the last 40 years to improve our understanding of the amygdala, an almond shaped structure located in the human medial temporal lobe. This region is composed of 13 nuclei across species that are classified into three main groups according to their distinct histochemistry, cytoarchitectonics, and structural connectivity (Sah et al., 2003). The first group is the basolateral complex, which consists of the lateral amygdala (LA), the basolateral amygdala (BLA) and the accessory basal (AB) or basomedial amygdala. The second superficial group contains cortical nuclei and the nucleus of the lateral olfactory tract. Finally, the third centromedial group includes the medial (MeA) and central amygdala (CeA) nuclei (Sah et al., 2003). Other accessory regions that do not belong to these three amygdala nuclear groups have also been identified, such as the intercalated cell masses and the amygdalohippocampal area (Sah et al., 2003). Cortical and thalamic inputs primarily enter the basolateral complex of the amygdala (Sah et al., 2003). The CeA represents the main output nucleus of the amygdala (Duvarci et al., 2011) although the BLA also directly projects to several brain

areas such as the medial prefrontal cortex (mPFC) (Bouwmeester et al., 2002a) and the ventral hippocampus (vHIPP) (Felix-Ortiz and Tye, 2014).

The amygdala is as heterogeneous in its function as it is anatomically; it plays a complex role in emotional and social behavior (LeDoux, 2000; Adolphs, 2010), stress responses (Roozendaal et al., 2009), learning and memory (McGaugh, 2004; Roozendaal et al., 2009), anxiety (Rauch et al., 2003), and conditioning to appetitive or aversive stimuli (Shabel and Janak, 2009). The crucial involvement of the amygdala in the fear circuitry is arguably the most well studied. In particular, the BLA nucleus and its efferent output projection, the CeA, are thought to be the main nuclei mediating fear learning and anxiety (Campeau and Davis, 1995; Fanselow and LeDoux, 1999; Maren, 1999b; Quirk and Mueller, 2008; Lee et al., 2015). As such, they also participate in modulating stress responses during exposure to fearful stimuli (Jankord and Herman, 2008). Our work will focus primarily on the BLA, however some aspects of CeA anatomy and function will also be reviewed.

1.2.1 Anatomy of the rodent basolateral and central amygdala

The BLA is part of the basolateral complex and sits ventral to the lateral amygdala (LA) (Sah et al., 2003). There are three classes of neurons in the BLA with contrasting cell body, dendrite and axon morphologies and approximately 80-85% of the neurons in the BLA are glutamatergic cells with spiny dendrites (McDonald, 1982). Class I neurons are the most common cell type in the BLA and have large cell bodies, as well as dendrites that are densely covered with spines (McDonald, 1982). The morphology of Class I neurons may be pyramidal, semi-pyramidal, or stellate (McDonald, 1982). Pyramidal and

semi-pyramidal neurons have one or two thick apical dendrites and thinner basal dendrites, whereas stellate shaped cells have dendrites of roughly equal thickness that extend radially in all directions from the cell body (McDonald, 1982). Class II and III neurons represent a very small minority of cells in the BLA with smaller, spherical or irregular shaped cell bodies. Their dendrites may lack spines, or have very small spines on a few dendrites (McDonald, 1982). Principal BLA neurons densely innervate the dendritic spines of surrounding pyramidal cells, as well as the peri-somatic and distal dendrites of spine-sparse GABAergic interneurons, many of which express the calcium binding protein parvalbumin (PV) (Muller et al., 2006).

Reciprocally, most of the glutamatergic pyramidal cells within the BLA are the post-synaptic targets of inhibitory interneurons, which can significantly influence their activity (Spampanato et al., 2011). The interneurons in the BLA comprise 20% of the neuron population and are characterized by their expression of calcium binding proteins and/or neuropeptides, such as PV and somatostatin, respectively (Muller et al., 2006; Spampanato et al., 2011). Around half of the interneurons in the BLA are PV-positive and exclusively colocalize with calbindin. The remaining four populations of interneurons express calbindin with somatostatin or cholecystokinin (CCK), and calretinin with vasoactive intestinal peptide and/or CCK (Spampanato et al., 2011).

Interneurons of the BLA with distinct morphological and neurochemical properties display post-synaptic target specificity (Woodruff and Sah, 2007). For instance, PV-positive cells with basket-type morphology synapse on the peri-somatic region of pyramidal cells, including the soma, axon initial segment, and proximal dendrites, whereas somatostatin-positive cells innervate the distal dendrites that have small

diameters (Spampanato et al., 2011). Parvalbumin interneurons with 'chandelier' morphology form another class of axo-axonic synapses with pyramidal cells, which innervate the largest portions of the axon initial segment (Gabbott et al., 2006). In addition, PV-positive cells form synapses with interneurons from their own or other groups (Spampanato et al., 2011).

The information that is shaped by the interplay of excitatory and inhibitory inputs on principal BLA neurons is then relayed to the CeA via excitatory projections (Muller et al., 2006; Jankord and Herman, 2008). The lateral border of the CeA is adjacent to the basolateral complex and both the CeA and BLA are heavily interconnected (Sah et al., 2003). Unlike the BLA, the CeA predominantly consists of GABAergic projection neurons, as well as inhibitory interneurons (Jankord and Herman, 2008; Babaev et al., 2018). Neurons of the CeA project widely to the BNST, nucleus of the solitary tract (NTS) and locus coeruleus (LC), as well as other critical regions that modulate both HPA activity and prefrontal activity (Herman et al., 2003; Hoover and Vertes, 2007; Jankord and Herman, 2008; Samuels and Szabadi, 2008).

1.2.2 Physiology of the rat basolateral amygdala

Excitatory (i.e., pyramidal) and inhibitory BLA neurons may also be distinguished based on their electrophysiological properties. *In vitro* whole-cell patch clamp recordings performed in the BLA of adult rats show that pyramidal cells have resting membrane potentials between -70 to -75 mV (Washburn and Moises, 1992; Duvarci and Pare, 2007) and typically display little to no spontaneous firing activity (Pare and Gaudreau, 1996). When 450 ms depolarizing current pulses (0.5 nA) are injected, these cells fire an initial

burst of multiple action potentials. Delivery of shorter 150 ms current pulses (1 nA) evokes a similar firing pattern and reveals broad afterhyperpolarization (Washburn and Moises, 1992). Longer (600 ms) and large depolarizing current steps (50 pA) evoke significantly more action potentials in pyramidal neurons, with varying degrees of spike-frequency adaption depending on the total current injected (Rau et al., 2015).

Interneuron firing properties are incredibly diverse and quite different from those of pyramidal cells in the BLA in that they do not show spike-frequency accommodation (Rainnie et al., 2006). Parvalbumin cells in adult rats have more positive resting membrane potentials (-60 mV) than pyramidal cells and fire short action potentials *in vitro* (0.6 – 0.7 ms half-width) following 750 ms current injections (25 pA increments) (Rainnie et al., 2006). The firing patterns of these PV-positive neurons are burst-firing or stuttering, but other interneuron subtypes display regular and fast-firing patterns (Rainnie et al., 2006). Thus, glutamatergic and inhibitory neurons of the BLA can be easily distinguished in electrophysiological recordings based on their firing patterns.

1.2.3 Major afferent and efferent amygdala connections in the adult rat

Anatomical tracing studies have been key in unveiling the numerous projections to and from the amygdala (Sah et al., 2003). The amygdala receives most of its sensory information from the cerebral cortex (McDonald, 1998; Sah et al., 2003). The BLA in particular, receives afferent input from the thalamus, layers II-VI of the mPFC, the CA1 hippocampal formation, vHIPP, as well as brainstem areas, such as the LC and NTS (Sah et al., 2003; Gabbott et al., 2005; Garcia-Medina and Miranda, 2013; Godsil et al., 2013). The structural connections between the BLA, vHIPP (CA1 region and ventral subiculum)

and mPFC form an interconnected corticolimbic network (See Section 1.2.3.1) (Godsil et al., 2013). Within the amygdala, the BLA receives mainly excitatory projections from the LA and few inputs from the CeA (Pitkanen et al., 1995; Jolkkonen and Pitkanen, 1998; Sah et al., 2003). External inputs from thalamic and cortical areas that supply multimodal sensory information, as well efferents from hypothalamic and brainstem structures that regulate autonomic and behavioral functions, converge onto the LA, which processes and relays the incoming information to the BLA (Sah et al., 2003). The LA may be stimulated in electrophysiology experiments to evoke synaptic responses in the BLA (Thompson et al., 2008). The functional roles of intra-amygdalar microcircuits and the corticolimbic circuitry have been described in the context of fear and anxiety processing and will be reviewed in Section 1.3.

The CeA similarly receives input from the cortex and thalamus, brainstem and subiculum, in addition to projections from the hypothalamus and all other amygdala nuclei (Sah et al., 2003). As eluded to earlier, the CeA constitutes the main output nucleus of the amygdala because it conveys most of the outputs from the BLA and other amygdala nuclei to the hypothalamic PVN via the BNST (Fig. 1) (Jankord and Herman, 2008; Gilpin, 2012; Radley, 2012). The MeA sends direct projections that target a different region of the BNST (Jankord and Herman, 2008). The inhibitory projection neurons of the CeA target the GABAergic regions in the BNST, thus leading to the disinhibition of the BNST-to-PVN pathway and net activation of PVN neurons (Jankord and Herman, 2008). The CeA also projects to the NTS and LC in the brainstem in order to regulate stress responses, as well as other processes, such as arousal (Jankord and Herman, 2008; Samuels and Szabadi, 2008). The LC is a major noradrenergic nucleus that receives CRF

projections from the CeA (Van Bockstaele et al., 1998) before targeting hypothalamic (e.g., PVN), limbic (e.g., amygdala) and cortical areas (e.g., PFC) (Samuels and Szabadi, 2008; Moret and Briley, 2011). Interestingly, there is considerable evidence that dysfunction in the activity of the LC and amygdala is observed in depression (Yang et al., 2010; Moret and Briley, 2011). The major efferent projections from the amygdala to the PVN, PFC and vHIPP are illustrated in Figure I-1.

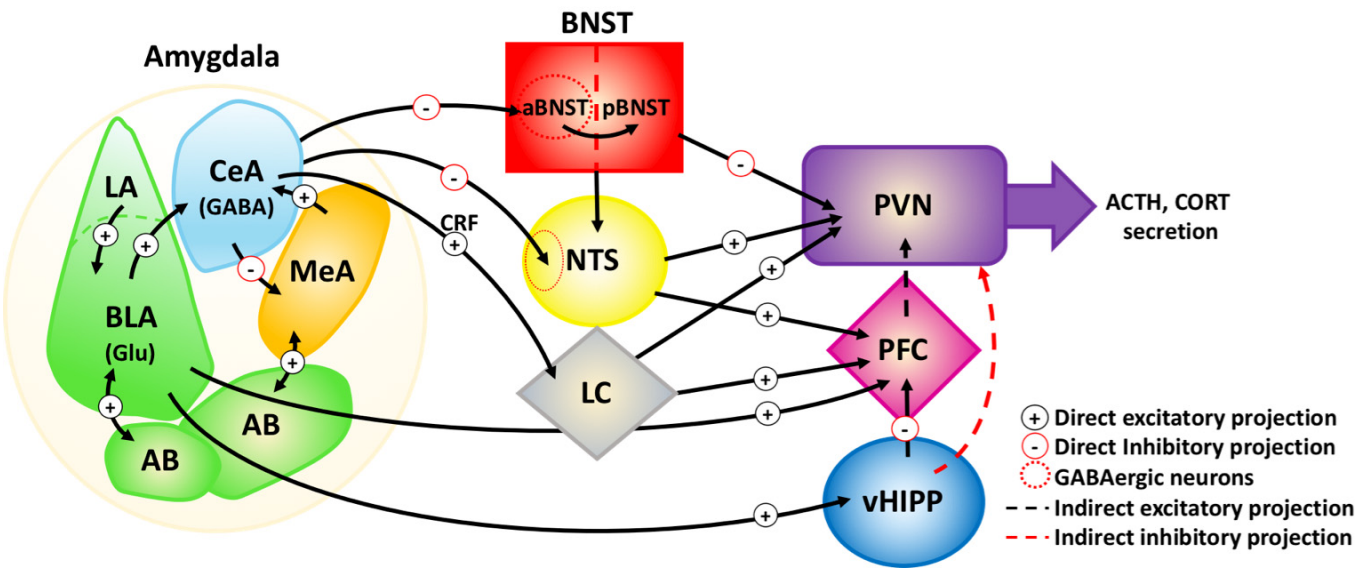


Figure I-1. Schematic illustration of many of the efferent projections from the amygdala to the PVN, PFC and vHIPP. The BLA, which consists of predominantly glutamatergic (Glu) neurons, indirectly activates PVN neurons to stimulate glucocorticoid release (Muller et al., 2006; Jankord and Herman, 2008). The CeA sends inhibitory inputs onto GABAergic neurons in the anterior BNST (Dong et al., 2001) and NTS (Herman et al., 2003), thus leading to the disinhibition of the BNST-to-PVN pathway and net activation of PVN neurons (Jankord and Herman, 2008; Radley, 2012). CRF projections from the CeA stimulate LC activity (Van Bockstaele et al., 1998). Figure adapted from Herman et al. 2003 (Herman et al., 2003) and Sah et al. 2003 (Sah et al., 2003). Abbreviations: aBNST: Anterior BNST, AB: Accessory basal or basomedial nuclei, BLA: Basolateral amygdala, BNST: Bed nucleus of the stria terminalis, CeA: Central amygdala, CRF: Corticotropin-releasing factor, Glu: Glutamate, LA: Lateral amygdala, LC: Locus coeruleus, MeA: Medial amygdala, NTS: Nucleus of the solitary tract, pBNST: Posterior BNST, PFC: Prefrontal cortex, PVN: Paraventricular nucleus, vHIPP: Ventral hippocampus

1.2.3.1 The corticolimbic circuitry

The BLA, ventral hippocampus (CA1/subiculum), and the mPFC are anatomically connected in what is broadly termed a corticolimbic network (Godsil et al., 2013). These projections allow for tightly controlled emotional and memory regulation (Godsil et al., 2013). When evaluating projections from the BLA, it is necessary to consider the anterior and posterior regions separately, as they have different patterns of projections, specifically to the mPFC (Hoover and Vertes, 2007; Giustino and Maren, 2015; Beyeler et al., 2018). The anterior BLA (aBLA) mainly innervates the prelimbic (PL) mPFC, whereas the posterior BLA (pBLA) projects to both PL and infralimbic (IL) areas (Giustino and Maren, 2015). The majority of BLA afferents to the mPFC are excitatory, found ipsilaterally and contact dendritic spines of neurons in layers II-VI (Krettek and Price, 1977; McDonald, 1991; Bacon et al., 1996; Hoover and Vertes, 2007; Dilgen et al., 2013). Interestingly, a small percentage (~5%) of the BLA axons monosynaptically target PV-positive cells in the mPFC (Gabbott et al., 2006). Projections from both the PL and IL originate in layers II-VI and terminate across the rostral-caudal BLA (Gabbott et al., 2005). However, one study noted that the density of projections from the IL to the BLA is comparably weak to labelling from the PL (Vertes, 2004). The aBLA and pBLA are reciprocally connected with the CA1 region and ventral subiculum of the hippocampus (Pitkanen et al., 2000; Godsil et al., 2013; Huff et al., 2016). The vHIPP efferents to the PL and IL are unidirectional, since they do not appear to receive reciprocal mPFC projections (Gabbott et al., 2005; Hoover and Vertes, 2007).

1.2.4 Development of the amygdala in humans and rats

Studies across species have revealed that amygdala development is protracted, and many changes in morphology and functioning of the BLA occur postnatally in both humans and rodents. Thus, environmental insults, such as ELS, during critical periods of amygdala neurodevelopment could lead to deviations in the normal maturational trajectory that subsequently affect the integrity of the amygdala. The human amygdala, including the basolateral complex, emerges early in embryonic life (Muller and O'Rahilly, 2006) and is well established at birth (Avino et al., 2018). A structural MRI study reported that the amygdala undergoes a 40% increase in volume from the ages of 8 to 18 years in neurotypical individuals (Schumann et al., 2004). Amygdala volume appears to peak between the ages of 9-11 years (Uematsu et al., 2012). The dramatic increase in amygdala volume from youth into early adulthood could be due to several factors, including synaptogenesis, dendritic growth and the maturation of immature neurons (Avino et al., 2018). A recent immunohistochemistry study by Avino et al. estimated the total number of mature (Nissl-stained) and immature (B cell lymphoma 2-positive) neurons in the amygdala of individuals aged 2 to 48 years. Between these ages, they found a linear 11% increase in the number of mature amygdala neurons, concomitant with a decline in the number of immature neurons in the ventral aspects of the amygdala (Avino et al., 2018). Remarkably, the increase in the number of mature neurons in the entire amygdala is primarily driven by increases in the number of BLA neurons (30%), and less so by neuron increases in other amygdala nuclei such as the LA, which only exhibit a 3% increase in mature neuronal counts (Avino et al., 2018).

As in humans, the rat BLA forms in embryonic development (Berdel et al., 1997) and most neurons are generated between E14 and E17 (Bayer, 1980). The total volume of the BLA increases until the third postnatal week (Rubinow and Juraska, 2009; Chareyron et al., 2011). After PND35 and until adulthood, total BLA volume remains stable (Rubinow and Juraska, 2009). The density of neurons in the BLA decreases rapidly from E20 to PND14 (Berdel et al., 1997), which might be in part due to the enhancement in its total volume during this period. Total neuron and glia numbers are unchanged from PND20 to PND35, but decrease by 13% between PND35-90 (Rubinow and Juraska, 2009). A recent study performed an extensive analysis of the morphological properties of principal BLA neurons at PND7, 21, 28 and 60 in rats and discovered that these cells undergo dramatic structural changes, particularly during the first postnatal month (Ryan et al., 2016). For instance, neuron soma size nearly doubles between PND7-28 and total dendritic length increases by three-fold until PND21 (Ryan et al., 2016). Dendritic arborization and spine density only reach maturity by PND28 (Ryan et al., 2016), which is comparable in age to an early adolescent human (Quinn, 2005). Development of inhibitory parvalbumin cells is also delayed, since they only appear in the BLA around PND14 and mature by PND30 (Berdel and Morys, 2000). PV interneurons and excitatory cells in the BLA are ensheathed by specialized lattice structures called perineuronal nets (PNN), which contain chondroitin sulfate proteoglycans and extracellular matrix components (Kwok et al., 2011). Interestingly, PNN also develop postnatally in the rat BLA and increase in number until PND28 (Gogolla et al., 2009).

Many of the morphological changes occurring in the rat BLA during postnatal development coincide with alterations in neuron physiology and synaptic plasticity.

Passive membrane properties of principal BLA neurons, such as input resistance and membrane time constant, decrease with age and reach maturity at the end of the first postnatal month, in parallel with their structural development (Ehrlich et al., 2012). The waveform and patterning of action potentials evoked by 250 ms depolarizing current ramps (85-555 pA range) in principal BLA cells also exhibit developmental changes (Ehrlich et al., 2012). More precisely, the half-width of action potentials decreases significantly between PND7 and PND28, indicating that action potentials become faster with age (Ehrlich et al., 2012). Furthermore, there is a negative shift in action potential threshold from PND7 (-33 mV) to PND28 (-40 mV). Spike trains elicited from principal cells with 1 s depolarizing current injections (16 – 800 pA range) reach maximal firing frequency by PND14 (Ehrlich et al., 2012).

In regards to developmental changes in BLA synaptic plasticity, high-frequency stimulation of LA inputs to the BLA results in modest long-term potentiation (LTP) on PND7-10, whereas after that and until PND19, long-term depression (LTD) is predominant (Thompson et al., 2008). The LTD state can be reverted to immature LTP via the application of $GABA_A$ receptor antagonists, suggesting that developmental changes in GABAergic transmission likely modulate synaptic plasticity in the neonatal BLA (Thompson et al., 2008). Indeed, many aspects of GABA transmission in the BLA only mature at the end of the first postnatal month (Ehrlich et al., 2013). In contrast to earlier findings, and without any pharmacological blockade, successful LTP in the BLA was demonstrated as early as PND20 in male and female rats (Bender et al., 2017). In adulthood, most *in vitro* studies that examine LTP formation in the BLA apply $GABA_A$ receptor antagonists, since GABAergic inhibition suppresses evoked excitatory post

synaptic potentials, or EPSPs (Rainnie et al., 1991; Thompson et al., 2008; Li et al., 2011; Suvrathan et al., 2014). It is unclear why before PND10, blockade of $GABA_A$ receptors is not required for LTP formation, but it may simply be because GABA circuits are still immature and unable to elicit feed-forward inhibition (Thompson et al., 2008). Around the end of the second postnatal week in the BLA (Ehrlich et al., 2013), there is a fundamental switch of GABAergic transmission from depolarizing to hyperpolarizing that has also been documented in the medial PFC (mPFC) and hippocampus (Ganguly et al., 2001; Le Magueresse and Monyer, 2013). This early excitatory GABA function might facilitate weak LTP in the young BLA in the absence of $GABA_A$ receptor antagonists (Thompson et al., 2008).

In addition, synaptogenesis in the BLA, as detected by presynaptic synaptophysin immunoreactivity, peaks at PND14 and stabilizes by PND30 (Morys et al., 1998). Increases in total synapse number during postnatal development may reflect the maturation of inputs to the BLA, particularly those coming from the mPFC which develop between PND13-21 (Bouwmeester et al., 2002b; Arruda-Carvalho et al., 2017). The maturation of BLA-mPFC projections is discussed in more detail below.

1.2.4.1 Amygdala-prefrontal circuit development

A rich body of human and rodent literature has identified robust structural and functional connections between the amygdala and PFC (Bouwmeester et al., 2002b; Bouwmeester et al., 2002a; Kim and Whalen, 2009; Gee et al., 2013b). The developmental trajectory of amygdala-PFC circuitry in humans is hierarchical in nature, because the amygdala displays early functionality relative to the PFC (Tottenham and

Gabard-Durnam, 2017). Functional magnetic resonance imaging (fMRI) data have shown that the amygdala in children (ages 4 to 9) exhibits strong reactivity to emotional stimuli (i.e., fearful faces) before mature connections with the PFC are established (Gee et al., 2013b). Furthermore, functional connectivity in response to fear between the amygdala and PFC switches from positive in early childhood to negative at age 10, and becomes progressively more negative into young adulthood (ages 18-22) (Gee et al., 2013b). The exact nature of these reciprocal connections remains unclear, although some studies have suggested that negative amygdala-PFC functional connectivity in adolescence and adulthood results from the development of active inhibitory PFC influence on the amygdala (Hariri et al., 2003; Kim et al., 2003; Hare et al., 2008). In summary, it is conceivable that early amygdala activity is able to instruct development of the PFC. As the PFC matures, top-down signaling increases with age and likely contributes to the observed valence switch in connectivity (Gee et al., 2013b; Tottenham and Gabard-Durnam, 2017).

In neonatal rats (PND10-12), the onset of fear learning relies on the postnatal maturation of anatomical connections between the BLA and mPFC (Raineki et al., 2010b; Tallot et al., 2016). Development of this emotional circuit is predominantly achieved in the second and third postnatal weeks (Bouwmeester et al., 2002b; Bouwmeester et al., 2002a; Arruda-Carvalho et al., 2017). Specifically, BLA efferents to the infralimbic (IL) and prelimbic (PL) regions of the mPFC emerge between PND7-9 and reach an adult-like innervation pattern in the mPFC by PND13 (Bouwmeester et al., 2002a). Most of the BLA-mPFC projections discovered in preweaning (PND18-20) rats are intra-hemispheric, but few inter-hemispheric connections also exist at this age (Verwer et al., 1996). The number

of BLA to PFC projections continues to increase throughout adolescence and only stabilizes in adulthood (Cunningham et al., 2002). The descending mPFC to BLA projections mature later, between PND13-21 (Bouwmeester et al., 2002b; Bouwmeester et al., 2002a) and remain relatively stable until they undergo pruning in late adolescence (PND45) (Cressman et al., 2010). Taken together, these anatomical studies provide evidence for a sequential type of maturation between the ascending and top-down components of this bidirectional circuitry, as described previously in humans (Tottenham and Gabard-Durnam, 2017).

1.2.4.2 Sex and hemispheric differences in amygdala development

As has been unveiled by the studies presented in this thesis and others, effects of ELS in a number of brain regions, including the amygdala, are often sexually dimorphic and hemispheric-dependent (Walker et al., 2017; White and Kaffman, 2019). To shed light on whether these differences are indeed stress-induced, it is necessary to explore some key questions. First, are differences already present during the normal developmental trajectory of amygdala development? If so, when and how do they arise? Few studies have aimed to answer these questions, although there is some evidence to suggest that sex and laterality influence amygdala development at different stages. In a comprehensive structural MRI study by Uematsu et al., it was found that female human amygdala volume (including white and grey matter) peaked in pre-adolescence, 18 months earlier than in males, regardless of amygdala side (Uematsu et al., 2012). In addition, the total growth period between 1 month and 25 years of age lasted longer in males than in females, and likely contributed to the observed larger male amygdala

volume (Uematsu et al., 2012). Neufang et al. found that amygdala grey matter volume is increased in human males compared to females (ages 8-15), only on the left side, and varies according to pubertal stage and circulating testosterone levels (Neufang et al., 2009). These findings suggest that gonadal steroid levels might play an important role in governing sexually dimorphic amygdala development. Hemispheric differences in total amygdala volume are seen in males during development, but the direction of this asymmetry is related to the age range and type of amygdala tissue imaged. For instance, in contrast to results reported by Neufang et al., right asymmetry of amygdala volume was observed in males, but not females, from infancy until adulthood (Uematsu et al., 2012). It is important to note that Uematsu et al. sampled a much longer age window and measured both amygdala white and grey matter, which likely accounted for such discrepancies between studies (Neufang et al., 2009; Uematsu et al., 2012). Fetal testosterone has been shown to positively correlate with gray matter volume in the left, but not the right, amygdala of typically developing boys (aged 8-11 years old) (Lombardo et al., 2012), suggesting that early organizing effects of sex hormones, such as androgens, may contribute to lateralized amygdala volume. The growth rate of the amygdala is also lateralized, as the left side grows faster than the right in both males and females (Uematsu et al., 2012).

The rodent amygdala, especially the BLA, has been the subject of far fewer studies examining sex and hemispheric differences during development. Some work focusing on the MeA has shown increased total structural volumes, dendritic volumes and neuron numbers in prepubertal (PND26-29) male compared to female rats (Cooke et al., 2007), independent of amygdala side. In an earlier study, the same group of researchers found

that sexual dimorphism for MeA volume in prepubertal rats was lateralized to the left amygdala (Cooke and Woolley, 2005). They also found that MeA neurons in males had more frequent miniature excitatory postsynaptic currents (mEPSCs) and a greater number of excitatory synapses on dendritic spines compared to females, again only on the left side (Cooke and Woolley, 2005). Enhanced MeA neuron numbers in males persist until adulthood (PND60) (Morris et al., 2008). For the developing rat BLA, no sex differences or interactions with amygdala hemisphere were found for total volume or number of neurons on PND20, 35 or 90 (Rubinow and Juraska, 2009). There was also no significant effect of sex on maturation of physiological properties of BLA pyramidal neurons in the first postnatal month (between PND7-28) (Ehrlich et al., 2012). Only in adulthood do some sex and hemispheric differences emerge in the BLA under normal circumstances. For instance, male adult rats have more dendritic spines on pyramidal BLA neurons compared to females (Rubinow et al., 2009), and greater numbers of PV-positive cells in the left compared to the right BLA (Butler et al., 2018). Thus, effects of sex and laterality in the rat emerge earlier in the developing MeA than in the BLA, highlighting that these amygdala nuclei are fundamentally different, both anatomically and functionally (Sah et al., 2003). If there are little underlying sex or laterality differences during the normal BLA developmental trajectory, sex and hemispheric-dependent effects of ELS on the young rat BLA as we demonstrate in this thesis could likely be a direct consequence of the stress exposure.

1.3 The role of the amygdala in fear and anxiety circuits

Emotions such as fear and anxiety are induced by threats to well-being or survival and trigger defensive psychological, physiological and behavioral responses (Steimer, 2002; Tovote et al., 2015). Distinct and overlapping circuits in the amygdala and connections with the mPFC and hippocampus, in particular, are critical for the processing of fear and anxiety and generating adaptive outcomes (Tovote et al., 2015). While fear is considered to be an acute emotional response to an actual threat, anxiety is characterized by chronic hyperactivity in the corticolimbic circuitry, which may or may not require the presence of potential threats (Steimer, 2002; McNaughton and Corr, 2004; Duval et al., 2015). Of particular interest to my thesis is how the amygdala and corticolimbic circuitry may be modified following ELS exposure to lead to excessive or pathological fear and anxiety. We predict that early adversity will cause an anxiogenic phenotype that might be revealed upon acute stimulation of the corticolimbic circuitry, either by stimulating LA neurons or exposing animals to fear conditioning.

1.3.1 Local amygdala circuits regulating fear conditioning and extinction

Most of our understanding of the amygdala fear circuitry has emerged from seminal studies using Pavlovian conditioning, an experimental paradigm that is frequently implemented to examine fear learning in both humans and rodents (Fanselow and LeDoux, 1999; Maren, 2001; Carrere and Alexandre, 2015). During Pavlovian fear conditioning, a conditioned stimulus (CS), such as a tone or light, is repeatedly paired with an aversive unconditioned stimulus (US), typically a foot shock in rodents (Fanselow and LeDoux, 1999; Tovote et al., 2015). Eventually, the previously neutral CS becomes

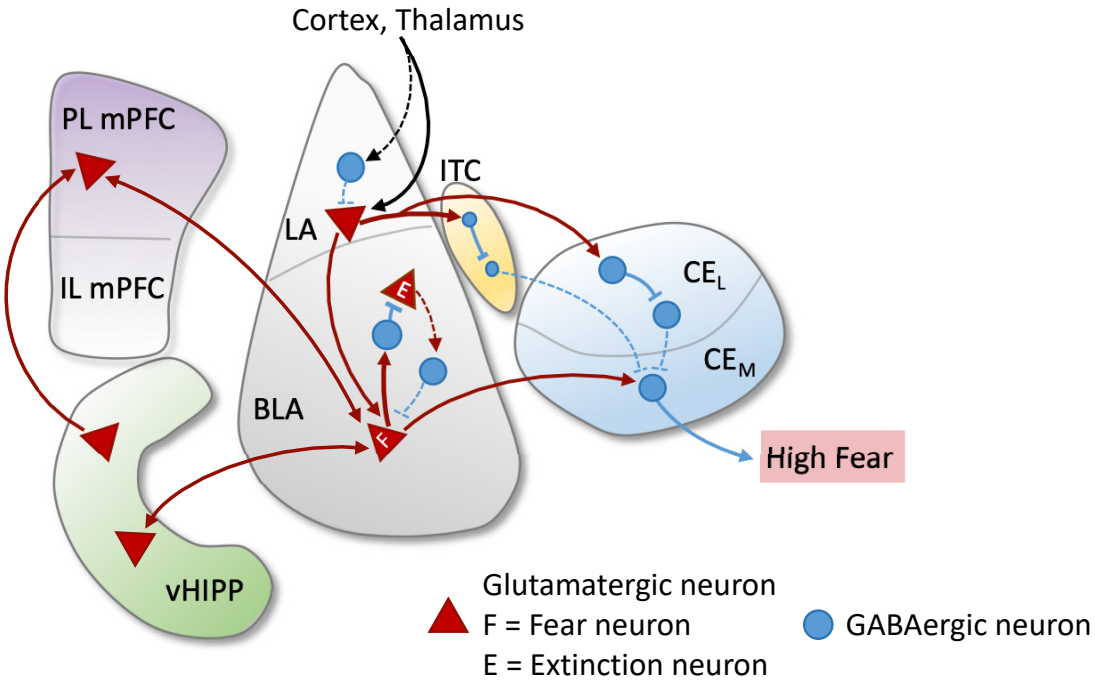
emotionally significant, and evokes a conditioned fear response (e.g., freezing) when presented alone (Phillips and LeDoux, 1992; Dityatev and Bolshakov, 2005; Shin and Liberzon, 2010). Fear expression may be decreased through fear extinction, which is considered a different learning process (Krabbe et al., 2018) that occurs in three phases known as acquisition, consolidation and retrieval (Quirk and Mueller, 2008; Lee et al., 2015). The expression of fear decreases during the acquisition phase of extinction, when the CS is repeatedly presented without the US (Quirk and Mueller, 2008; Lee et al., 2015). Once the fear memory is consolidated, it may be reinstated by presenting the CS in the same context in which extinction occurred (Quirk and Mueller, 2008) or renewed by placing animals in a novel context (Marschner et al., 2008; Maren et al., 2013). Importantly, the renewal of context-dependent fear memories is developmentally regulated in rats, since extinguished fear responses can only be reinstated after the third postnatal week (Kim and Richardson, 2007). Before this time, fear experience is erased and there is no fear recall when rats are placed in a novel context (Kim and Richardson, 2007). Dysregulated fear conditioning and poor extinction are a hallmark of anxiety disorders in humans, and many pathological conditions in animals. In cases of poor extinction, animals show increased expression of fear to the CS during retrieval (Quirk and Mueller, 2008).

The LA and BLA are involved in the acquisition and consolidation of conditioned fear (Fanselow and Kim, 1994; LeDoux, 2000; Duvarci and Pare, 2014). Multimodal sensory information regarding the CS and US from the thalamus and cortex predominantly converges onto projection and GABAergic neurons of the LA, but also directly to BLA neurons (Romanski and LeDoux, 1993; Ehrlich et al., 2009; Duvarci and

Pare, 2014). Feedforward inhibition to projection neurons of the LA is reduced, which enables LTP at excitatory synapses, a fundamental component of associative fear learning (Bissiere et al., 2003; Tully et al., 2007; Tovote et al., 2015)(See Section 1.3.3). This form of synaptic plasticity activates LA projections terminating on glutamatergic cells in the BLA (Ehrlich et al., 2009). To facilitate excitatory output to the CeA and for successful fear acquisition, the BLA engages separate disinhibitory mechanisms involving PV-positive and somatostatin-positive interneurons (Wolff et al., 2014). During the CS, PV-expressing cells are stimulated and inhibit somatostatin interneurons, which results in dendritic disinhibition of BLA projection neurons (Wolff et al., 2014). Conversely, presentation of the US inhibits both PV and somatostatin interneurons and leads to the disinhibition of projection neurons in dendritic and perisomatic zones and enhanced fear learning (Tovote et al., 2015). In addition to processing and relaying sensory information to the CeA, studies suggest that the BLA encodes (Gale et al., 2004) and stores permanent fear memories (Fanselow and LeDoux, 1999; Maren, 2001; Repa et al., 2001; Gale et al., 2004). The encoding of fear memories is dependent upon connections with the PL mPFC (See Section 1.3.2) (Arruda-Carvalho and Clem, 2014). The CeA is then responsible for initiating the behavioral responses related to fear (Ehrlich et al., 2009; Duvarci et al., 2011). Regulatory control over the CeA is exerted by the GABAergic intercalated cell masses located adjacent to the BLA that act to suppress fear responses (Sotres-Bayon and Quirk, 2010; Arruda-Carvalho and Clem, 2015). Interestingly, the inhibitory projections from the CeA to the brainstem are enhanced during fear retrieval and may act to regulate the expression of fear (Jungling et al., 2015).

Disruption of BLA activity not only impairs fear acquisition and conditioned fear responses (Amano et al., 2011), but also fear extinction (Herry et al., 2006). Within the BLA, functionally distinct populations of neurons encode fear acquisition (fear neurons) and fear extinction (extinction neurons). These neurons are differentially connected with the mPFC and vHIPP, in particular, to regulate many aspects of fear processing (Herry et al., 2008) as is illustrated in Figure I-2 and will be discussed below.

A) Fear Conditioning



B) Fear Extinction

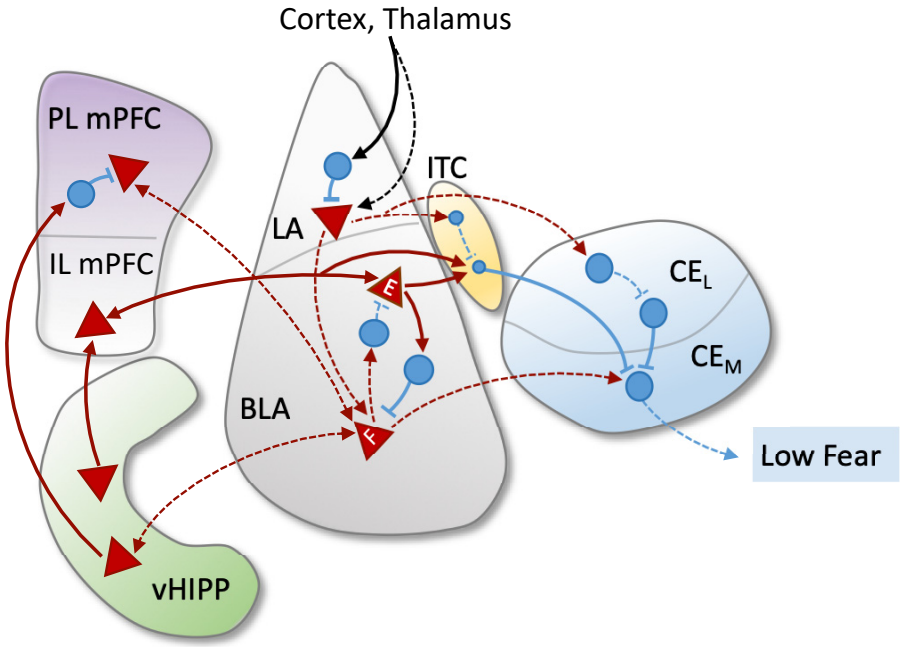


Figure I-2. Amygdala and corticolimbic pathways involved in fear conditioning and extinction. Strengthened and weakened connections are represented by the solid and dashed lines, respectively. A) During fear conditioning, multimodal sensory information from the cortex and thalamus primarily converges onto lateral amygdala (LA) projection neurons and activates descending projections to fear neurons in the BLA, as well as inputs to GABAergic neurons in the intercalated cell masses (ITC). BLA fear neurons are also stimulated by projections from the ventral hippocampus (vHIPP) and prelimbic medial prefrontal cortex (PL mPFC). Output neurons in the medial division (Ce_M) of the central amygdala are disinhibited by GABAergic neurons in the ITC and lateral division of the central amygdala (Ce_L) and activated by BLA fear neurons, leading to high fear responses. B) During fear extinction, incoming sensory information mostly converges onto LA inhibitory interneurons, leading to suppressed activity of LA projection neurons and decreased BLA fear neuron activity. Projections from the vHIPP inhibit PL mPFC activity and stimulate infralimbic mPFC activity (IL mPFC). The IL region activates glutamatergic extinction neurons in the BLA, which stimulate neurons in the ITC and inhibit BLA fear neurons, thus resulting in the inhibition of CeM output neurons and reduced fear responses. Figure adapted from Arruda-Carvalho et al. 2015 (Arruda-Carvalho and Clem, 2015), Duvarci and Pare 2014 (Duvarci and Pare, 2014), Ehrlich et al. 2009 (Ehrlich et al., 2009), Sotres-Bayon et al. 2012 (Sotres-Bayon et al., 2012), Sotres-Bayon and Quirk 2010 (Sotres-Bayon and Quirk, 2010), Lee et al. 2013 (Lee et al., 2013) and Zimmermann et al. 2019 (Zimmermann et al., 2019).

1.3.2 Corticolimbic fear processing

Fear behavior does not solely rely on amygdala microcircuits; the corticolimbic network provides a much finer control of fear conditioning and extinction (Arruda-Carvalho and Clem, 2015; Tovote et al., 2015; Ganella et al., 2018). Specifically, reciprocal connections between the PL mPFC and the BLA fear neurons become activated during fear conditioning (Sotres-Bayon et al., 2012). Inactivation of the BLA in fear conditioned rats reduces spontaneous activity of PL projection neurons and PL conditioned tone responses, suggesting that during conditioning, most fear-related input to PL projection neurons comes from the BLA and enhances PL activity (Sotres-Bayon et al., 2012). Under normal circumstances, LA neurons exhibit transient conditioned responses lasting a few hundred milliseconds (Quirk et al., 1995; Sotres-Bayon et al., 2012). By contrast, BLA fear neurons show prolonged conditioned tone responses on the order of tens of seconds that closely resemble PL neuron response patterns (Burgos-Robles et al., 2009; Amano et al., 2011). As a result, the PL mPFC is believed to transform BLA inputs and maintain conditioned fear responses (Burgos-Robles et al., 2009). Indeed, inactivation of the PL dramatically reduces the expression of conditioned fear (Corcoran and Quirk, 2007). In addition, fear responses may be modulated by plasticity within the descending PL-BLA circuit (Arruda-Carvalho and Clem, 2014). Conditioned animals display excitatory post-synaptic strengthening in the PL, but not IL inputs to BLA pyramidal neurons, which is believed to contribute to fear memory encoding and increased behavioral responses (Arruda-Carvalho and Clem, 2014). Fear neurons in the BLA also receive direct excitatory input from the vHIPP to increase conditioned fear responses (Sotres-Bayon et al., 2012). With respect to fear extinction, the IL mPFC plays a vital role (Arruda-Carvalho and Clem,

2015; Bloodgood et al., 2018). A sub-population of BLA neurons is recruited during fear extinction and connects with the IL mPFC and ventral hippocampus (Herry et al., 2008). The IL integrates BLA input, as well as input from the vHIPP, and projects back to the BLA, both directly and indirectly through the amygdala intercalated cell masses, to ultimately strengthen extinction learning and reduce fear (Arruda-Carvalho and Clem, 2015; Ganella et al., 2018). Following fear extinction, the vHIPP inhibits spontaneous activity of PL inputs to the BLA, thereby reducing fear expression (Sotres-Bayon et al., 2012).

1.3.3 Synaptic plasticity in the amygdala underlies fear learning

Electrophysiological recordings both *in vivo* and *in vitro* have demonstrated that fear conditioning induces lasting changes in amygdala neuron activity (Marek et al., 2013). This has been extensively studied in the LA, whereby neurons exhibit increased spike firing and field potentials in response to a CS that was previously associated with a US, but not to a CS that remains unpaired (Quirk et al., 1995). Activity-dependent plasticity in the LA is essential for associative learning and is thought to reflect enhanced synaptic strength at excitatory sensory inputs onto projection neurons (Blair et al., 2001). As such, enhanced inputs to the BLA are important to maintain fear learning. Long-term potentiation (LTP) is a form of experience-dependent neural plasticity that increases the strength of synaptic connections (Blair et al., 2001; Rodrigues et al., 2004). It represents a powerful cellular mechanism to study fear learning in the amygdala (Dityatev and Bolshakov, 2005). LTP induced by electrical stimulation at glutamatergic afferents that carry CS information closely resembles endogenous LTP-like processes that occur in the

amygdala following fear conditioning (Rogan et al., 1997), supporting the hypothesis that LTP mediates fear learning (Fanselow and Kim, 1994; Blair et al., 2001).

1.3.3.1 NMDA receptors and synaptic plasticity in the amygdala

Strong evidence has long supported a fundamental role for NMDA (N-methyl-d-aspartate) receptors (NMDARs) in synaptic plasticity and fear conditioning (Rogan et al., 1997; Rodrigues et al., 2004; Dityatev and Bolshakov, 2005; Kim and Jung, 2006; Dalton et al., 2012). Infusing NMDAR antagonists in the LA or BLA prior to fear conditioning prevents fear acquisition (Rodrigues et al., 2004). Similarly, blockade of NMDARs in the same amygdala regions abolishes LTP formation (Huang and Kandel, 1998). NMDARs are ligand-gated ionotropic channels that mediate excitatory glutamatergic transmission. The NMDAR at resting membrane potential is blocked by Mg^{2+} that may only be removed during postsynaptic depolarization (Luscher and Malenka, 2012). However, LTP induction requires the concurrent activation of the pre- and postsynaptic cell, such that presynaptically released glutamate binds to the NMDAR while the postsynaptic neuron is depolarized (Blair et al., 2001; Luscher and Malenka, 2012). Only then, can calcium fully enter the channel and trigger a series of molecular cascades in the postsynaptic cell that generate activity-dependent synaptic plasticity (Bekkers and Stevens, 1993; Blair et al., 2001; Luscher and Malenka, 2012).

NMDARs are heteromultimers containing GluN1, GluN2 (A-D) and GluN3 (A and B) subunits (Cull-Candy et al., 2001). NMDAR subunit composition in the brain is developmentally regulated, with GluN2B expression beginning in embryonic life (Monyer et al., 1994). GluN1 and GluN2A are expressed at birth, but GluN2A expression is nearly

undetectable and steadily increases until it reaches peak levels in the brain on PND20 (Monyer et al., 1994). Within the amygdala, most of the NMDAR in the LA at birth are composed of GluN1/GluN2B multimers, and as development progresses, GluN2A largely replaces GluN2B subunits (Lopez de Armentia and Sah, 2003). Both GluN2A and GluN2B subunits are necessary for LTP formation in the adult LA (Muller et al., 2009). In contrast, in the CeA, NMDAR subunits do not change composition over the course of development and remain as GluN1/GluN2B multimers into adulthood (Lopez de Armentia and Sah, 2003). There is limited information on the development of NMDAR subunits in the BLA, specifically. Within BLA pyramidal cells of juvenile and adolescent rats (PND25-40), the majority of NMDARs are likely heterotrimeric and assembled from GluN1, GluN2A and GluN2B subunits (Delaney et al., 2013). In these same animals, the presence of the GluN2B subunit in the BLA is required for LTP formation (Delaney et al., 2013). Others have suggested that both GluN1 and GluN2B in the adult BLA are required for fear conditioning and the associated long-term changes in synaptic plasticity (Walker and Davis, 2008; Delaney et al., 2013). Thus, in the BLA, synaptic plasticity may be mediated by different NMDAR subunits as a function age. Synaptic plasticity in the developing BLA may also be regulated by other components that modulate neurotransmission, such as specialized extracellular matrix structures known as perineuronal nets.

1.3.3.2 Perineuronal nets regulate synaptic plasticity

Perineuronal nets (PNNs) are lattice structures consisting primarily of chondroitin sulfate proteoglycans and extracellular matrix components that have recently attracted much attention for being important mediators of neuroplasticity in the developing brain

(Kwok et al., 2011; Reichelt et al., 2019). In the rodent visual cortex, the preferential formation of PNNs around PV interneurons has been implicated in the functional maturation of inhibitory circuits and the closure of critical periods of synaptic plasticity at the end of the first postnatal month (Hensch, 2003). Consistent with this idea, degradation of PNNs in the adult visual cortex results in atypical or “juvenile” ocular dominance plasticity (Pizzorusso et al., 2002) and enhanced LTP formation (de Vivo et al., 2013). In addition to limiting functional plasticity, cortical PNNs in adult rodents restrict spine motility and stabilize structural plasticity (de Vivo et al., 2013).

Within the mouse BLA, PNNs emerge on PND16 and reach mature levels by PND28 (Gogolla et al., 2009). By adulthood, the majority of PNNs encapsulate PV interneurons as well as excitatory cells (Morikawa et al., 2017). Interestingly, the appearance of PNNs in the mouse BLA on PND23 coincides with a developmental switch in fear learning, such that conditioned fear memories are subject to context-dependent renewal following extinction training (Gogolla et al., 2009). The degradation of PNNs on all BLA cell types in adulthood reenables the permanent erasure of fear memories, a process normally only observed in neonatal mice, and decreases LTP formation at LA inputs (Gogolla et al., 2009). These results suggest that in the adult BLA, PNNs actively protect fear memories from being extinguished by facilitating synaptic strengthening (Gogolla et al., 2009). It is worth noting that loss of PNNs in the adult visual cortex enhances synaptic plasticity (de Vivo et al., 2013), while similar manipulations induce the opposite effect in the amygdala (Gogolla et al., 2009). Thus, PNNs in different brain regions may serve very different roles in synaptic transmission.

1.3.4 Lateralized amygdala emotional functionality

Studies in both humans and rodents have shown that the amygdala exhibits marked hemispheric asymmetry, generally favoring higher activity in the right amygdala under pain conditions (Neugebauer and Li, 2003; Ji and Neugebauer, 2009) and fear memory processing (Baker and Kim, 2004) compared to the left amygdala. For instance, using a chronic arthritis pain model, Ji and Neugebauer observed increased baseline and evoked firing of right, but not left, CeA neurons in adult male rats (Ji and Neugebauer, 2009). Right lateralized activity of the entire human amygdala was also detected with fMRI in response to gastric pain (Lu et al., 2004).

The human neuroimaging literature suggests that the right amygdala is preferentially involved in the evaluation and encoding of negative emotionally arousing memories, while the left amygdala is primarily activated during conscious or cognitive emotional processing (Baas et al., 2004; Butler et al., 2018). Sex differences in laterality also exist, as the encoding of emotional memories specifically activates the right amygdala in men, but the left amygdala in women (Canli et al., 2002). These results were corroborated by Cahill et al. using both PET and fMRI (Cahill et al., 2001; Cahill et al., 2004). While lateralization of amygdala responses is well documented in humans, fewer studies have been conducted in rodents. However, they tend to confirm the involvement of the right amygdala in fear conditioning in male rats (Baker and Kim, 2004) because unilateral amygdala lesions performed after, but not before, fear conditioning significantly impair freezing behavior during fear retention, only when the lesion is made on the right side (Baker and Kim, 2004). Other studies have confirmed that expression of the

immediate early gene, c-Fos, was elevated in the right compared to the left BLA and CeA in fear conditioned animals (Scicli et al., 2004).

It is currently unclear which processes mediate lateralization of function in the amygdala. Differences in anatomical properties (Butler et al., 2018) and synaptic transmission (Adamec et al., 2005a; Orman and Stewart, 2007) between hemispheres may contribute to lateralized fear responses. For instance, adult male rats have greater numbers of inhibitory PV interneurons in the left compared to the right BLA, which could result in reduced left BLA activity (Butler et al., 2018). In addition, the levels of protein kinase C beta II, a signaling molecule implicated in the formation of long-term fear memories, were also found to be significantly elevated in the right relative to the left BLA of rats randomly presented with tone and shocks (Orman and Stewart, 2007). Exposing male rats to predator stress, a fearful stimulus, increases synaptic plasticity in the right BLA (Adamec et al., 2001) and the magnitude of changes in plasticity appears to strongly predict anxiogenic-like behaviors (Adamec et al., 2005b). The hemispheric bias in synaptic transmission is likely NMDAR-dependent, as systemic injection of an NMDAR antagonist prevents predator-stress induced potentiation of evoked synaptic responses in the right, but not the left, BLA (Adamec et al., 2005a).

1.3.5 Amygdala and corticolimbic circuits in anxiety

Compared to the circuits mediating fear responses, the local and long-range amygdala circuits underlying anxiety are seemingly more complex (Tovote et al., 2015) and, although not a direct focus of this thesis, merit some discussion. It is well known that amygdala lesions in humans and rodents produce anxiolytic phenotypes (Korn et al.,

2017; Babaev et al., 2018) and conversely, that anxiety disorder is associated with amygdala hyperexcitability (Rauch et al., 2003). In particular, activation of the BLA in rodents promotes anxiety-like behavior (Tye et al., 2011). Recent studies have suggested that the increase in anxiogenic behavior might be due to the activation of a subpopulation of pyramidal cells in the BLA terminating on unidentified inhibitory interneurons in the medial region of the CeA (Babaev et al., 2018). In contrast, other excitatory projections from the BLA to the lateral division of the CeA have been found to suppress anxiety-like behavior (Tye et al., 2011). So, within the BLA, subpopulations of excitatory glutamatergic neurons have both an anxiogenic as well as anxiolytic action. In addition, many BLA inhibitory interneurons, notably PV-expressing cells, are also implicated in the anxiety circuitry (Babaev et al., 2018) because they actively modulate projection cell activity and are recruited upon the delivery of anxiogenic drugs for instance (Hale et al., 2010).

Some well-defined long-range connections between the BLA, mPFC and vHIPP also participate in generating anxiety-like behaviors (Tovote et al., 2015). Direct BLA inputs to both the mPFC and CA1 vHIPP critically mediate the expression of anxiety-related behaviors (Felix-Ortiz et al., 2016; Yang and Wang, 2017). For instance, optogenetic inhibition of anterior BLA inputs onto either mPFC or CA1 vHIPP neurons decreases anxiety-like behavior of adult male mice in the open field and elevated plus maze, while direct activation of these pathways increases anxiety-related behaviors and reduces social interaction (Felix-Ortiz et al., 2013; Felix-Ortiz et al., 2016). Recent work in adult male mice has specified that anterior BLA inputs to the ventral CA1 region are anxiogenic, whereas inputs from the posterior BLA are anxiolytic (Pi et al., 2020). This further emphasizes the heterogeneity of BLA function along its rostral-caudal axis and the

need to consider the anterior and posterior BLA as separate entities. Connections between the vHIPP and mPFC are also closely linked to anxiety. For example, upon exposure to anxiogenic environments, firing in the mPFC increases and is synchronized with vHIPP activity (Adhikari et al., 2010, 2011). This anxiety-related increase in functional activity in the vHIPP-mPFC circuit is required for normal anxiety-like behavior and is dependent upon direct vHIPP inputs to the mPFC (Padilla-Coreano et al., 2016).

Importantly, studies in both rodents (Yan et al., 2017; Johnson et al., 2018) and humans (Burghy et al., 2012; Gee et al., 2013a) have demonstrated that functional connectivity within the corticolimbic circuitry is sensitive to ELS exposure (See Section 1.4.3). Any disruptions in development of anxiety-related pathways could negatively affect long-term anxiety behaviors. Indeed, increased resting-state fMRI connectivity between the whole amygdala and vHIPP in male mice subjected to unpredictable postnatal stress correlates with anxiety-like behavior in adulthood (Johnson et al., 2018). In adolescent humans exposed to early adversity, reduced resting-state fMRI between the amygdala and the PFC predicts higher anxiety symptoms (Burghy et al., 2012).

1.3.6 Development of amygdala-related fear behaviors in rodents

The protracted postnatal development of the structure and connections of the amygdala makes this nucleus particularly sensitive to environmental stressors occurring during the neonatal and juvenile periods. Modifications in the organization of amygdala connections might contribute to changes in the maturation of emotional behavior (Ryan et al., 2016). During the first week and a half of postnatal life, rat pups have limited hearing and vision and quickly learn to preferentially recognize maternal odors which promotes

caregiver attachment (Sullivan, 2001; Tallot et al., 2016). Pup-caregiver attachment is initially programmed by an influx of norepinephrine, secreted by the LC, into the olfactory bulb (Sullivan and Wilson, 1991; Santiago et al., 2017). Many studies using conditioning paradigms in neonatal rodents have therefore used olfactory cues as the CS (Jovanovic et al., 2013). In these instances, PND8 rats are able to pair an odor and a foot shock (US), but the conditioned response is approach rather than avoidance to the shock (Sullivan et al., 2000). Once pups leave the nest around PND10, this same odor-shock conditioning can produce avoidance responses (Thompson et al., 2008) (Moriceau and Sullivan, 2006), although when conditioning occurs in the presence of the mother, approach rather than avoidant responses to the CS are observed, showing the ability of the mother to modify the emotional valence of stimuli (Moriceau and Sullivan, 2006; Santiago et al., 2017; Sullivan, 2017). Studies have demonstrated that maternal presence decreases pup CORT levels and amygdala activity, which are believed to prevent neonatal conditioned threat responses (Santiago et al., 2017; Sullivan, 2017). This is supported by the fact that in PND8 pups, infusion of CORT into the amygdala causes premature amygdala activity and fear conditioning (Moriceau et al., 2006). Activation of the amygdala, as measured by uptake of labelled 2-deoxygucose, is enhanced following odor-shock conditioning on PND12, but not on PND8, again suggesting that amygdala neural activity is increased once pups become progressively independent to program avoidance conditioning (Sullivan et al., 2000; Moriceau and Sullivan, 2006). The emergence of odor fear conditioning on PND10 also coincides with developmental changes in BLA synaptic plasticity (Thompson et al., 2008). Specifically, high frequency

stimulation of the LA induces LTD in the BLA on PND10, whereas before that on PND7, modest LTP is present (Thompson et al., 2008).

As the BLA structure and connectivity matures, so do behavioral responses, such as freezing and fear-potentiated startle. Conditioned freezing in rats emerges on PND16-18 (Hunt et al., 1998; Barnet and Hunt, 2006; Ryan et al., 2016) and reaches adult-like levels by PND23-27 (Jovanovic et al., 2013). Conversely, fear-potentiated startle to a tone is observed after weaning on PND23, but not earlier on PND16 in the rat (Hunt et al., 1994).

Fear extinction outcomes and cellular mechanisms across development are also fundamentally different. In neonates (PND16), extinction training promotes the permanent erasure of fear memories, as examined by a lack of freezing during fear retrieval the following day (Kim and Richardson, 2007, 2008). By contrast, the same protocol in PND23 rats reinstates extinguished fear, as seen in adulthood (Kim and Richardson, 2010) (Kim and Richardson, 2007, 2008). This switch appears to be mediated by developmentally-regulated changes in NMDAR activity in the brain (Langton et al., 2007), as their blockade only disrupts fear extinction on PND23, while PND16 extinction is unaffected (Langton et al., 2007). PNN may also play a significant role in protecting fear memories from erasure at later developmental stages because as mentioned earlier, enzymatic “dissolution” of PNN in the BLA of adult rats reinstated the phenotype of erasure of fear memory that is normally only observed in young neonatal rats (Gogolla et al., 2009). Lastly, inactivation of the amygdala (BLA, LA, CeA) disrupts extinction on PND17 but not on PND24 (Kim and Richardson, 2008), suggesting that extinction becomes more dependent on the mPFC once reciprocal connections between the BLA

and mPFC are established (Sotres-Bayon and Quirk, 2010) (Kim and Richardson, 2008; Jovanovic et al., 2013).

1.4 Early-life stress and the amygdala

Having reviewed the amygdala's normal developmental trajectory and its role in regulating stress responses and emotions, it is important to understand how exposure to ELS during critical periods of amygdala development could promote later fear and anxiety-related disorders (Burghy et al., 2012; Pagliaccio et al., 2015) and whether these mechanisms are sex dependent. This section will examine reports of early stress-induced amygdala changes in males and females during infancy through adolescence. As will be made evident, a paucity of data still exists with respect to sex-specific proximal ELS effects, therefore some long-term consequences of ELS on the amygdala will also be discussed for comparison with our data presented in this thesis. Finally, the behavioral dysfunctions associated with early stress-related amygdala changes will be described.

1.4.1 Structural alterations in the amygdala following early-life stress

Due to the high density of glucocorticoid receptors present in the amygdala (Geuze et al., 2012), this structure is extremely sensitive to the effects of both acute (Mitra et al., 2005; Rodriguez Manzanares et al., 2005; Duvarci and Pare, 2007; Kim et al., 2014) and chronic stress exposure (Vyas et al., 2002; Vyas et al., 2004; Vyas et al., 2006; Tottenham et al., 2010; Rau et al., 2015). The amygdala is one of the few structures that generally increases in volume in response to chronic stress (Vyas et al., 2002; Mehta et al., 2009; Tottenham et al., 2010). In humans, early life adversity has conflicting effects on

amygdala volume depending on the developmental timing of stress exposure, as well as the duration and intensity of the stress (Lupien et al., 2009; Fox et al., 2010; Gee and Casey, 2015). Children reared in orphanages display significantly larger bilateral amygdala volumes (Mehta et al., 2009; Tottenham et al., 2010), while children who suffered from physical abuse or early neglect were found to have significantly smaller left amygdala volumes (Hanson et al., 2015). Similarly, children exposed to maternal depressive symptomology since birth (i.e., prolonged stress) exhibit increased amygdala volume, while hippocampal volume remains unaffected (Lupien et al., 2011). Interestingly, a recent study found that self-reported childhood neglect was associated with sex-specific and lateralized changes in the adolescent amygdala, with boys, but not girls, having larger right amygdala volumes (Roth et al., 2018). In adulthood, greater exposure to chronic stressors during childhood, measured using an index of cumulative risk exposure, is positively correlated with enlarged amygdala volumes (Evans et al., 2016).

Consistent with many human studies, the rodent BLA undergoes hypertrophy in response to chronic stress. Juvenile chronic restraint stress (6h/day from PND20-41) increases BLA pyramidal neuron dendritic length in male and female rats (Eiland et al., 2012). Similarly, chronic immobilization stress (CIS, 2h/day for 10 days) in adult rats enhances dendrite arborization of pyramidal and stellate neurons in the BLA (Vyas et al., 2002). Interestingly, the same stress paradigm does not impact neuron morphology in the CeA (Vyas et al., 2003). Chronic stress also alters neuron morphology in the hippocampus by reducing spine density and arborization, however these changes are reversible, unlike those observed in the BLA (Vyas et al., 2003; Vyas et al., 2004). Even after animals are given a 21-day stress-free period following CIS, the dendritic arbors in

their BLA continue to increase in size, indicating that the amygdala may be 'sensitized' and thus more resistant to recovery following chronic stress (Vyas et al., 2004; Gee and Casey, 2015).

While the effects of chronic stress on the amygdala have been well documented in adults, less understood are the effects of chronic ELS on the structure of the neonatal and juvenile amygdala. Prenatal stress was shown to increase BLA volume, as well as neuron and glia density in male juvenile (PND25) rat offspring (Kraszpulski et al., 2006). Even though these structural differences appear to dissipate by adulthood, it is suggested that the accelerated BLA growth trajectory could impair developing reciprocal connections with the mPFC and enhance fear behaviors in the long-term (Kraszpulski et al., 2006). ELS in the form of early weaning (on PND14) causes precocious myelination in the BLA of adolescent male mice (Ono et al., 2008) and restricted maternal care between PND8-12 reduces the intensity of PNN staining in the BLA of PND23 male and female rats (Santiago et al., 2018), suggesting that early environmental insults can modify the maturity stage of PNN in the developing BLA. Collectively, these findings indicate that the consequences of ELS on the amygdala can be observed early, although the proximal effects of ELS on the neonatal amygdala structure are still unknown. We specifically address this knowledge gap in Chapter II by examining BLA volume and morphology as early as PND10. Importantly, we found that early stress-induced alterations in amygdala structural integrity occur in parallel with enhanced amygdala reactivity and neuron excitability as will be described in Chapter III.

1.4.2 Effects of early-life stress on amygdala reactivity and excitability

Humans with a history of ELS typically have larger amygdala volumes that presumably contribute to heightened amygdala reactivity to emotional stimuli (Tottenham et al., 2010). Retrospective (Dannlowski et al., 2013; van Harmelen et al., 2013) and recent prospective studies (Javanbakht et al., 2015; Evans et al., 2016) of adults exposed to early adversity have identified exaggerated amygdala reactivity to negative emotional cues. In line with these findings, children and adolescents (all right-handed, males and females) that were previously institutionalized display right-lateralized hyperreactive amygdala responses to fear faces (Gee et al., 2013a). Youths having experienced caregiver deprivation or emotional neglect similarly display enhanced amygdala activation during the analysis of detailed emotional faces, but only on the left side (Maheu et al., 2010). In this case, left as opposed to right-lateralized amygdala activity may be due to the type of task used that engaged more extensive and conscious emotional processing (Maheu et al., 2010). Interestingly, the earlier the onset of childhood maltreatment, the more severe the hyperreactive amygdala responses to emotional stimuli, which further emphasizes the importance of the timing of postnatal ELS exposure in predicting amygdala outcomes (McCrory et al., 2013). It is worth noting that adverse rearing conditions increase amygdala reactivity to fearful relative to neutral faces in children (Tottenham et al., 2011), a response normally only observed in naïve adults (Thomas et al., 2001), suggesting that ELS enables precocious amygdala development.

Amygdala reactivity in rodents is similarly enhanced following exposure to chronic stress during early life and adolescence (Raineki et al., 2012; Malter Cohen et al., 2013; Rau et al., 2015). More specifically, amygdala neural activity, measured with c-Fos

immunohistochemistry, is increased after exposure to acute forced swim in adolescent male and female rats subjected to ELS (Raineki et al., 2012). ELS in the form of altered maternal care between PND2-21 also increases the expression of c-Fos protein in the BLA of juvenile and adolescent male mice (PND26-34) exposed to a threatening context (Malter Cohen et al., 2013). ELS-induced changes in the development of the amygdala might result from dysregulated HPA axis activity that is associated with ELS. For example, the emergence of amygdala fear reactivity is accelerated by increasing CORT levels systemically or by injecting CORT directly in the amygdala of male and female neonatal rats (Moriceau et al., 2006). Following adolescent social isolation, adult male rats show hyperexcitability of BLA pyramidal neurons (Rau et al., 2015). In summary, the few existing studies on the effects of ELS on the juvenile or adult amygdala emphasize amygdala hyperexcitability and increased emotional behavior. However, several questions remain unanswered, for instance whether ELS-induced changes in intrinsic neuron excitability could produce alterations in synaptic plasticity (Xu et al., 2005), seeing as chronic stress in adulthood increases LTP formation in the amygdala (Dalton et al., 2012; Suvrathan et al., 2014). Moreover, it remains unknown whether similar amygdala functional recruitment already occurs in neonatal and juvenile rats with the ELS exposure and whether these changes are sex- and hemispheric-dependent. These questions are addressed in Chapters II and IV.

1.4.3 Early-life stress effects on amygdala-prefrontal connectivity

The predominant postnatal maturation of the BLA-PFC circuit makes it very vulnerable to adverse early life experiences (Bouwmeester et al., 2002b; Bouwmeester

et al., 2002a; Gee et al., 2013a; Tottenham and Gabard-Durnam, 2017). Importantly, elegant studies have documented that heightened amygdala reactivity in previously institutionalized youths accelerates the development of more “mature” amygdala-PFC connectivity as assessed by fMRI in response to an emotional task (Gee et al., 2013a). Early life adversity also weakens resting state amygdala-PFC functional connectivity in adolescence (Burghy et al., 2012) and adulthood (Kim et al., 2013), suggesting that ELS exposure programs lasting changes in the functional integrity of this circuit. Reduced functional connectivity between the amygdala and anterior cingulate cortex is also observed in children with high levels of prior cumulative stress exposure (Pagliaccio et al., 2015).

Neuroimaging studies in rodents exposed to ELS have revealed equally disrupted patterns of amygdala functional connectivity (Holschneider et al., 2016; Yan et al., 2017). Early adversity reduces rs-fMRI connectivity between the BLA and mPFC in adolescent and adult male rats (Yan et al., 2017), although it is unknown how it affects the functional integrity and development of the BLA-mPFC pathway in neonates. This topic is addressed in our work reported in Chapter III. Importantly, a recent study used high resolution *ex vivo* diffusion tensor imaging to visualize bilateral BLA-mPFC projections in PND56 rats and compare naïve to ELS-exposed rats. The results of this study showed an increased number of tracts crossing the midline (Bolton et al., 2018), indicating that ELS might cause aberrant structural, as well as functional connectivity.

1.4.4 Early-life stress and amygdala-related emotional outcomes

Early adversity produces robust changes in the amygdala and amygdala-prefrontal pathway across species that associate with emotional difficulties throughout the life-span (Ellis et al., 2004; Burghy et al., 2012; Gee et al., 2013a; Hanson et al., 2015). Stress-induced amygdala alterations might well represent the neural underpinnings of anxiety and mood disorders. Children reared in orphanages with greater amygdala volumes display more internalizing behaviors and anxiety (Tottenham et al., 2010), which are risk factors for the development of future psychopathologies (Hofstra et al., 2002). Indeed, roughly half of the children tested in this study met diagnostic criteria for at least one psychiatric illness, of which ~20% were anxiety disorders (Tottenham et al., 2010). Higher levels of self-reported childhood neglect in adolescent males associate with enhanced right amygdala activity and elevated anxiety symptoms (Roth et al., 2018). Adults with anxiety disorders and a history of ELS also display enhanced amygdala activity to threatful cues (Bremner et al., 2003b; Bremner et al., 2003a; Bremner et al., 2005). Indeed, atypically large amygdala volumes in ELS-exposed individuals might allow for greater processing of negatively valenced information and increased amygdala sensitivity to fearful cues (Tottenham et al., 2010; Gee et al., 2013b). It is suggested that the heightened amygdala reactivity observed in early development might actually reduce the functional connectivity between the amygdala and the PFC (Burghy et al., 2012; Gee et al., 2013a), leading to a phenotype of higher trait anxiety levels and disease symptomatology (Burghy et al., 2012; VanTieghem and Tottenham, 2017). In addition, as mentioned previously, ELS might modify the number of fiber tracts crossing the midline that target cortical regions, as well as modify their integrity (Bolton et al., 2018). This is

important since previous reports have shown that the structural integrity of white matter tracts between the amygdala and the PFC is inversely correlated with anxiety symptoms in adult humans (Kim and Whalen, 2009; Burghy et al., 2012).

In rodents, exposure to chronic stress during the developmental period also has lasting effects on the male adolescent and adult amygdala, as evidenced by increased anxiety and conditioned fear responses in these animals (Stevenson et al., 2009; Eiland et al., 2012; Molet et al., 2014)(see also our work in Chapter II). Adolescent male rats reared under suboptimal conditions display increased BLA neural activity and enhanced anxiety and depressive-like behaviors (Raineki et al., 2012). Importantly, when the amygdala is temporarily deactivated, depressive-like behaviors in ELS animals are reversed, suggesting a causal link between amygdala functioning after ELS and behavioral dysfunctions (Raineki et al., 2012). Similarly, enhanced BLA neural activity observed in juvenile and adolescent mice exposed to ELS increases fear responses to a threatening context (Malter Cohen et al., 2013). Hypertrophy of BLA neurons in male and female adolescent mice following chronic juvenile stress associates with enhanced anxiety-like behavior in the elevated plus maze (Eiland et al., 2012). In line with the human literature (Gee et al., 2013a), reduced rs-fMRI connectivity between the BLA and mPFC after ELS is linked to long-term decreases in social interactions and increased depressive behaviors in adult rats (Yan et al., 2017). In summary, most models of ELS exposure both in humans and rodents have demonstrated that it creates a phenotype of enhanced anxiety and depression as well as reduced social behavior that is likely to be mediated, at least in part, by morphological and functional changes in the amygdala-mPFC circuitry. Variations in the magnitude of the effects, the type of behavior mostly impaired and the

possibility for resilience might depend on a large number of factors including the genetic and epigenetic makeup of the individual as well as the timing and nature of the early stressors (Tottenham and Sheridan, 2009; Gee and Casey, 2015; McCarthy et al., 2017). In designing preclinical studies to examine the underlying mechanisms of ELS effects, several models of early adversity have been used and will be briefly discussed below in addition to our chosen model of ELS.

1.5 Animal models of early-life stress

Children cannot easily and ethically be assigned to select rearing environments for the longitudinal study of ELS-induced neurobehavioral consequences (Zeanah et al., 2003; Nelson et al., 2007). In addition, the human literature relies on non-invasive techniques, such as neuroimaging, to study relationships between early maltreatment and amygdala-related emotional outcomes (Tottenham et al., 2010). Animal models of ELS overcome these challenges and enable the investigation of precise cellular and molecular mechanisms that might contribute to the pathogenesis of psychiatric disorders (Walker et al., 2017). Several models of ELS have been used in neonatal rats (Walker and Woodside, 2015), including acute 24 h maternal deprivation (Hofer et al., 1993) or repeated maternal separation during the first 2 weeks of life in rodents (Zimmerberg and Sageser, 2011). These models are used to assess deficits in the quantity of maternal care towards the pups and less so deficits in the patterns and/or quality of maternal care (McLaughlin et al., 2016; Walker et al., 2017). While still useful, these models do not lead to continuous changes in the quality and consistency of maternal care and are associated

with a significant intrusion of the experimenter in the mother-pup interactions (Walker et al., 2017).

There is strong evidence in both the human and animal literature to suggest that consistent and effective maternal care confers resilience to disease, whereas suboptimal maternal care is associated with impaired mental and cognitive health in the offspring (Essex et al., 2011; VanTieghem and Tottenham, 2017; Walker et al., 2017; Hambrick et al., 2019). A more naturalistic model of ELS should therefore impact both the patterns and quantity of maternal care, to simulate an important element of maternal neglect in the human condition (Molet et al., 2014). The limited bedding (LB) paradigm restricts the amount of nesting material available in the cage from PND 2 to 9, which causes stress to the mother and results in fragmented maternal care (Ivy et al., 2008; Walker et al., 2017). Frequent shifts in behaviour and shortened periods of nursing elicit chronic stress among the pups (Molet et al., 2014; McLaughlin et al., 2016; Walker et al., 2017). This is supported by the fact that in some studies, pups reared in LB conditions display increased basal plasma CORT levels, adrenal hypertrophy and increased excitatory inputs onto CRH neurons in the PVN (Gilles et al., 1996; Avishai-Eliner et al., 2001; Ivy et al., 2008; Molet et al., 2014).

1.6 Hypotheses and aims

Exposure to ELS is clearly recognized to be an important risk factor for the development of vulnerability to mental pathologies and behavioral impairments throughout the life span. While many studies have documented persistent and sex-specific effects of ELS, very few have investigated proximal effects and tentative mechanisms during the neonatal period. Since the amygdala is a lateralized structure that exhibits exquisite sensitivity to chronic stress (Baas et al., 2004; Vyas et al., 2004; Vyas et al., 2006) and could lead to dysregulation in the HPA axis and anxiety-related disorders (Herman et al., 2003; Dityatev and Bolshakov, 2005; Ulrich-Lai and Herman, 2009; Shin and Liberzon, 2010), the goal of this dissertation is to determine whether ELS already modifies amygdala function in neonatal life, how it affects the basolateral amygdala in particular and how permanent these effects can be. We also wanted to explore the possibility that the effects of ELS are modulated by sex, as the development of the brain is generally considered to be sexually dimorphic. Therefore, we explored the effects of chronic ELS on the neonatal (PND10) and juvenile BLA (PND18-29) of male and female rats. Our general hypothesis was that chronic stress in the form of exposure to limited bedding conditions (LB) during the first 10 days of life would induce morphological and functional changes in the developing BLA, decrease BLA-mPFC resting-state fMRI connectivity, and enhance synaptic plasticity and neuron excitability, possibly via altered indices of GABA functioning. A secondary hypothesis was that these effects are sex-dependent and lateralized. Finally, we proposed that structural, functional and resting-state fMRI connectivity BLA alterations would underlie sex-specific increases in fear

behavior in offspring exposed to ELS. To address our hypotheses, we answered the following specific questions:

Aim 1: Does chronic ELS in the form of altered maternal care induce prolonged morphological and functional changes in the BLA of neonatal rats?

Here, we documented morphological (volume, dendritic arborization and spine density) and functional (electrophysiological recordings) changes occurring in the neonatal amygdala as a result of LB exposure during the first 10 days postnatally. Two time points were examined: at the end of the stress exposure (PND10) and preweaning (PND18-21). Since ELS is known to modify the activity of the HPA axis, we also tested whether amygdala changes were mediated by altered neonatal HPA activity. This aim is explored in Chapter II.

Aim 2: Does ELS affect BLA-mPFC functional connectivity and laterality?

In the previous study (Aim 1), we demonstrated that the effects of ELS on the BLA can be observed very early, but precisely how LB exposure affects amygdala function and its connectivity to other brain structures in the emotion regulation circuitry is still unknown. To address this aim, we examined resting-state fMRI connectivity in preweaning (PND18) and adult male offspring *in vivo* and hypothesized that LB rearing would reduce BLA-mPFC connectivity in the long-term, and disrupt BLA connectivity with other structures as well. We also considered morphometric properties of the BLA, mPFC and HIPP and their volumetric relationships. These experiments are described in Chapter III.

Aim 3: Does ELS modify BLA synaptic plasticity and neuron excitability in a sex- and hemispheric-dependent manner?

To expand upon our previous findings that were acquired for the most part in males, we probed for lateralized effects and sex differences of LB by investigating synaptic plasticity, neuron excitability and NMDAR subunit expression in the juvenile BLA (PND22-29). This aim is explored in Chapter IV.

Aim 4: Are sex-specific and asymmetrical changes in excitatory BLA neuron activity after ELS possibly driven by modified expression of perineuronal nets and indices of GABA functioning?

Here, we sought to unravel the potential mechanisms by which ELS produces sex and asymmetrical effects on juvenile amygdala activity (Aim 3). We hypothesized that increased BLA synaptic plasticity and neuron excitability in LB juvenile males would be associated with altered GABA neurotransmission. More specifically, we proposed that LB exposure reduces the activity of PV-positive cells and accelerates the development of perineuronal nets around GABAergic cells, including PV interneurons. This aim is addressed in Chapter IV.

Aim 5: Are ELS-induced alterations in the BLA associated with sex-dependent impairments in anxiety-like and fear behaviors?

Finally, we determined whether ELS-induced changes in the developing amygdala and BLA-mPFC circuitry lead to lasting sex-selective impairments in behavioral regulation

related to anxiety and fear, using tests such as the elevated plus maze, open field, social interaction (Chapter II) and fear conditioning (Chapter II, III, IV).

We anticipate that these experiments will increase our understanding of the sex-specific mechanisms by which ELS contributes to affective disorders in the adult by identifying a potential facet of the emotion and extended stress circuitry that is altered by early chronic stress exposure. Studying how ELS produces lateralized and sex-dependent effects during a critical period of brain development could guide further investigation into sex-dependent mechanisms and allow for more targeted and improved treatment of stress-and emotionality-related disorders.

Chapter II

Morphological and functional changes in the preweaning basolateral amygdala induced by early chronic stress associate with anxiety and fear behavior in adult male, but not female rats

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Progress in Neuro-Psychopharmacology and Biological Psychiatry

2018. 81: 25-37

https://doi.org/10.1016/j.pnpbp.2017.09.025

Abstract

Suboptimal maternal care is a form of chronic early-life stress (ELS) and a risk factor for mental illness and behavioral impairments throughout the life span. The amygdala, particularly the basolateral amygdala (BLA), exhibits exquisite sensitivity to ELS and could promote dysregulation of stress reactivity and anxiety-related disorders. While ELS has profound impacts on the adult or adolescent amygdala, less is known regarding the sensitivity of the preweaning BLA to ELS. We employed a naturalistic rodent model of chronic ELS that limits the amount of bedding/nesting material (LB) available to the mother between postnatal day (PND) 1-9 and examined the morphological and functional effects in the preweaning BLA on PND10 and 18-22. BLA neurons displayed dendritic hypertrophy and increased spine numbers in male, but not female, LB pups already by PND10 and BLA volume tended to increase after LB exposure in preweaning rats, suggesting an accelerated and long-lasting recruitment of the amygdala. Morphological changes seen in male LB pups were paralleled with increased evoked synaptic responses recorded from BLA neurons *in vitro*, suggesting enhanced excitatory inputs to these neurons. Interestingly, morphological and functional changes in the preweaning BLA were not associated with basal hypercorticosteronemia or enhanced stress responsiveness in LB pups, perhaps due to a differential sensitivity of the neuroendocrine stress axis to the effects of LB exposure. Early changes in the synaptic organization and excitability of the neonatal amygdala might contribute to the increased anxiety-like and fear behavior observed in adulthood, specifically in male offspring.

Introduction

Optimal mother-infant interactions critically modulate mental and cognitive health in the offspring (McEwen, 2006; Fox et al., 2010) conferring resilience to the development of affective disorders (Franklin et al., 2012), whereas impaired maternal care associated with chronic early-life stress (ELS) (Essex et al., 2011; Baram et al., 2012) increases vulnerability to mood disorders and substance abuse (Weber et al., 2008). Altered regulation in the hypothalamic-pituitary adrenal (HPA) axis in the offspring (Essex et al., 2011) as well as functional changes in several activated brain structures participating in the extended stress circuitry, notably the amygdala, might be implicated in the effects of ELS (Jankord and Herman, 2008; Flak et al., 2012). The basolateral (BLA) and central amygdala (CeA) are involved in the processing and implicit learning of fear and anxiety (LeDoux, 2000; Shin and Liberzon, 2010). As such, they also play a key role in modulating stress responses during exposure to fearful stimuli (Schulkin, 2006; Jankord and Herman, 2008).

The amygdala is extremely sensitive to the effects of both acute (Mitra et al., 2005) and chronic stress exposure (Vyas et al., 2002; Vyas et al., 2003; Vyas et al., 2006). For instance, chronic immobilization stress (CIS) in adult rodents enhances dendrite arborizations of BLA, but not CeA neurons (Vyas et al., 2002; Vyas et al., 2003). CIS also alters neuron morphology in the hippocampus, although in this structure, the changes are reversible, whereas this does not appear to be the case in the BLA (Vyas et al., 2002; Vyas et al., 2004). Functionally, chronic stress in adult rodents also induces BLA neuron hyperexcitability that associates with an anxiety-like phenotype (Rosenkranz et al., 2010).

Exposure to chronic stress during early life and adolescence has lasting effects on the adult amygdala, increasing excitability of BLA pyramidal neurons (Rau et al., 2015) and conditioned fear (Stevenson et al., 2009). However, it is unknown whether similar amygdala recruitment already occurs in neonatal rats with the ELS exposure. Recent work demonstrated that global and gene-specific DNA methylation is significantly modified in the amygdala of postnatal day (PND) 30 rats following chronic ELS (Doherty et al., 2016) and adolescent rats exhibited enhanced amygdalar neural activity when exposed to ELS (Raineki et al., 2012). These findings indicate that the consequences of ELS on the amygdala can be observed early, although the proximal effects of ELS on the neonatal amygdala are still unknown. In this study, we used the limited bedding (LB) paradigm as a naturalistic model of ELS (Molet et al., 2014; McLaughlin et al., 2016) to examine neonatal amygdalar function and morphological changes induced by ELS in the preweaning period. We examined the potential contribution of altered neonatal HPA activity in mediating amygdala changes as ELS is known to modify the activity of the HPA axis (Jankord and Herman, 2008) ranging from sensitized to blunted basal and stress responses depending on the life period tested (McLaughlin et al., 2016; Walker et al., 2017). Finally, we determined whether ELS-induced changes in the preweaning amygdala are sex dependent and lead to impairment in behavioral regulation related to anxiety and fear in adult male and female rats.

Methods

Animals:

Untimed-pregnant (gestation day 15-16) Sprague-Dawley female rats (Charles River, St-Constant, QC, Canada) were individually housed under controlled conditions of light (12 hr light:12 hr dark, lights on at 08:00 h), temperature (22-24°C), and humidity (70–80%) and provided access to rat chow and water *ad libitum.* The day of birth was considered PND0 and litters were culled to 8-10 pups on PND1. No more than 1-2 pups per sex and litter were used for each experimental data point. All experimental procedures were approved by the University Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care.

Limited bedding paradigm and maternal behavioral observations:

The LB paradigm was used between PND1-9 according to a protocol adapted from Baram and colleagues (Molet et al., 2014) with cage changes on PND4 and PND7. On PND1, mothers and their litters were randomly assigned to the limited bedding (LB) or normal bedding (NB) condition. LB mothers and their litters were placed on an aluminum mesh platform 2.5 cm above the cage floor. Approximately 1.5 cm of bedding was added below the platform to cover the cage floor. The dams were given one-half of one paper towel for nesting material. The NB cages received a 2.5 cm layer of woodchips and one-half of one paper towel. On PND10, all LB mothers/litters were returned to NB conditions. All litters were kept with their biological mother for the duration of the experiments. The weights of all pups and dams were recorded on PND1, 4, 7, 9, 14 and 18 and animals

were otherwise left undisturbed until experiments and tissue collection on PND10 and PND18-22.

Across all scored experimental cohorts (n=3, 13 litters/bedding condition), maternal behavior (active/passive nursing, pup grooming, pup retrieving, self-grooming, sleeping, eating, drinking and wandering) is reported between PND5-6 using three 72 min observation periods (two sessions in the light phase and one in the dark phase) as previously described (McLaughlin et al., 2016). Behavior was recorded every minute for each 72 min observation session. The fragmentation of overall behavior, i.e., the degree to which maternal behaviour occurs in many short bouts (Baram et al., 2012), during each observation period was determined using a behavioral consistency score in which a score of “1” was given when behavior changed from one epoch (minute) to the next, and “0” when there was no change in the type of behavior exhibited (Ivy et al., 2008).

Stress procedures:

Neuroendocrine responses to stress were compared between NB and LB pups using exposure to 60 min of immobilization (PND10) (McLaughlin et al., 2016) or restraint (PND20). For immobilization stress on PND10, male pups (6-7/group/time point) were placed side-by-side on a plastic surface with two 2 cm-wide strips of adhesive tape placed across the back of each pup while head and limb movements remained unrestricted (McLaughlin et al., 2016). On PND20, pups (6-26/group/time point) were placed in plastic restraint cone bags for 60 min as an acute restraint stress. Pups were sacrificed right before stress onset (0 min) and at the end of stress (60 min) exposure. Trunk blood samples were collected into tubes containing EDTA (60 mg/mL) and plasma was stored

at −20°C before determination of ACTH and corticosterone (CORT) concentrations. Brains of pups in the no stress (0 min) group were collected, rapidly frozen on dry ice and stored at -80°C prior to being sliced for determination of amygdalar volume.

Preweaning amygdalar volume:

Frozen PND10 and 20 brains were sectioned coronally on a cryostat at 18 and 20 µm, respectively, and sections were collected onto Superfrost Plus slides (ThermoFisher, Montreal, QC). The serial sections were dried under vacuum overnight and stored at -80˚C until stained with Cresyl Violet. The basolateral amygdala (BLA) and central amygdala (CeA) regions were identified by referring to the rat brain atlas of Paxinos and Watson (adult) (Paxinos and Watson, 2005) and Sherwood and Timiras (neonatal) (Sherwood and Timiras, 1970). These structures were contoured on 12 sections/brain for PND10 pups (6 male and female pups/group) and 20 sections/brain for PND20 pups (6 male and female pups/group), using Stereoinvestigator software (Cavalieri method, MicroBrightField, Williston, VT) with the experimenter blind to bedding group. The grid points (20 µm grid spacing) overlapping the contoured areas were counted and converted into volume estimates using the Cavalieri probe after accounting for the non-consecutive section interval and section thickness (Gundersen and Jensen, 1987).

Morphological analysis of BLA neurons:

Naïve NB or LB male and female pups (PND10 or PND20) were anesthetized with ketamine/xylazine (0.1 mL, subcutaneous) and perfused transcardially with 0.9% ice-cold saline-heparin (5USP units/mL heparin) for 5 min. Following perfusion, brains were kept

in Golgi-Cox solution (1.04% $K_2Cr_2O_7$, 1.04% $HgCl_2$, and 0.83% K_2CrO_4 diluted in distilled water, all reagents from Fisher Scientific, Fair Lawn, NJ) for 14 days in the dark at room temperature before being transferred in 30% sucrose at 4°C for 2-7 days (Gibb and Kolb, 1998). Coronal sections (200 μm) were cut using a vibratome (Leica, Concord, ON) and immersed into a 6% sucrose solution. Sections were placed serially onto 2% gelatin-coated slides, dried and stained with 100% ammonium hydroxide followed by fixative (Carestream GBX fixer, diluted 1:1 in distilled water, Sigma-Aldrich, St Louis, MO). Sections were dehydrated in serial alcohol rinses, cleared in xylene and coverslipped. Golgi-Cox stained Class I BLA neurons were selected for analysis on the basis of morphological criteria described previously (McDonald, 1982).

Neurons were manually traced by an experimenter blind to bedding condition, with the Neurolucida software (MicroBrightField, Williston, VT) using a Zeiss Imager M1 microscope with a 100x objective and a Hamamatsu camera. Digital images were captured with 20x and 100x objectives. 1-4 neurons were analyzed per animal (8-10 male and female pups/group), for a total of 21 male and 16-18 female (PND10) and 16 male and 16 female (PND20) neurons per experimental group. Branched structure analyses were performed in Neuroexplorer (MicroBrightField, Williston, VT) on reconstructed neurons to determine total dendritic length, number of branch points and number of dendritic spines. To calculate spine density (spines/μm), the total number of spines was divided by the total dendritic length for each neuron.

Whole-cell and field recording of preweaning BLA neurons:

In vitro electrophysiological whole-cell or field recordings of BLA neurons were performed on slices from PND18-22 preweaning male pups, either from the NB or LB condition. The brains were rapidly collected after light isoflurane anesthesia and coronal slices (250 μm) containing the amygdala were cut using a Vibratome (Leica, Concord, ON) in hyperosmotic, ice-cold carbogenated (95% O_2 and 5% CO_2) sucrose-substituted artificial cerebrospinal fluid (S-aCSF) containing (mM) 252 sucrose, 2.5 KCl, 0.1 $CaCl_2$, 4 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, and 1.25 NaH_2PO_4 (pH 7.35, 360–370 mOsm). Slices were subsequently incubated in carbogenated normal aCSF (125 mM NaCl instead of sucrose; 310–320 mOsm) at 32°C for 1 hr and then kept at room temperature for at least 1 hr before the onset of recordings.

Whole-cell recordings of preweaning BLA neurons were performed as previously described (Tse et al., 2011). Amygdala slices were transferred to a recording chamber perfused continuously with carbogenated aCSF, and TTX (0.5 μM) to prevent the generation of action potentials and isolate miniature synaptic activities. Recordings were obtained with borosilicate glass patch pipettes containing (mM) 132.5 Cs-gluconate, 17.5 CsCl, 10 HEPES, 2 $MgCl_2$, 0.5 ethylene glycol tetraacetic acid (EGTA), 4 ATP, 5 QX-314, and 0.5% neurobiotin (pH 7.2). Neurons were voltage-clamped at -60 mV for recording miniature excitatory postsynaptic currents (mEPSCs). The access resistance of the patch pipettes was continuously monitored, and only recordings with stable and low (<15MΩ) access resistance were used. All recorded neurons were labeled with neurobiotin (Ryan et al., 2013) to identify whether whole-cell recordings were obtained from spiny or aspiny

BLA neurons. Only labelled neurons with visible spines on 50% or more of the dendrites were included in the electrophysiological analysis.

Evoked field excitatory postsynaptic potentials (fEPSPs) were induced by a bipolar stimulating electrode that was placed in the LA, adjacent to the BLA (Wiltgen et al., 2009) and recorded by an aCSF-filled glass electrode (Fig. 5D). Bicuculline (5 μM), a $GABA_A$ receptor antagonist, was added in the perfusing solution during fEPSP recordings. All recordings were amplified by MultiClamp 700B and stored in a PC for offline analysis using the Mini Analysis Program 6.0.3 for whole-cell recordings (Synaptosoft, Decatur, GA) and Clampfit for fEPSP recordings (Axon, Molecular Devices, Sunnyvale, CA).

Neurobiotin labeling:

Cells were labelled with 0.5% neurobiotin in the intracellular solution during whole-cell recording (Ryan et al., 2013). Immediately after recording, slices were transferred to 4% paraformaldehyde (PFA) in 0.1 M Phosphate buffer (pH 7.4) and fixed overnight at 4ºC. The sections were rinsed with 0.1 M PBS containing 0.1% Triton X-100 (3 x 10 min) and incubated in a quenching solution of 0.1 M PBS, 0.1% Triton X-100 and 0.2% H_2O_2 for 2 hr at room temperature. After rinsing with PBS, the slices were incubated in a VectaStain Avidin/Biotinylated Enzyme Complex (ABC) solution (Vector, laboratories, Burlingame, CA) for 90 min at room temperature in 0.1 M PBS alone, and incubated for 2-3 min in a 3,3'-diaminobenzidine (DAB, 0.05%) solution in H_2O_2. The reaction was monitored and stopped by washing in PBS. The slices were then mounted onto 2% gelatin-coated slides, partially dried overnight in a closed, damp paper towel-lined

Tupperware box, dehydrated through a graded series of alcohols, cleared with xylene for 10 min and coverslipped with Permount mounting medium.

Hormonal assays:

Plasma ACTH and CORT concentrations were measured using commercially available radioimmunoassay (RIA) kits as previously described (McLaughlin et al., 2016). For ACTH RIA (MP Biomedicals, Santa Ana, CA, Cat# 07-106102) 50 µl samples were assayed in duplicates and the limit of detection of the assay was 7 pg/ml. For CORT RIA (MP Biomedicals Santa Ana, CA, Cat# 07-120102) 5 µl of samples were assayed in duplicates and the limit of detection of the assay was 0.031 µg/dl.

Adult behavioral tests:

Behavioral testing was performed on adult (PND60-78) offspring from NB and LB mothers (n=4 mothers/group) by an experimenter blind to group. A total of 16 male (7-9/group) and 16 female (8/group) rats were weaned on PND21 and transferred to a reverse 12 h light/dark cycle room (lights off at 08:00 h). Animals were same-sex, group-housed and handled weekly by the same experimenter. One week before testing, animals were individually housed (with environmental enrichment) in the same room, mainly to increase interaction with a novel partner in the social interaction test (File, 1990). Animals were tested in a battery of tasks, separated up to a maximum of one week apart, to assess anxiety-related behavior. All behavioral tests were performed in the dark under red light between 09:30 h and 14:00 h and rats were left to acclimatize in the experimental room for at least 30 min prior to test onset. All equipment (EPM, open field, fear chamber

(including floor bars and waste tray)) was cleaned with Peroxyguard between trials. Seeing as the hormonal variations during the estrous cycle of female rats might influence behavior (Marcondes et al., 2001), vaginal smears were obtained from all females for estrous cycle stage determination at the end of the EPM, social interaction and fear retrieval tasks. We did not intend to test the females in a particular phase of their estrous cycle, however the interval between the various behavioral tests was close to the duration of two estrous cycles (7-10 days), resulting in a similar ratio of females in a given phase at each determination. Behavior was recorded by video camera and scored using the TopScan (Clever Sys Inc, Reston, VA) tracking software, with the exception of the fear conditioning experiments, which were analyzed using the FreezeScan (Clever Sys Inc, Reston, VA) software.

Elevated plus maze: To evaluate anxiety, animals were placed at the junction of the open and closed arms of the EPM apparatus (110 cm × 10 cm arms, two open arms and two closed arms) facing the open arm opposite to the location of the experimenter (Walf and Frye, 2007). Animals remained in the EPM for a total duration of 5 min. Number of entries into and time spent in each arm and the center zone of the apparatus were compared between groups.

Open field and social interaction: Social interaction has been repeatedly used as a behavioral assay for anxiety-related behavior, because it is significantly reduced after administration of anxiogenic drugs (Overstreet et al., 2002). Adult rats (PND64-PND71) were paired with peripubertal male and female rats (PND40-52) that were always group-

housed. No peripubertal animal was tested more than once per day. To reduce novelty and increase social interaction, all animals (including peripubertal rats) were habituated to the open field arena for 5 min for two days before testing (File and Hyde, 1978). The time spent (sec) in the center area vs the periphery of the arena was documented on the first habituation day to evaluate anxiety-related behavior in the open field. On day three, the test animal was marked with a permanent marker on the head and placed at the opposite corner from the peripubertal animal in the arena. The animals were left to explore and interact in the open field for 5 min and social contact behavior was assessed.

Cued fear conditioning, extinction and retrieval: Cued fear conditioning was compared between adult male and female NB and LB rats using a protocol adapted from Stevenson et al. (Stevenson et al., 2009). On day 1, animals underwent fear conditioning in operant boxes with a metal rod floor delivering the shocks. A tone was used as the conditioned stimulus or CS (amplitude: 80 dB; duration: 30 s; variable inter-trial interval (ITI): 2 min) and an electric shock constituted the unconditioned stimulus or US (intensity: 0.5 mA for males, 0.7 mA for females; duration: 0.5 s, co-terminating with the tone). The CS parameters were the same on all three days of testing. On day 1, animals were placed in the chamber for 5 min and then presented with two habituation tones alone. After an ITI of 2 min, six tone-shock pairings were presented. On day 2, the expression of fear conditioning was assessed by the presence of freezing while in the same context as day 1. Freezing was defined by the lack of all movements except those related to breathing (Stevenson et al., 2009). Animals were subjected to fear extinction, which consisted of presenting 8 tones alone (all parameters same as day 1) after a 1 min habituation period.

On day 3, animals were assessed for fear retrieval by presenting six tones alone after a 1 min acclimation. Freezing behavior was determined for all 3 testing days using the FreezeScan (Clever Sys Inc, Reston, VA) software.

Statistical analyses:

Maternal behavior, hormonal data, and fEPSP were analyzed using a two-factor analysis of variance (ANOVA), with appropriate within and between subject factors. Morphological data were analyzed using two- and three-way between-subject ANOVAs. Simple main effect tests were used to dissect any significant interactions from the ANOVAs. Whole cell recordings and behavioral data, such as EPM, open field and social interaction, were analyzed using separate unpaired, one-tailed Student's t-tests, as appropriate. Fear conditioning (day 1) and extinction (day 2) data were analyzed using a two-factor analysis of variance (ANOVA), with appropriate within and between subject factors. To contrast freezing between tones, Bonferroni *post-hoc* analyses were used for the fear extinction (day 2) data. Fear retrieval (day 3) was analyzed using unpaired, one-tailed Student's t-test. Data are presented as means ± standard error of the mean (SEM). Statistical significance was set at $P < 0.05$.

Results

Effect of bedding conditions on maternal behavior and pup weight gain

Analysis of maternal behavior on PND5-6 (13 litters/bedding condition) was performed using two-way ANOVAs, with bedding and light cycle as between-subjects factors.

Behavior was divided into three main categories: nursing (both active and passive), pup grooming and fragmentation (Table 1). LB mothers spent significantly more time nursing compared to NB mothers (bedding effect: $F(1,48) = 7.61$, $p < 0.01$), and all mothers tended to nurse more during the light phase (light cycle effect: $F(1,48) = 53.92$, $p < 0.001$).

Consistent with results documented by others (Molet et al., 2014), pup grooming behavior was similar between LB and NB mothers and not altered by light cycle. Analyses of fragmentation scores yielded no significant main effect of bedding ($F(1,48) = 1.53$, $p = 0.22$) and no bedding x light cycle interaction ($F(1,48) = 3.06$, $p = 0.087$) although mothers generally changed behaviors more frequently during the dark compared to the light phase (light cycle effect: $F(1,48) = 29.56$, $p < 0.001$).

Student's t-tests used to analyze delta body weight found that LB pups displayed significantly reduced weight gain between PND1-9 ($t(36) = 2.922$, $p < 0.005$) compared to NB pups, as observed previously (McLaughlin et al., 2016). There were no significant differences in the weight gain of NB vs LB pups between PND9-18 ($t(13) = 0.933$, $p = 0.18$). Despite this, the effects of LB rearing on pup body weight were still significant when comparing weight gain between PND1-18 ($t(14) = 2.218$, $p < 0.05$) (Table 1).

Maternal Behavior Score	*NB*		*LB*	
	Light phase	**Dark phase**	**Light phase**	**Dark phase**
Nursing	14.85 ± 1.06	4.15 ± 1	17.04 ± 1.32	9 ± 1.63
Pup grooming	1.92 ± 0.39	1.69 ± 0.36	2.23 ± 0.37	2.53 ± 0.48
Fragmentation	6.46 ± 0.94	12.38 ± 1.08	6.88 ± 0.49	9.92 ± 0.65
Delta Body Weight (g)	*NB*		*LB*	
PND9-1	18.80 ± 0.63		15.82 ± 0.84 **	
PND18-9	31. 86 ± 1.71		29.81 ± 1.29	
PND18-1	52.18 ± 2.57		44.96 ± 1.61 *	

Table II-1. Maternal behavior scores and body weight changes in normal and limited bedding pups. Mean scores of normal (NB) and limited (LB) bedding mothers for total nursing, pup grooming, and fragmentation behaviors between PND5-6 and delta body weight of pups. The mean light phase observation scores were compared to scores during the dark phase period for each bedding condition. Two-way ANOVAs of bedding condition (NB vs LB) by light cycle (light vs dark phase) showed a significant bedding effect ($p<0.01$) for nursing, and a significant light cycle effect ($p<0.001$) for nursing and fragmentation (n = 13 litters/group pooled from three separate experiments). Student's t-tests used to analyze delta body weight found significant reductions in body weight gain in LB compared to NB pups between PND 9-1 ($p<0.005$) and 18-1 ($p<0.05$) (n = 2-4 litters/group pooled from 2-5 separate experiments). Values represent mean ± SEM. *$p<0.05$, **$p<0.005$ NB vs. LB.

Effect of bedding conditions on stress responsiveness in offspring

As shown in Figure 1, a two-way ANOVA with bedding and time as between-subjects factors revealed that basal ACTH and CORT concentrations were not affected by bedding condition on PND10 and PND20. For plasma ACTH, we observed no significant effect of bedding or time on PND10, while on PND20 the effect of time was significant ($F(1,23) = 148.52$, $p < 0.001$). Two-way ANOVA for plasma CORT on PND10 revealed no significant effect of bedding, but a significant effect of time ($F(1,21) = 37.12$, $p < 0.001$) and bedding x time interaction ($F(1,21) = 4.67$, $p < 0.05$). Both NB and LB rats showed significant stress responses ($p < 0.05$), but the response of LB compared to NB pups was blunted ($p < 0.01$). In PND20 pups, both NB and LB pups exhibited a significant CORT response to stress (time effect: $F(1,57) = 99.09$, $p < 0.001$), but no main effect of bedding or bedding x time interaction.

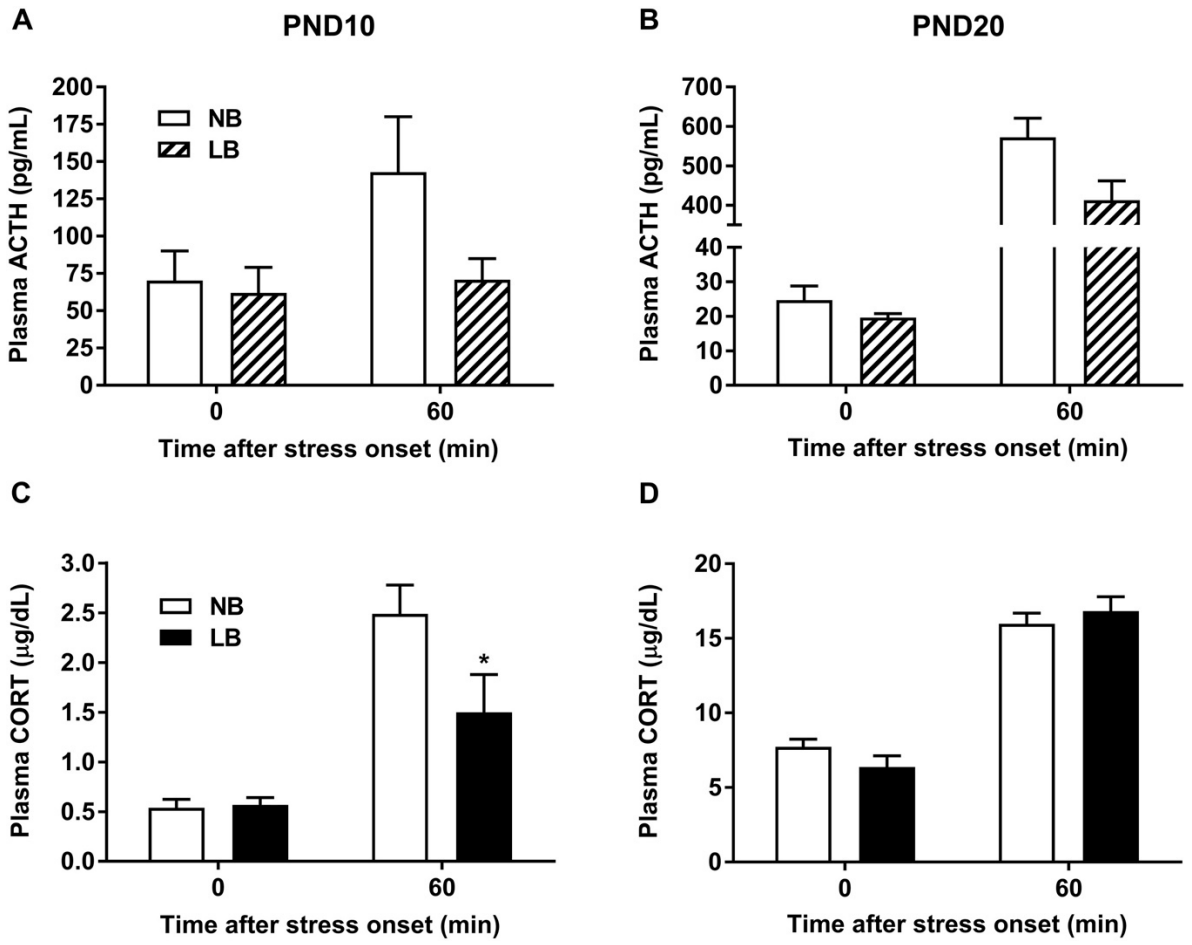

Figure II-1. Plasma ACTH and corticosterone (CORT) stress-induced concentrations (60 min, restraint stress) in normal bedding (NB, white bars) and limited bedding (LB, patterned and black bars) in postnatal days (PND) 10 (A,C) and 20 (B,D) pups. On PND10, LB pups displayed a significantly blunted stress-induced CORT release, 60 min post-stress ($p<0.05$). Two-factor ANOVAs yielded a significant main effect of time in PND20 pups for both ACTH and CORT responses ($p<0.001$). Values represent mean ± SEM of n = 6-7 pups per group (PND10) and 6-26 pups/group (PND20). *$p<0.05$ NB vs LB

Prolonged effects of bedding condition on amygdala morphology in male, but not female rats

Early stress-induced changes in the volume of the BLA and CeA were determined because of the critical implication of these areas in fear conditioning (Campeau and Davis, 1995) and stress circuitry (Jankord and Herman, 2008), respectively. We predicted that LB would enhance preweaning BLA volume, as does chronic stress in adult rodents (Vyas et al., 2006). No predictions were made for CeA volume, because the neuronal volume in this structure is not typically affected by chronic stress (Vyas et al., 2003). Two-way ANOVAs were conducted with bedding and sex as between-subject factors for the BLA and CeA. As depicted in Figure 2, there was a significant effect of sex on BLA volume on PND10 ($F(1,20) = 9.42$, $p < 0.01$), but no effect of bedding or bedding x sex interaction. Male pups had significantly larger BLA volumes compared to females. On PND20, in addition to observing a significant effect of sex ($F(1,20) = 7.25$, $p < 0.05$), with greater BLA volumes in males compared to females, we also found a trend towards increased BLA volume in LB compared to NB pups ($F(1,20) = 3.95$, $p = 0.061$). For CeA volume, there were no significant effects of sex, bedding or bedding x sex interaction on PND10. The CeA volume on PND20 was not modified by bedding ($F(1,20) = 1.73$, $p = 0.2$) or sex, and did not display a bedding x sex interaction.

The dendritic length, branching, total spine numbers and spine density of BLA neurons for each age group are displayed as graphs in the top panels of Figure 3 (males) and Figure 4 (females). The bottom panel of Figure 3 depicts magnified digital images of BLA neurons in males (PND10 and 20), whereas the bottom panel of Figure 4 includes images of BLA neuron dendritic segments to show differences in spine numbers in PND20 male

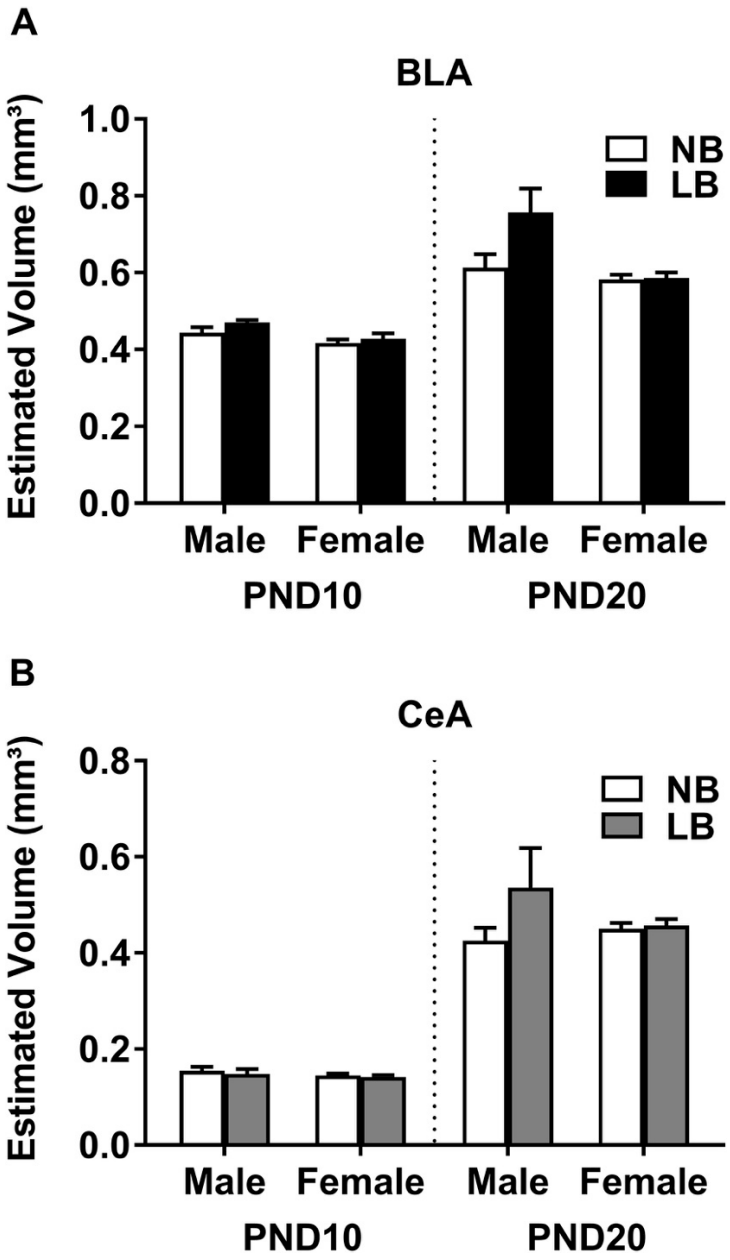

Figure II-2. Estimated basolateral (BLA) (A) and central (CeA) (B) amygdala volumes in normal bedding (NB, white bars) and limited bedding (LB, shaded bars) male and female pups on postnatal days (PND) 10 and 20. A two-way ANOVA revealed a trend for slightly enhanced BLA volume in LB pups on PND20 ($p=0.06$). Values represent mean ± SEM of n = 6 pups/group.

and female pups. We first performed two-way ANOVAs with bedding and age as between-subjects factors. For males, there were significant bedding x age interactions for all morphological parameters (length: $F(1,70) = 13.06$, $p < 0.001$, total spines: $F(1,70) = 41.54$, $p < 0.001$, branching: $F(1,70) = 8.37$, $p < 0.01$) except spine density, which showed significant main effects of bedding ($F(1,70) = 19.21$, $p < 0.001$) and age ($F(1,70) = 167.99$, $p < 0.001$). Simple effects tests revealed that dendritic length (Fig. 3B), total spine number (Fig. 3A) and branching (Fig. 3D) were enhanced in LB males compared to NB males, but only on PND20 ($p < 0.001$). Spine density was generally increased in LB males compared to NB males ($p < 0.001$, Fig. 3C). Females did not exhibit significant main effects of bedding for any of the parameters ($p > 0.05$), but there were main effects of age for branching ($F(1,62) = 6.45$, $p < 0.05$, Fig. 4D) and total spine number ($F(1,62) = 108.37$, $p < 0.001$, Fig. 4A). Dendritic length showed a near significant bedding x age interaction ($F(1,62) = 3.80$, $p = 0.056$), which was likely associated with greater total length in NB females on PND20 (Fig. 4B). Spine density displayed a significant bedding x age interaction in females ($F(1,62) = 4.96$, $p < 0.05$) and a simple effects test revealed that LB females had significantly fewer spines on PND10, compared to NB females ($p < 0.05$, Fig. 4C).

We next interrogated sex differences using a three-way ANOVA and found significant bedding x age x sex interactions for all morphological parameters (length: $F(1,132) = 15.40$, $p < 0.001$, total spines: $F(1,132) = 14.01$, $p < 0.001$, branching: $F(1,132) = 6.58$, $p < 0.05$) except spine density ($F(1,132) = 2.66$, $p = 0.1$). Spine density showed significant bedding x age ($F(1,132) = 5.22$, $p < 0.05$) and bedding x sex effects ($F(1,132) = 12.77$, $p < 0.001$). Simple effects tests revealed that on PND10, NB males had significantly fewer

spines compared to NB females ($p < 0.05$). On PND20, NB and LB males had significantly longer dendrites compared to NB ($p < 0.01$) and LB females ($p < 0.001$), respectively. LB males also had significantly more branch points ($p < 0.001$) and total spines ($p < 0.001$) compared to LB females on PND20. Simple effects tests revealed significantly greater spine density in NB females compared to NB males ($p < 0.001$).

Males

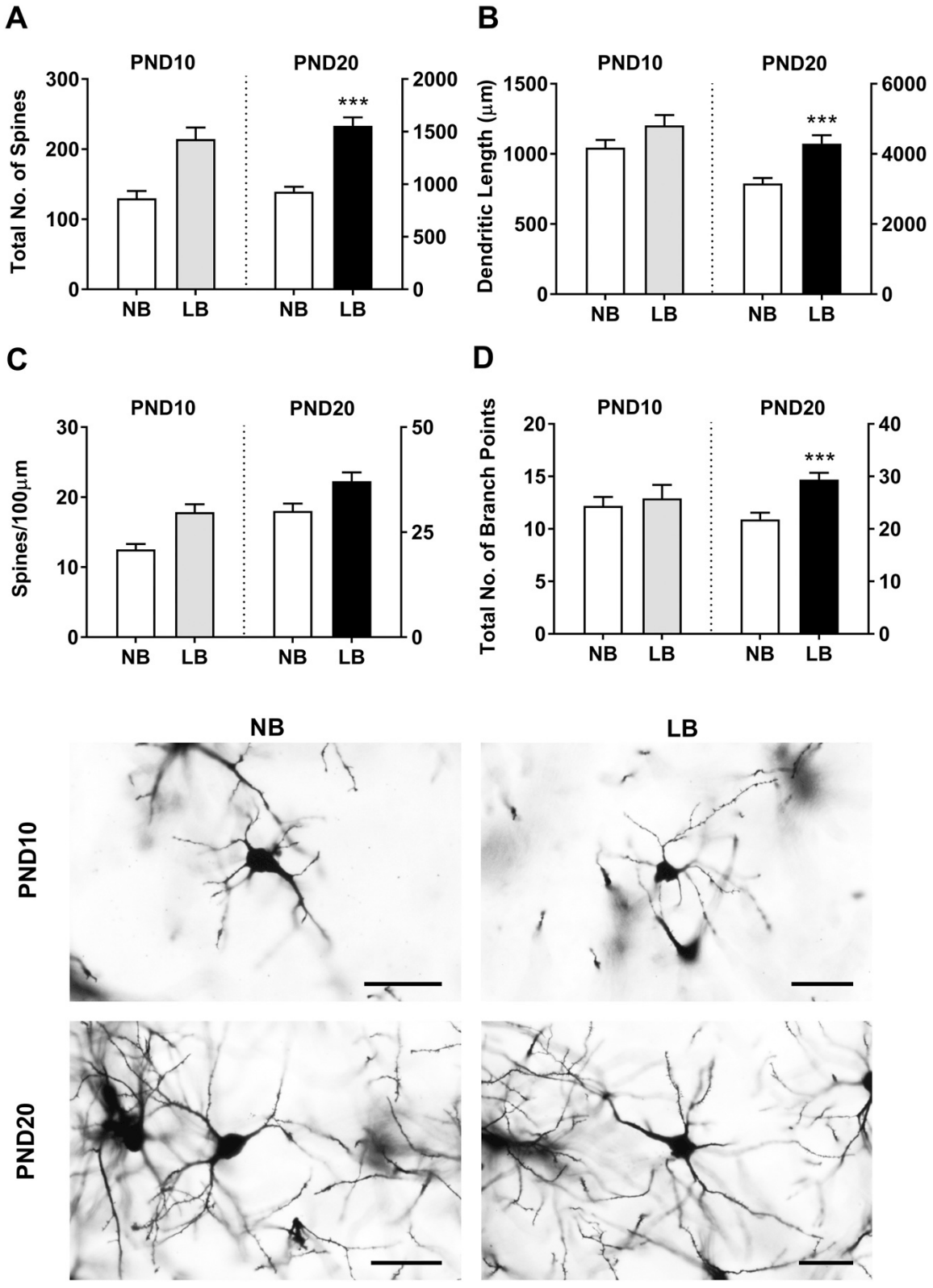
A
PND10
PND20
Total No. of Spines
300
200
100
0
2000
1500
1000
500
0
***
NB
LB
NB
LB
B
PND10
PND20
Dendritic Length (μm)
1500
1000
500
0
6000
4000
2000
0
***
NB
LB
NB
LB
C
PND10
PND20
Spines/100μm
30
20
10
0
50
25
0
NB
LB
NB
LB
D
PND10
PND20
Total No. of Branch Points
20
15
10
5
0
40
30
20
10
0
***
NB
LB
NB
LB
NB
LB
PND10
PND20

Figure II-3. Rearing under limited bedding conditions impacts basolateral amygdala neuron morphology in preweaning male rats. Effects of bedding condition on number of spines (A), mean dendritic length (B), spine density (C), and number of branch points (D) in normal bedding (NB, white bars) and limited bedding (LB, shaded bars) basolateral amygdala (BLA) neurons on postnatal days (PND) 10 (n = 21 neurons/group, 2-4 neurons/animal, 8 animals/group) and 20 (n = 16 neurons/group, 1-3 neurons/animal, 9 animals/group). On PND20, LB males displayed increased total spine numbers, dendritic length, and branching compared to NB pups. Spine density was increased in LB males compared to NB males ($p < 0.001$). Bottom panel: Magnified digital images (20x objective) of Golgi-stained NB and LB BLA neurons in PND10 and 20 male pups. Scale bars = 50 μm. ***$p<0.001$; two-way ANOVA. Values represent mean ± SEM.

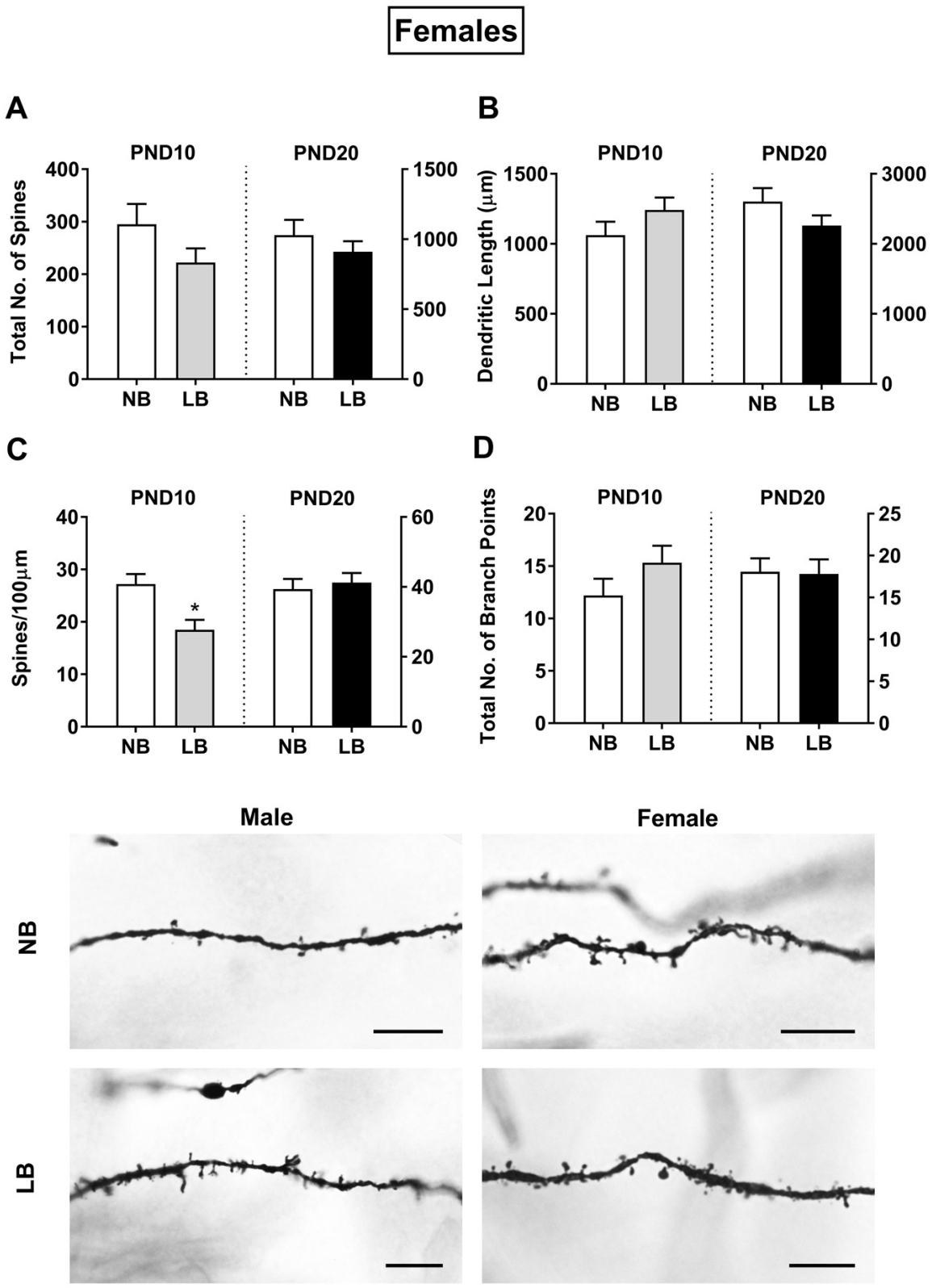
Females
A
PND10
PND20
Total No. of Spines
400
300
200
100
0
1500
1000
500
0
NB
LB
NB
LB
B
PND10
PND20
Dendritic Length (μm)
1500
1000
500
0
3000
2000
1000
0
NB
LB
NB
LB
C
PND10
PND20
Spines/100μm
40
30
20
10
0
60
40
20
0
*
NB
LB
NB
LB
D
PND10
PND20
Total No. of Branch Points
20
15
10
5
0
25
20
15
10
5
0
NB
LB
NB
LB
Male
Female
NB
LB

Figure II-4. ELS exposure does not induce lasting morphological changes in the BLA of female preweaning rats. Effects of bedding condition on number of spines (A), mean dendritic length (B), spine density (C), and number of branch points (D) in normal bedding (NB, white bars) and limited bedding (LB, shaded bars) basolateral amygdala (BLA) neurons on postnatal days (PND) 10 (n = 16-18 neurons/group, 2-4 neurons/animal, 8 animals/group) and 20 (n = 16 neurons/group, 1-2 neurons/animal, 10 animals/group). Spine density was significantly reduced on PND10 in LB females, compared to NB females. Bottom panel: Magnified digital images (100x objective) of Golgi-stained NB and LB BLA neurons to show differences in spine numbers in PND20 male and female pups. Scale bars = 10 μm. *$p<0.05$; two-way ANOVA. Values represent mean ± SEM.

Consequences of LB conditions on electrophysiological responses in the male preweaning amygdala

In view of our morphological data demonstrating lasting effects on the BLA in male, but not female LB offspring, we concentrated on investigating functional changes using electrophysiological recordings in male pups. As shown in Figure 5, Student's t-tests showed no significant bedding group differences in the frequency ($t(22) = -0.602$, $p = 0.27$), amplitude ($t(20) = -0.226$, $p = 0.41$), rise time ($t(20) = -0.095$, $p = 0.46$) or decay time constant ($t(20) = 0.223$, $p = 0.41$) of action potential independent mEPSCs.

We next examined the effect of LB on evoked synaptic function (fEPSP) in the BLA. Recordings of fEPSP were performed and stimulus intensity was changed accordingly to generate increasing fiber volley sizes ranging from 0.1 to 0.4 mV (Fig. 5E). A two-factor ANOVA was computed using bedding and fiber volley amplitude as a between- and within-subjects factor, respectively. There were significant main effects of bedding ($F(1,26) = 4.79$, $p < 0.05$), with LB neurons displaying higher fEPSP slopes, and fiber volley amplitude ($F(3,78) = 33.36$, $p < 0.001$), but no bedding x fiber volley amplitude interaction ($F(3,78) = 2.26$, $p = 0.088$) (Fig. 5F).

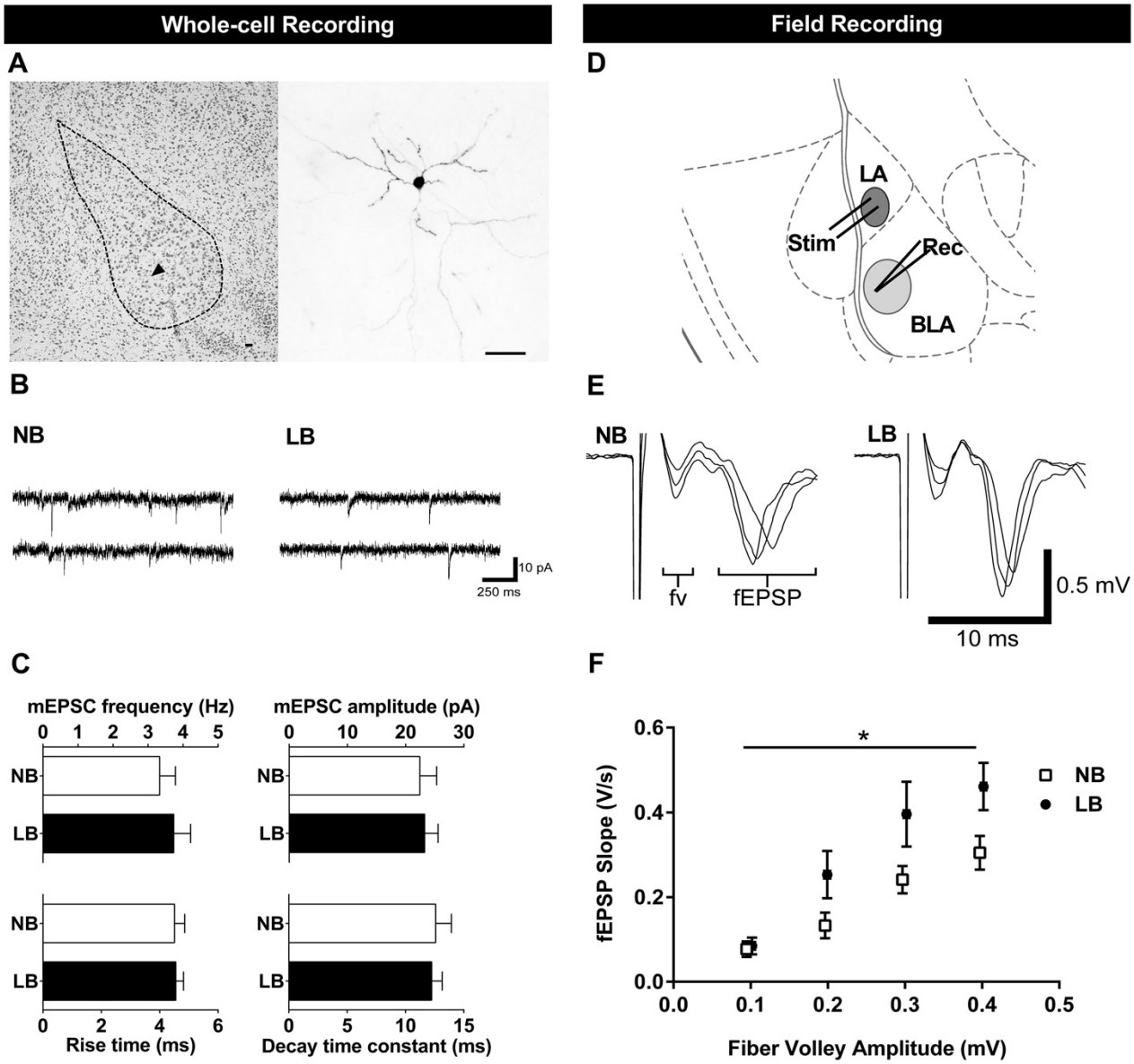
Whole-cell Recording
Field Recording
A
D
LA
Stim
Rec
BLA
B
E
NB
LB
10 pA
250 ms
fv
fEPSP
0.5 mV
10 ms
C
F
mEPSC frequency (Hz)
mEPSC amplitude (pA)
Rise time (ms)
Decay time constant (ms)
fEPSP Slope (V/s)
Fiber Volley Amplitude (mV)
*

Figure II-5. Consequences of limited bedding exposure on electrophysiological responses in the basolateral amygdala (BLA) between postnatal days (PND) 18 and 22. (A) Left panel: Cresyl violet stained section with the BLA contoured at 4x magnification. Arrowhead shows approximate location of neurobiotin labeled neuron. Right panel: Neuron from the BLA labeled with neurobiotin during whole-cell recording at 20x magnification. Scale bars = 50 μm. (B) Representative whole-cell recording traces from BLA neurons of normal (NB) and limited bedding (LB) pups. (C) There were no group differences in the functional properties (averaged frequency, amplitude, rise time and decay time constant) of miniature excitatory postsynaptic currents (mEPSCs) in BLA neurons (n = 11-13 neurons/group, 1-2 neurons/animal, 8-11 animals/group). (D) Recordings of evoked field excitatory postsynaptic potentials (fEPSP) were performed by placing the stimulating electrode in the lateral amygdala (LA) and the recording electrode in the BLA. (E) Representative average traces of fEPSP at fiber volley sizes 0.3-0.5. (F) Two-way ANOVA found that LB exposure significantly increased fEPSP slope (n = 14 slices/group, 1-3 slices/animal, 6 animals/group). *$p<0.05$

Sex and bedding differences in anxiety-related behaviors

We tested whether increased total dendritic spine number and excitatory inputs in the neonatal BLA of LB pups would be associated with increased anxiety-like behavior in adult male rats (Table 2). As sex differences have been reported for the adult consequences of ELS, both male and female offspring were tested (Prusator and Greenwood-Van Meerveld, 2015). When tested in the EPM, 75 and 62.5% of adult female NB and LB rats, respectively, were in the proestrus or estrus stage of their cycle. The remaining rats were in the metestrus or diestrus stage of their cycle. All anxiety-related behaviors were evaluated using Student's t-tests. The number of entries into and time spent in the different arms and center area of the EPM were not different between groups for both male and female rats. However, male LB rats spent significantly more time in the closed arms of the maze, compared to NB rats ($t(14) = -2.457$, $p < 0.05$) and less time in the center area of the maze ($t(14) = 2.319$, $p < 0.05$). In contrast, female LB rats spent significantly less time in the closed arms ($t(14) = 2.208$, $p < 0.05$) compared to their NB counterparts.

The open field test provides another measure of anxiety while also evaluating habituation processes over two consecutive days. On the third day, social interaction with an unknown peripubertal conspecific was evaluated. On day 1, LB male offspring spent significantly more time in the periphery ($t(14) = -1.992$, $p < 0.05$) and less time in the center ($t(14) = 1.963$, $p < 0.05$) of the open field, compared to NB males (Table 2). No group differences were observed in females. On day 2, the open field was no longer novel and no effect of bedding condition was observed for either sex.

Social contact behavior between adult and same-sex peripubertal rats was evaluated after habituation (Table 2). Adult LB male rats spent significantly less time in contact with a peripubertal rat ($t(14) = 2$, $p < 0.05$) compared to NB rats, while there was no bedding effect in females. Vaginal smears obtained after the social interaction tests indicated that 87.5 and 75% of NB and LB females, respectively, were in the proestrus or estrus stage of their estrous cycle. The remaining rats were in the diestrus stage of their cycle.

	Males		Females	
	NB	**LB**	**NB**	**LB**
Elevated plus maze				
Open				
No. of entries	4.71 ± 0.94	3 ± 0.60	7.75 ± 1.53	8.88 ± 0.90
Time (s)	70.64 ± 23.16	34.12 ± 6.87	101.93 ± 15.46	138.26 ± 17.50
Closed				
No. of entries	7 ± 1.25	9.11 ± 0.82	6.5 ± 0.50	6.25 ± 0.86
Time (s)	113.34 ± 30.80	188.43 ± 12.72 *	101.51 ± 12.73	63.28 ± 11.73 *
Center				
No. of entries	10.71 ± 1.43	10.78 ± 1.10	11.75 ± 1.25	13.88 ± 0.67
Time (s)	116.12 ± 14.91	77.60 ± 9.03 *	97.56 ± 9.82	98.62 ± 9.65
Open field day 1				
Periphery				
Time (s)	255.53 ± 9.49	276.74 ± 5.85 *	281.21 ± 4.12	279.19 ± 5.10
Center				
Time (s)	44.68 ± 9.56	23.68 ± 5.86 *	18.86 ± 4.12	20.88 ± 5.10
Social Contact				
Time (s)	103.07 ± 7.90	81 ± 7.54 *	83.5 ± 12.02	84.7 ± 14.41

Table II-2. Male offspring subjected to chronic early-life stress display an anxiogenic phenotype. Behavior scores of adult rats from the elevated plus maze, open field (day 1) and social interaction tests showed sex differences following exposure to limited bedding (LB) conditions. Male LB offspring exhibited increased anxiety-like behavior in all the above-mentioned tasks, compared to their normal bedding (NB) counterparts ($p < 0.05$; t-test). Females did not show these behavioral outcomes ($p > 0.05$; t-test). Values represent mean ± SEM of n = 7-9 rats/group (males) and 8 rats/group (females). *$p < 0.05$, NB vs LB.

Effect of LB conditions on adult fear conditioning and retrieval

Amygdala-dependent cued fear conditioning was compared between NB and LB adult male and female offspring, followed by fear extinction on day 2 and fear retrieval on day 3 (Figure 6). Fear conditioning (day 1) and extinction (day 2) data were analyzed at each sex level using separate two-way ANOVA, with bedding as a between-subjects factor, and tone as a within-subjects factor. Fear retrieval (day 3) data were assessed with Student's t-tests. During fear conditioning (day 1), male LB rats exhibited more freezing behavior compared to NB male rats ($F(1,14) = 4.76$, $p < 0.05$), but there was no significant bedding effect in females. Conditioning was successful as there was an effect of tone for both males ($F(5,70) = 24.56$, $p < 0.001$) and females ($F(5,70) = 15.72$, $p < 0.001$), but no significant bedding x tone interaction.

Extinction of fear was observed for both males and females on day 2 since a two-way ANOVA yielded a significant main effect of tone in both sexes, (males: $F(7,98) = 2.17$, $p < 0.05$; females: $F(7,98) = 2.16$, $p < 0.05$) but no effect of bedding or bedding x tone interaction in either sex. Bonferroni contrasts between tones showed that males and females froze less during tone 8, compared to tones 3 and 6, respectively ($p < 0.05$).

On day 3 (fear retrieval), LB males froze more to the tones than NB males during fear recall ($t(10) = -4.579$, $p < 0.001$). A near significant result was also noted in females ($t(10) = -1.613$, $p = 0.069$). On day 3 of testing, 75% of NB and LB females were in the proestrus or estrus stage of their estrous cycle. The remaining rats were in the metestrus or diestrus stage of their cycle.

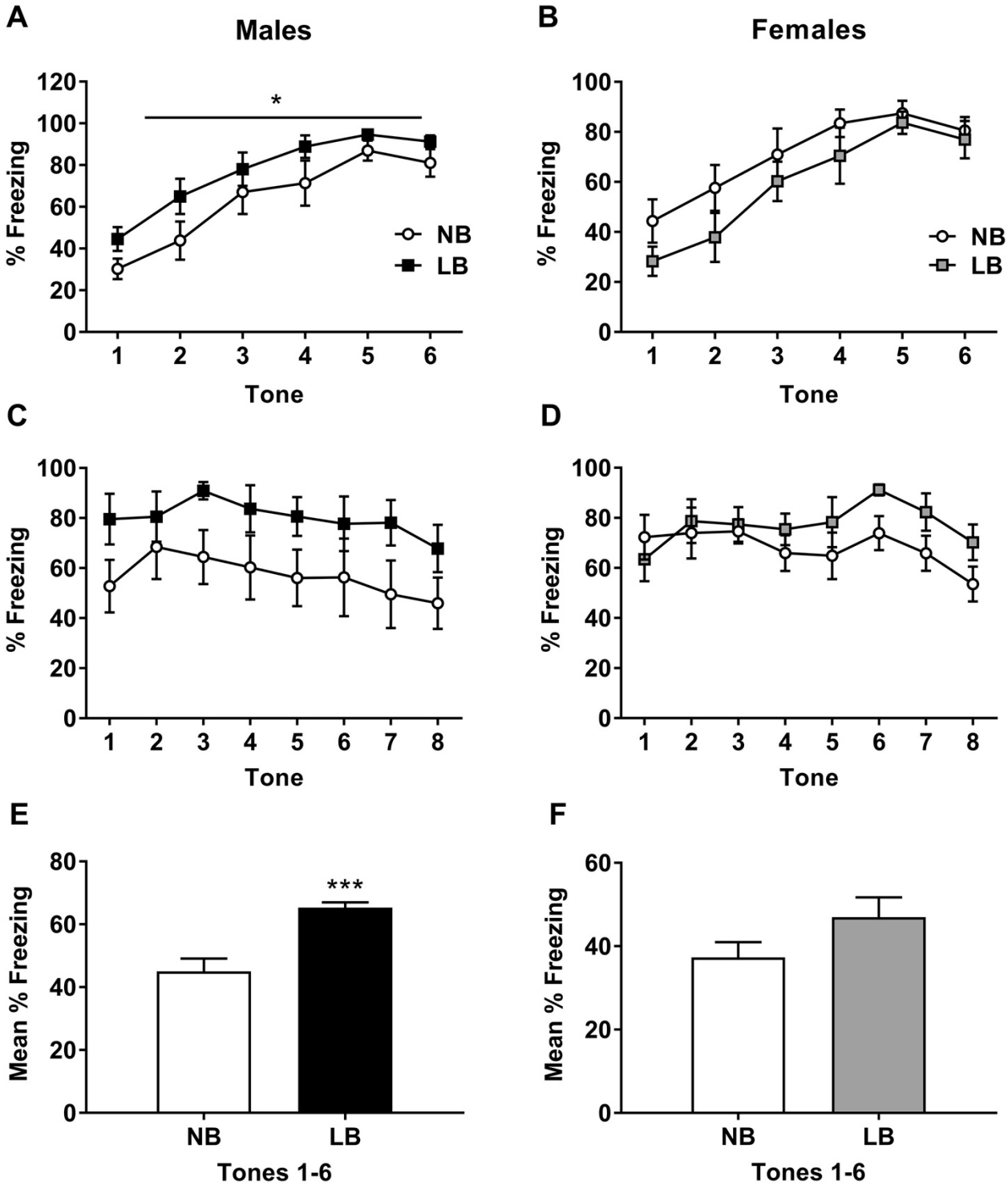
A
Males
% Freezing
Tone
NB
LB
*
B
Females
% Freezing
Tone
NB
LB
C
% Freezing
Tone
D
% Freezing
Tone
E
Mean % Freezing
***
NB
LB
Tones 1-6
F
Mean % Freezing
NB
LB
Tones 1-6

Figure II-6. Fear conditioning and retrieval are enhanced in adult male offspring reared under limited bedding conditions. Percentage of freezing behavior by normal bedding (NB, white circles and bars) and limited bedding (LB, shaded squares and bars) male and female rats during individual 30 s tones for fear conditioning (A, B) and extinction (C, D). Percentage of freezing during tones 1-6 was averaged for fear retrieval (E, F). (A) LB males exhibited significantly more freezing behavior during fear conditioning, compared to NB males ($p<0.05$; ANOVA). (B) This was not observed in females ($p>0.05$; ANOVA). (C, D) Two-way ANOVAs did not reveal significant bedding effects during fear extinction in either sex ($p>0.05$). (E) LB males displayed significantly more freezing behavior than NB males during fear retrieval ($p<0.001$; t-test). (F) On day 3, no significant group differences were noted in females ($p>0.05$; t-test). Values represent mean ± SEM of n = 7-9 rats/group (males) and 8 rats/group (females). *$p<0.05$, ***$p<0.001$

Discussion

In this study, we sought to document the early effects of exposure to the LB procedure on morphological and functional changes in the preweaning amygdala and regulation of stress responsiveness as indices of early chronic stress exposure. Our novel findings were that LB induced sexually dimorphic consequences in BLA morphology, with male rats showing increased spine density and dendritic length of neurons on PND10, reaching significance by PND20. These morphological changes were associated with, and might have been leading to functional changes in BLA excitability documented on PND18-20 in male pups. Interestingly, there were no significant morphological effects of LB in female neonates, apart from the fact that spine density was transiently reduced compared to NB females on PND10. Accordingly, emotional and social consequences of neonatal LB exposure were more pronounced in adult male, compared to female offspring.

Sex differences in BLA morphological outcomes following LB exposure

Under control (NB) conditions on PND10, females displayed significantly greater BLA neuron spine density compared to their male counterparts, although these sex differences normalized by PND20. In adults however, 3-5 month old and aged male rats exhibit more spines than females (Rubinow et al., 2009), suggesting that the direction of sex differences in BLA spine density are age-related. Several factors might mediate these sex differences in BLA morphology and the relative importance of these might also vary as a function of age and developmental period. Both androgens and estrogens have been

reported to affect dendritic spine proliferation, in a sex and region-specific manner (Womble et al., 2002; Tsurugizawa et al., 2005; Hajszan et al., 2008; Bender et al., 2017). In particular, androgens have been found to increase spine synapse formation in the hippocampus of male rats independently of aromatization (Hajszan et al., 2008). The early effects found in our study would point to organizational, rather than activational effects of sex steroids. Alternatively, sex-specific differences in maternal care (Oomen et al., 2009; van Hasselt et al., 2012), nutrition and metabolism (Yam et al., 2017), or epigenetic differences (Lucassen et al., 2013; Rodgers et al., 2015; Holland et al., 2016) might also be implicated.

Consistent with earlier findings in adult male rodents exposed to chronic stress (Vyas et al., 2002; Vyas et al., 2006), BLA neurons of preweaning LB male pups displayed significantly greater total dendritic length and increased total dendritic spine number compared to NB pups. Ten days after return to NB conditions, most BLA morphological parameters remained altered in males, suggesting that the amygdala, and specifically the BLA, exhibits persistent dendritic remodeling beyond the period of early stress exposure. Although we have not yet documented whether morphological changes induced by LB are maintained in adult animals, it is possible that the adolescence period provides yet another window for recovery of normal morphology in LB animals. BLA neurons from LB males also exhibited significantly greater dendritic arborizations compared to NB neurons on PND20, consistent with the fact that differences in branching may only become apparent near the peak of BLA dendritic arbor maturity, which occurs at the end of the first postnatal month (Ryan et al., 2016). Whereas LB significantly affected morphology of BLA neurons in males, no such effects were detected in females, except for a transient

decrease in spine density on PND10. These results highlight a striking contrast in how the BLA of males and females reacts to the LB paradigm, and more generally that females might be engaging protective mechanisms immediately during and after chronic stress exposure to prevent morphological changes and potentially protect long-term behavioral outcomes as seen in our study and others (Loi et al., 2017). Sexually dimorphic epigenetic, cellular and behavioral outcomes resulting from variations in the caregiving environment have been widely documented (Hill et al., 2014; Blaze and Roth, 2017; Loi et al., 2017; Walker et al., 2017), although only few studies have focused on the developing amygdala. Perturbations in amygdala-locus coeruleus and olfactory bulb network (Moriceau et al., 2009a), as well as enhanced amygdala neural activity during mother-pup interaction (Raineki et al., 2010a) have been reported in Long-Evans LB neonatal pups, but without sex differences in the responses. Similarly, sex differences in the effect of aversive caregiving on BDNF expression were not observed in neonates, but emerged later, by PND30 and in adulthood (Hill et al., 2014), demonstrating that only a few sexually dimorphic responses such as those documented in the present study are found in the preweaning amygdala. It remains possible that, once challenged after weaning or in adulthood, morphological and functional characteristics of the BLA would show higher vulnerability in LB females than their male counterparts.

Several local factors might regulate the structural plasticity observed in male preweaning BLA neurons, including changes in BDNF production and methylation as reported in LB rodents (Bath et al., 2013; Hill et al., 2014; Roth et al., 2014) and/or inflammatory processes and activated microglia. Interestingly, chronically-stressed adult rats display long-term upregulation of BDNF in the BLA that persists beyond termination

of stress exposure and correlates with dendritic hypertrophy (Vyas et al., 2004; Lakshminarasimhan and Chattarji, 2012).

There is now good evidence that in the healthy developing and adult brain, microglial cells modulate and support neuronal functioning by making transient contacts with dendritic spines, axon terminals, and several synapse-associated elements (Tremblay et al., 2010; Eyo and Wu, 2013). Microglia also mediate experience-dependent synaptic plasticity in developing mice for instance (Tremblay et al., 2010), by engulfing synapses (Paolicelli et al., 2011) and modifying spine number and size. In adult rats, chronic stress enhances microglial activation in the CeA, but not BLA (Tynan et al., 2010), highlighting cellular differences between the two subnuclei. The extent to which this occurs in development is unknown, but it is possible that reduced microglial activation in the preweaning BLA following LB might contribute to decrease synaptic pruning and thus, allow for increased spine numbers in males.

In agreement with previous studies (Rubinow and Juraska, 2009), we found no sex difference in the BLA (and CeA) volume at either PND10 or 20. However, we observed a modest enhancement of amygdala volume in LB male pups on PND20, concomitant with, and perhaps as a consequence of, the dendritic hypertrophy and increased spine number in the male BLA. The volume change was not significant in the CeA. The differential subregional effect of LB is difficult to explain but might result from the differential expression and activation of tissue Plasminogen activator (tPA) by CRH found in the CeA and not in the BLA (Pawlak et al., 2003; Matys et al., 2004; McEwen et al., 2016). tPA, a secreted protease, is required for stress-induced spine loss in the hippocampus and medial amygdala (McEwen et al., 2016) and could potentially be actively involved in

synaptic pruning in the neonatal CeA after LB exposure. Similar changes in cellular effector molecules in various brain structures as well as differential afferent inputs might determine the direction of stress-induced morphological effects to either increase (e.g. amygdala) or reduce (e.g. hippocampus, prefrontal cortex) spine numbers. In summary, our data suggest that LB-induced synaptic remodeling specifically in male offspring might also induce functional plasticity in the preweaning BLA.

Functional changes in the BLA induced by LB

Using *in vitro* electrophysiology, we investigated the effect of LB on the functional properties of excitatory synaptic inputs in preweaning male BLA neurons. We focused on males because LB-induced morphological changes were primarily seen in male and not female pups. Using patch clamp recordings, we did not see differences in the functional properties of mEPSCs between experimental groups. The discrepancy between increased dendritic spine number and the lack of change in mEPSC properties may suggest the presence of immature non-functional synapses or low release probability. However, we unravelled the heightened input-output relationship in evoked fEPSP experiments, supporting our hypothesis of stronger excitatory inputs on BLA neurons in LB neonates. Our findings are in accordance with a number of studies showing increased neuronal firing and excitability of pyramidal BLA neurons after chronic stress in adults (Rosenkranz et al., 2010) or after exposure to ELS (Rau et al., 2015). Chronic ELS also induces amygdala seizures in preweaning and adolescent offspring (Dube et al., 2015). While modest long-term potentiation (LTP) is reported in the BLA following tetanic stimulation of the lateral amygdala prior to PND10, after that, long-term depression (LTD)

appears to be predominant (Thompson et al., 2008). Changes in synaptic plasticity occurring during this early life period are also important for the encoding of fearful stimuli (Thompson et al., 2008). The contribution of increased synaptic inputs to BLA neurons after LB exposure might thus alter the timing of transition between LTP and LTD in the BLA. This possibility is currently being investigated. Interestingly, increased neonatal BLA excitability after LB in our current study contrasts with the reported reduction in amygdalar neuronal excitability following prenatal stress (Ehrlich and Rainnie, 2015), emphasizing the importance of timing of stressor exposure on the developmental trajectory of amygdala (van Bodegom et al., 2017).

Morphological and functional changes observed in the neonatal amygdala of LB male pups were not associated with significant changes in basal ACTH or CORT secretion between NB and LB neonates, in agreement with some, but not other reports (Walker et al., 2017). Furthermore, in previous experiments conducted in our laboratory, we probed for indices of chronic exposure to elevated glucocorticoids and found no significant group differences in the thymus or adrenal gland weights of PND10 pups, suggesting that basal CORT levels are likely unchanged during LB exposure (PND1-9). Here, we document that LB pups exhibited blunted CORT stress responsiveness compared to NB pups on PND10, in line with previous findings from our laboratory (McLaughlin et al., 2016). On PND20 and 10 days after termination of the LB condition, stress responses were similar between experimental groups, indicating recovery of function in the HPA axis. We suggest that blunted HPA responses at the time of LB exposure serve to protect the metabolic resources of the LB pups because these pups display reduced body weight compared to NB pups and mounting stress responses is

energetically costly (Myers et al., 2014). In contrast to the adult literature demonstrating the critical role of CORT on BLA plasticity under acute or chronic stress conditions (McEwen et al., 2016), our results suggest that tonic increases in CORT are not required for BLA plasticity in neonates, either because of the involvement of other CORT-independent processes or due to the developmental patterns of glucocorticoid receptors in this area, which may not be affected by early stress (Avishai-Eliner et al., 1999).

Adult consequences of early LB exposure on emotionally-relevant behavioral tests

Emotional dysregulation and enhanced anxiety-like behavior has been associated with BLA neuron dendritic hypertrophy (Vyas et al., 2004), hyperexcitability (Rau et al., 2015) and functional synaptic plasticity (Rodriguez Manzanares et al., 2005) in adult rodents. Consistent with a recent study (Prusator and Greenwood-Van Meerveld, 2015), our results revealed that in contrast to adult females, adult male LB offspring showed increased anxiety-like behavior in the EPM and open field and increased fear behavior compared to NB offspring. They also exhibited less engagement to social contact, highlighting the prevalent role of the amygdala in mediating social, in addition to anxiety behaviors (Allsop et al., 2014). We suspect that altered BLA neuron functioning, which we found to occur as early as PND18, underlies emotional impairments and increased anxiety-like behavior in males exposed to LB because the behavioral outcomes of chronic ELS are reduced when normal BLA cell functioning is restored (Rau et al., 2015). In contrast, a recent study using a different window of maternal maltreatment (PND8-12) found reduced Fos activation in the BLA (but not CeA) in response to a social challenge in peri-weaning and adolescent rats (Rincon-Cortes and Sullivan, 2016). Differences in

the timing of ELS exposure and/or the endpoint used to assess BLA excitability might explain discrepancies with the present study.

During fear conditioning and retrieval, LB adult males displayed significantly more freezing behavior compared to NB males, suggesting that ELS exposure enhances fear learning and the recall of fear memory, without significantly impairing the rate of fear extinction. Freezing during fear extinction was not increased in LB male pups, although it was increased during fear recall, suggesting a discrepancy between actual extinction and behavioral responses (that is, freezing) occurring during fear extinction. We also suspect that enhanced fear retrieval in LB rats may simply be the result of poor extinction (Quirk and Mueller, 2008). Indeed, adult male LB mice have also been found to exhibit deficits in the ability to discriminate between cue-on and "safe" episodes in-between cue exposure, 24 h after fear learning (Arp et al., 2016). It is possible that LB animals have dysfunctions in the ventro mPFC-to-BLA circuit because silencing this pathway weakens fear extinction (Bukalo et al., 2015). While emotional behavior impairments were significant in adult male LB offspring, no behavioral consequences of LB were observed in female offspring, in parallel with the lack of significant morphological alterations in BLA morphology in females. A large recent literature review found that impaired behavioral performance in adult rodents that had been subjected to adverse early life conditions was observed in one third of the experimental endpoints reported for males, whereas this was only 25% for females (Loi et al., 2017). Earlier reports have also documented lower anxiety-like behavior in the elevated plus maze in female rats during either the proestrus or estrus stage of their cycle (Marcondes et al., 2001) and in our studies, a large proportion of both NB (75-87%) and LB (62-75%) female groups were tested during these

phases. The overrepresentation of females in proestrus/estrus might have contributed to lower anxiety and fear responses altogether, but similarly in both NB and LB groups. Indeed, we performed statistical analyses of our behavioral data as a function of estrous cycle and found no significant effects of estrous cycle stage on group performance.

Conclusions

In conclusion, our results demonstrate that exposure to LB conditions, which altered the quality of maternal care during the first 10 days of life, increases spine density in the BLA of male offspring already by PND10 and results in enhanced excitability before weaning. These early changes in BLA function are not associated with concurrent increased CORT secretion, but might be causal, at least in part to enhanced anxiety-like behavior, fear conditioning and recall and reduced social contact in adult male rats. There were no significant differences in any of these measures in female offspring, suggesting that females might be more resistant to the stress of LB compared to males or that there are compensatory mechanisms activated in females that prevent early hyperexcitability of the BLA during the pre-weaning period. These data offer insight into how the preweaning amygdala, and more specifically the BLA, may represent a sexually-dimorphic critical neural substrate for the development and progression of stress-induced psychopathologies.

Acknowledgements and Disclosures

This work was supported by an NSERC grant to CDW (Grant #138199) and an internal McGill-Faculty of Medicine studentship award to AG. The authors declare no competing financial interests. We thank Ms Hong Long and Ms Silvanna Verlezza as well as Dr Yiu Chung Tse for their excellent and dedicated technical expertise with the cellular and electrophysiological experiments, respectively. We would like to acknowledge the advice of Dr Joseph Rochford on behavioral experiments.

Conflict of Interest: The Authors declare no conflict of interest.

Connecting Statement to Chapter III

In chapter II, we showed for the first time that the neonatal BLA undergoes persistent and sex-dependent morphological and functional changes in response to LB exposure during the first 10 postnatal days. More specifically, we identified increased spine numbers and evoked synaptic responses in preweaning LB males, that associated with enhanced anxiety-like and fear behaviors in adulthood. We next wanted to address precisely how ELS exposure affects BLA function and connectivity with the mPFC, in particular, and whether alterations in this circuit could mediate impaired fear behaviors in adulthood.

Humans and adult rodents with a history of early adversity exhibit reduced functional connectivity in the BLA-mPFC circuit that strongly predicts emotional dysfunctions (Burghy et al., 2012; Gee et al., 2013a; Yan et al., 2017). The goal of the upcoming manuscript presented in Chapter III was to determine whether ELS can disrupt functional connectivity of the developing BLA with the mPFC, as well as with other regions, and increase fear behaviors in the long-term. We carried out these experiments in males, since our prior findings showed that the effects of LB were primarily observed in male LB offspring.

Chapter III

Reduced resting-state functional connectivity of the basolateral amygdala to the medial prefrontal cortex in preweaning rats exposed to chronic early-life stress

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Brain Structure and Function. 2018. 223, 3711–3729

https://doi.org/10.1007/s00429-018-1720-3

Abstract

Early-life stress (ELS) exposure has long-term consequences for both brain structure and function and impacts cognitive and emotional behavior. The basolateral amygdala (BLA) plays an important role in anxiety and fear conditioning through its extensive anatomical and functional connections, in particular to the medial prefrontal cortex (mPFC). However, how ELS affects amygdala function and connectivity in developing rats is unknown. We used the naturalistic limited bedding/nesting (LB) paradigm to induce chronic stress in the pups between postnatal day (PND) 1-10. Male normal bedding (NB, control) or LB offspring underwent structural and resting-state functional MRI (rs-fMRI) on PND18 and in adulthood (PND74-76). Adult male rats were tested for fear conditioning and extinction behavior prior to scanning. Seed-based functional connectivity maps were generated based on four BLA seeds (left, right, anterior and posterior). At both ages, LB induced different effects on anterior and posterior BLA networks, with significant reductions in rs-fMRI connectivity between the anterior BLA and mPFC in LB compared to NB offspring. BLA connectivity was lateralized by preweaning age, with the right hemisphere displaying more connectivity changes than the left. Weak negative volumetric correlations between the BLA and mPFC were also present, mostly in preweaning LB animals. rs-fMRI connectivity and volumetric changes were associated with enhanced fear behaviors in adult LB offspring. Activation of the LB-exposed neonatal amygdala described previously might accelerate the maturation of BLA-mPFC projections and/or modify the activity of reciprocal connections between these structures, leading to a net reduction in rs-fMRI connectivity and increased fear behavior.

Introduction

Early life stress (ELS) has long lasting consequences on cognitive and emotional health, increasing vulnerability to future psychopathologies (Essex et al., 2011). Functional and structural alterations in the emotion regulation circuitry are documented in humans after ELS and play a pivotal role in stress-related disorders, including anxiety, depression and PTSD (VanTieghem and Tottenham, 2017). One critical element of this "emotion" circuitry, the basolateral amygdala (BLA), can affect stress reactivity and anxiety through its wide connections to the medial prefrontal cortex (mPFC) and inputs onto the CA1 neurons and ventral regions of the hippocampus, in particular (Godsil et al., 2013; Marek et al., 2013). The BLA-mPFC projections have been well studied for their important role in fear conditioning and fear extinction (Marek et al., 2013; Duvarci and Pare, 2014). Connections from the BLA to the prelimbic (PL) mPFC are involved in fear memory expression (Sotres-Bayon and Quirk, 2010), whereas the BLA to infralimbic (IL) circuitry mediates fear extinction (Quirk and Mueller, 2008). Interestingly, there are reports of hemispheric lateralization of BLA functions, with the right amygdala showing stronger involvement in fear conditioning compared to the left BLA (Baker and Kim, 2004). The PFC demonstrates similar asymmetrical activity in conditioning processes (Haritha et al., 2013). Fear memory expression can be perturbed by inactivating the BLA in one hemisphere and the ipsilateral or contralateral mPFC, highlighting the importance of both inter- and intra-hemispheric emotional processing in this complex circuit (Stevenson, 2011).

Maturation of the BLA-PFC circuit is incomplete at birth and is mostly achieved during the postnatal and early juvenile periods (Bouwmeester et al., 2002b; Bouwmeester et al., 2002a). The development of anatomical connections in the emotional circuit allows for the onset of fear learning around PND10-12 in rats (Raineki et al., 2010b; Tallot et al., 2016). Projections from both the anterior and posterior BLA to the IL and PL portions of the mPFC emerge between PND7-9 and they reach an adult-like pattern of distribution in the layers of the mPFC by PND13 (Bouwmeester et al., 2002a). The development of reciprocal mPFC to BLA projections occurs later, mostly during the second week of postnatal life between PND13-21 (Bouwmeester et al., 2002b; Bouwmeester et al., 2002a), and provides support to a more sequential type of maturation between the ascending and top-down components of this reciprocal circuitry as suggested in humans (Tottenham and Gabard-Durnam, 2017).

The predominant postnatal maturation of the BLA-mPFC circuit (Cunningham et al., 2002), makes it very sensitive to environmental stressors, particularly during the first two weeks of life. In humans, studies have documented increased amygdala reactivity in ELS-exposed individuals in response to fearful stimuli and emotional learning tasks (Silvers et al., 2017; VanTieghem and Tottenham, 2017). Importantly, enhanced amygdala reactivity following periods of chronic ELS can accelerate the development of a more “mature” BLA-PFC connectivity (Tottenham and Gabard-Durnam, 2017) and impair resting state functional connectivity in humans (Burghy et al., 2012). Disruptions in the BLA-PFC circuitry likely result in higher trait anxiety levels and disease symptomatology (Burghy et al., 2012; VanTieghem and Tottenham, 2017).

Early life adversity also leads to weakened BLA-prefrontal connectivity in adult rodents (Yan et al., 2017) and humans (Kim et al., 2013), but it is unknown how it affects the development of the BLA-mPFC projections. A recent study from our laboratory showed that early stress in the form of exposure to limited bedding (LB), a well characterized preclinical model of early life stress (Molet et al., 2014; Walker et al., 2017), increased evoked synaptic responses recorded from BLA neurons *in vitro* in preweaning rats (Guadagno et al., 2018a). LB-reared pups also exhibited persistent dendritic hypertrophy and increased spine density of BLA neurons and a modest BLA volume enhancement (Guadagno et al., 2018a). These outcomes were associated with enhanced anxiety and fear behaviors in adulthood (Guadagno et al., 2018a). Enhanced amygdala neural activity in adolescent rats exposed to ELS was also documented after exposure to an acute stressor of forced swim (Raineki et al., 2012).

Together, these findings demonstrate that the effects of ELS on the BLA can be observed very early, at a critical time of maturation of the emotional circuit, but precisely how early stress affects amygdala function and its connectivity to other brain structures important for emotional processing and fear conditioning remains to be elucidated. In this study, we examined resting state functional MRI (rs-fMRI) connectivity in preweaning and adult offspring *in vivo* and tested the hypothesis that exposure to the stress of LB would reduce BLA-mPFC connectivity in the long-term, and impair BLA connectivity with other structures as well. Total structural volumes and volumetric correlations between the BLA, mPFC and HIPP were also considered. Finally, we proposed that rs-fMRI connectivity and structural alterations in the BLA-mPFC circuitry in particular, would underlie the increased fear behavior of LB offspring.

Methods

Animals:

Untimed-pregnant (gestation day 13-14) Sprague-Dawley female rats (Charles River, St-Constant, QC, Canada) were individually housed under controlled conditions of light (12 hr light:12 hr dark, lights on at 08:00 h), temperature (22-24°C), and humidity (70–80%) and provided *ad libitum* access to rat chow and water. The day of parturition was considered PND0 and litters were culled to 10 pups on PND1 with as many males as possible. Animals were weaned on PND21 and same-sex group housed with environmental enrichment. All experiments were carried out in males because we have documented earlier that the effect of LB on amygdala morphology and reactivity as well as the adult behavioral effects were observed primarily in male offspring (Guadagno *et al*, 2018). No more than 1-3 pups per litter were used for imaging and 3 pups per litter for behavioral experiments. All experimental procedures were approved by the University Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care.

Limited bedding paradigm:

In order to induce early chronic stress for the offspring, we used a protocol adapted from Baram and colleagues (Molet et al., 2014), which produces disruptions of maternal care as previously validated in our laboratory (McLaughlin et al., 2016; Guadagno et al., 2018a). Briefly, on PND1, mothers and their litters were randomly assigned to the limited bedding (LB) or normal bedding (NB) condition. LB mothers and their litters were placed

on an aluminum mesh platform raised 2.5 cm above the cage floor, with approximately 1.5 cm of bedding underneath the platform. The dams were given one-half of one paper towel as nesting material. The NB cages received a 2.5 cm layer of woodchips and one-half of one paper towel. Cages were changed on PND4 and 9 at which time mothers and pups were weighed. On PND10, all LB mothers/litters were returned to the normal bedding conditions. All litters were kept with their biological mother for the duration of the experiments.

Preweaning (PND18) and adult resting state connectivity

Image acquisition:

Approximately 24 h prior to scanning, preweaning (PND18, n=12/group from 7 mothers/group) and adult (PND74-76, n=6/group from 7 mothers/group) rats received treatment with Manganese Chloride ($MnCl_2$, 32 mg/kg, sc) (Eschenko et al., 2010; Sperry et al., 2017). $MnCl_2$ improves contrast in structural images by shortening the longitudinal relaxation time (T1) (Pan et al., 2011). Preweaning rats were scanned in 2 different cohorts from a total of 7 mothers/group that were considered as separate repeat studies, performed approximately one year apart (Cohort 1: n=8 pups/group from 3 mothers/group Cohort 2: n=4 pups/group randomly selected from 4 mothers/group). There were also two adult cohorts scanned within the same week that were combined and treated as part of a cross-sectional study: Cohort 3: n=4/group were scanned as preweaning (cohort 2) and adult animals, and Cohort 4: n=2/group randomly selected from 2 NB and 2 LB mothers scanned as adults only. Animals were weighted and lightly anesthetized with isoflurane

(PND18: <2%, Adults: <3%; 1L/min, in medical air) during the entire scan and the level of anesthesia was adjusted to maintain high oxygenation and a relative constant breathing rate (average: 42 breaths/min (PND18) and 53 breaths/min (Adults)). Resting-state fMRI measures changes in cerebral blood flow in the absence of explicit sensory or cognitive stimuli (Smitha et al., 2017; Lv et al., 2018). Previous studies in rodents and primates have suggested that anesthesia might affect neurovascular coupling (Magnuson et al., 2014; Wu et al., 2016). However, low dose (<2%) isoflurane anesthesia, which is most commonly used in rodent resting-state studies, does not affect neural activity and functional connectivity (Magnuson et al., 2014). Three RAREst (Rapid Acquisition and Relaxation Enhancement) fMRI scans (5 min each) were acquired first, followed by the FLASH (Fast Low-Angle SHot imaging) anatomical scan of longer duration (20 min) for a total scan time less than 40 min including set up in the scanner. Body temperature and respiration rate were monitored throughout the image acquisition in the scanner using an MR-compatible monitoring system (SA Instruments, Stony Brook, NY). Animals were kept warm with a 37°C air flow into the scanner. All scans were obtained on a horizontal bore Bruker 7T preclinical MRI (BioSpec 70/30 USR - Billerica, MA) with the actively shielded 120mm inner diameter gradient insert upgrade (650 mT/m in 150μs), AV-III electronics and ParaVision 5.1. Standard Bruker-issued pulses sequences were utilized for both anatomical and functional MRI. A different set of parameters was used for either age: on preweaning pups (PND18), we used a Bruker-issued 23mm volumetric transceiver with a mouse bed and the parameters of the scans were: anatomical scan: FLASH, FA (Flip Angle) 20 deg, TR/TE 21.5/5.1 ms, Matrix 256x192x112 pts, FOV 25.6x19.2x11.2 mm, Resolution 0.1x0.1x0.1 mm, NEX 2, TA 23 min. For the fMRI scans: RAREst, TR/TE

3000/28 ms, RARE factor 38, Matrix 64x50 pts, FOV 16x12.5x17.6 mm, nSlices 22, Resolution 0.25x0.25x0.8 mm, NEX 1, Repetitions 100, TA 5 min. In adult rats, a Bruker-issued 40mm volumetric transceiver with a rat bed was utilized. The same imaging pulses were used for the adults as for pups, with the following parameters modified: anatomical: FLASH, FOV 35.84x26.88x15.68 mm, Resolution 0.14x0.14x0.14 mm, TA 32 min 24 sec; fMRI: RAREst, FOV 19.2x15x19.8 mm, Resolution 0.3x0.3x0.9 mm.

Image processing:

Structural scans were bias-field corrected for each age group (PND18: n=12/group, Adults: n=6/group) and iteratively co-registered to generate cross-sectional, group average anatomical images (FLASH) using the Pydpiper2 toolkit (Friedel et al., 2014). FLASH sequences were manually inspected for the possibility of susceptibility artifacts. Three rs-fMRI acquisitions for each animal were pre-processed in the following fashion. All images were motion corrected and rigidly registered to their respective anatomical scan based on ANTs tools (http://stnava.github.io/ANTs/). Final transformation to the consensus anatomical average was achieved using the transformations estimated from the consensus process. Then, rs-fMRI images were corrected for slice time, band pass filtered between 0.01 and 0.15 Hz, spatially smoothed 2mm full width half maximum (FWHM) Gaussian kernel and corrected for transient drift by removing the first 5 frames using AFNI tools (https://afni.nimh.nih.gov/). Each rs-fMRI run then generated seed-based connectivity maps at four BLA seeds (left, right, anterior, posterior) using linear mixed effect model in FMRISTATS (http://www.math.mcgill.ca/keith/fmristat/). Seeds were located on the consensus

anatomical average and the BLA was identified by referring to the Paxinos atlas (Paxinos and Watson, 2005). Three unique connectivity maps were generated from FMRISTATS: effect size, standard-deviation and t-statistical maps. Seed-based connectivity maps were spatially smoothed 1.6mm FWHM. Subject-level seed-based connectivity maps from individual rs-fMRI runs were then combined using linear mixed effect models with FMRISTATS tools. The subject-level seed connectivity maps were transformed into the template space by applying affine and nonlinear transformations from the consensus average state. Group contrast was analyzed using a linear mixed effect model accounting for subject variability and white noises as random effects using FMRISTATS.

Manual Segmentation:

We examined BLA, PFC and HIPP volume in our experiments, as previous studies have documented modest ELS-induced changes in these structures (Molet et al., 2016; Sarabdjitsingh et al., 2017; Guadagno et al., 2018a). Four regions (left and right) were manually segmented using the Display software package (part of the MINC toolkit: http://www.bic.mni.mcgill.ca/ServicesSoftware/HomePage) on the pup and adult group average anatomical averages. The BLA, PL PFC, IL PFC and HIPP were delineated by referring to the Paxinos and Watson atlas (Paxinos and Watson, 2005). The BLA was segmented from Bregma -1.56 to -3.48 mm, over as span of 19 scan slices for pups and 20 for adults. Hippocampal regions segmented approximately included the CA1/CA2/CA3, dentate gyrus, stratum oriens/stratum radiatum/stratum lacunosum/stratum moleculare (SO/SR/SL/SM), and subiculum. The hippocampus was segmented on 63 slices for pups and adults, from Bregma -1.72 to -8.16. The entire PL

and IL mPFC were delineated on 19 slices for pups and adults (Bregma 5.16 to 2.52 mm). Labels were used to estimate the volume for each subject by summing the jacobian determinant voxel values from the consensus average to each subject within the label, yielding an estimate of the total volume of that structure for each subject. Whole brain volumes were also computed for each animal by the same method.

Adult fear conditioning and extinction:

Behavioral testing was performed before adult image acquisition took place on adult (PND67-69) male offspring from NB and LB mothers and analyzed by an experimenter blind to group. A total of 24 male rats (12/group from 7 mothers/group) were used for behavioral testing, including those rats that were subsequently scanned and constituted cohorts 3&4 for imaging. No specific criteria were used to determine which rats were scanned after behavior other than the fact that they would originate from different mothers within an experimental group. Rats were transferred to a reverse 12 hr light/dark cycle room 1 week before behavioral testing (lights off at 08:00 h) and individually housed for 3-5 days before fear testing. Cued fear conditioning and fear extinction tests were performed and analyzed as described previously (Guadagno et al., 2018a). On day 1, animals underwent fear conditioning in operant boxes with a metal rod floor delivering the shocks. A tone was used as the conditioned stimulus or CS (amplitude: 80 dB; duration: 30 sec; variable inter-trial interval (ITI): 2 min) and an electric shock constituted the unconditioned stimulus or US (intensity: 0.5 mA). The CS parameters were the same on both days of testing. Animals were placed in the chamber for 5 min and then presented with two habituation tones alone, followed by a 2 min ITI and five tone-shock pairings. On

day 2, the expression of fear conditioning was assessed by the presence of freezing while in the same context as day 1. Animals were subjected to fear extinction, which consisted of presenting 8 tones alone (all parameters same as day 1) after a 1 min habituation period.

Statistical analyses:

Group effects for resting state functional connectivity measures from the four BLA seeds were estimated using a voxel-wise linear mixed effect model, with effect size and standard deviation maps as the input files. The final t-statistical maps display the significance of the analysis and were corrected for multiple comparisons using Random Field Theory, set at a statistical threshold value of $p = 0.05$. Structure volume data were analyzed using two-factor analysis of variance (ANOVA) with brain side and bedding condition as between-subjects factors. Total brain volumes and body weight were analyzed using separate unpaired, one-tailed Student's t-tests. Left and right volumes were residualized for effects of total brain volume prior to generating correlation matrices. Fear conditioning (day 1) and extinction (day 2) data were analyzed using either a two or three-factor analysis of variance (ANOVA), with appropriate within and between subject factors. The day 1 and day 2 data were then analyzed using separate, unpaired, one-tailed Student's t-tests, to examine the degree of fear acquisition and extinction, respectively. The use of one-tail t-test was based on the prediction that LB would enhance fear behavior as observed in our previous study (Guadagno et al., 2018a). For day 1, a trial was defined as the presentation of a tone as well as the inter-trial interval following the tone. The increase in percent freezing was computed by subtracting the mean

freezing percentage during the last trial from the first trial. For day 2, we subtracted the percent freezing during tone 8 minus tone 1. Volume, weight and behavioral data are presented as means ± standard error of the mean (SEM). Statistical significance was set at $P < 0.05$.

Results

Functional connectivity in preweaning rats (PND18)

Significant differences in the projections from the anterior and posterior BLA to the mPFC have been reported (Fig. 1) and thus, we analyzed connectivity using seeds placed in either the anterior (aBLA, Bregma coordinates: -2.04) or posterior (pBLA, coordinates: -3.12) portion of the BLA using coordinates from the Paxinos and Watson atlas (Paxinos and Watson, 2005) verified with the atlas of the developing rat brain (Sherwood and Timiras, 1970). Furthermore, since there is evidence for a lateralized function of the adult BLA (Baker and Kim, 2004), we analyzed connectivity from seeds placed both in the left and right BLA. Resting state seed connectivity was analyzed in rostro-caudal sections between +5.64 mm to -7.44 mm from bregma. A summary of differences in pup connectivity between NB and LB pups is presented in Table 1 and connectivity maps are illustrated in Figures 2 and 3.

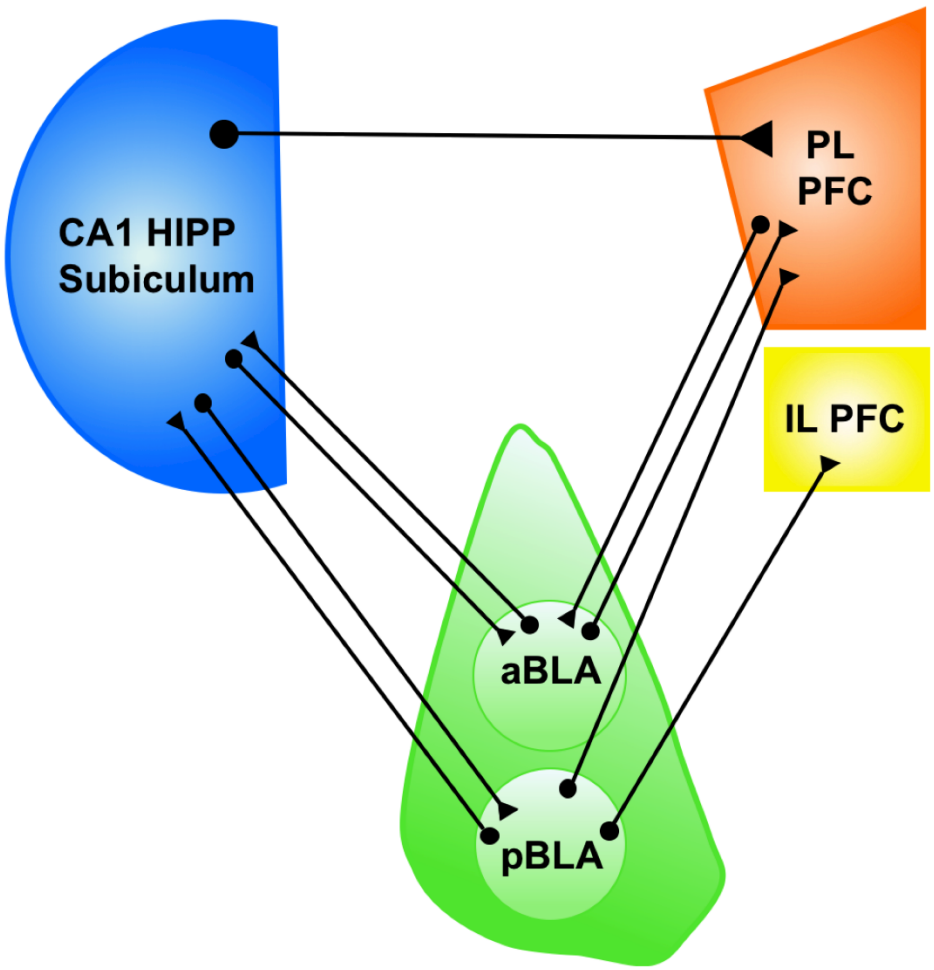

Figure III-1. Projections between the CA1, ventral hippocampus (HIPP) and subiculum, basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) in the adult rodent forming an interconnected corticolimbic network. The anterior BLA (aBLA) bidirectionally projects to the CA1 ventral HIPP and the prelimbic (PL) prefrontal cortex (PFC) (Hoover and Vertes, 2007; Giustino and Maren, 2015). Whereas the pBLA sends efferents to the PL and infralimbic (IL) PFC, the IL does not appear to project to the pBLA directly (Vertes, 2004; Sotres-Bayon and Quirk, 2010). The pBLA is bidirectionally connected with the CA1 ventral HIPP (Pitkanen et al., 2000; Huff et al., 2016). Figure adapted from (Godsil et al., 2013).

In general, differences in connectivity originating from the seeds in the right amygdala were more pronounced than from those in the left amygdala. There was a significant reduction in connectivity from the aBLA seeds (both left and right) to both prelimbic (PL) and infralimbic (IL) portions of the mPFC in LB compared to NB pups (Fig. 2). More specifically, the right aBLA showed reduced connectivity to both the ipsilateral and contralateral PL and IL PFC (Fig. 2b), whereas the left aBLA connectivity was only decreased to the contralateral PL PFC (Fig. 2a). Animals subjected to LB also displayed reduced connectivity from the left pBLA to most of the PFC, except for increased connectivity to the contralateral IL PFC (Fig. 3a). The right pBLA connectivity in LB compared to NB pups was only increased to the ipsilateral IL PFC (Fig. 3b).

Reciprocal connections between both the aBLA and pBLA are found with the CA1 region and ventral subiculum of the hippocampus (HIPP) in adult rats (Godsil et al., 2013) (Fig. 1). Dorsal and ventral hippocampal subfields were identified as described previously (Banasr et al., 2006). In preweaning pups, we observed that connectivity of the left aBLA was predominantly increased to the ipsilateral ventral CA1 region in LB vs NB pups, whereas the right aBLA displayed mostly reductions in connectivity to the ipsilateral ventral CA1 and DG/Subiculum regions (Fig. 2 and Table 1). For the pBLA seeds, the left seed displayed increased and decreased connectivity to the ipsilateral and contralateral ventral HIPP regions, respectively (Fig. 3a). The right pBLA seed showed predominantly decreased connectivity to the ipsilateral CA1 and DG ventral HIPP subfields (Fig. 3b). The effects on rs-fMRI connectivity in the dorsal HIPP were mixed, with the aBLA and left pBLA seeds displaying ipsilateral increases and contralateral reductions in connectivity

(Fig. 2 and 3a), and the right pBLA exhibiting mostly enhanced connectivity to the dorsal HIPP (Fig. 3b).

Indirect connections between the BLA, BNST and hypothalamic nuclei are also interesting because they modulate the hypothalamus-pituitary adrenal activity under conditions of stress as induced by the LB in our experiments (Jankord and Herman, 2008). Indeed, we found that BLA connectivity to hypothalamic and BNST regions was also affected by LB, as LB rats showed reduced connectivity to the BNST primarily from the left BLA (anterior and posterior portions) (Fig. 2a and 3a), while the right amygdala showed an increased connectivity to the BNST (Fig. 2b and 3b). We also found altered connectivity from BLA seeds to the ventromedial and lateral hypothalamus, preoptic areas and paraventricular nucleus. The right pBLA seed was the only region that showed an increased connectivity to the hypothalamic paraventricular nucleus in LB pups (Fig. 3b).

Animals subjected to LB rearing also exhibited changes in BLA connectivity to deep gray and midbrain regions, such as the nucleus accumbens (NAcc), ventral tegmental area (VTA), substantia nigra region (SNR) and periaqueductal gray (PAG). The effects were mostly observed for the right BLA seeds which exhibited a consistent bilateral reduction in connectivity to the VTA and SNR (Fig. 2b and 3b). Connectivity to the NAcc was significantly reduced from the left pBLA seed in LB pups (Fig. 3b), but no effect was observed with either the left or right aBLA. The left pBLA seed also showed decreased connectivity to the contralateral habenula and thalamic nuclei (Fig. 2b).

Finally, since the BLA heavily projects to other amygdala subregions, including the output neurons of the central amygdala (CeA) (Duvarci and Pare, 2014), we examined intra-amygdalar connectivity in LB animals. In preweaning animals, there was no LB effect

on connectivity of the anterior or posterior BLA with the CeA, but mixed effects with other subnuclei of the amygdala.

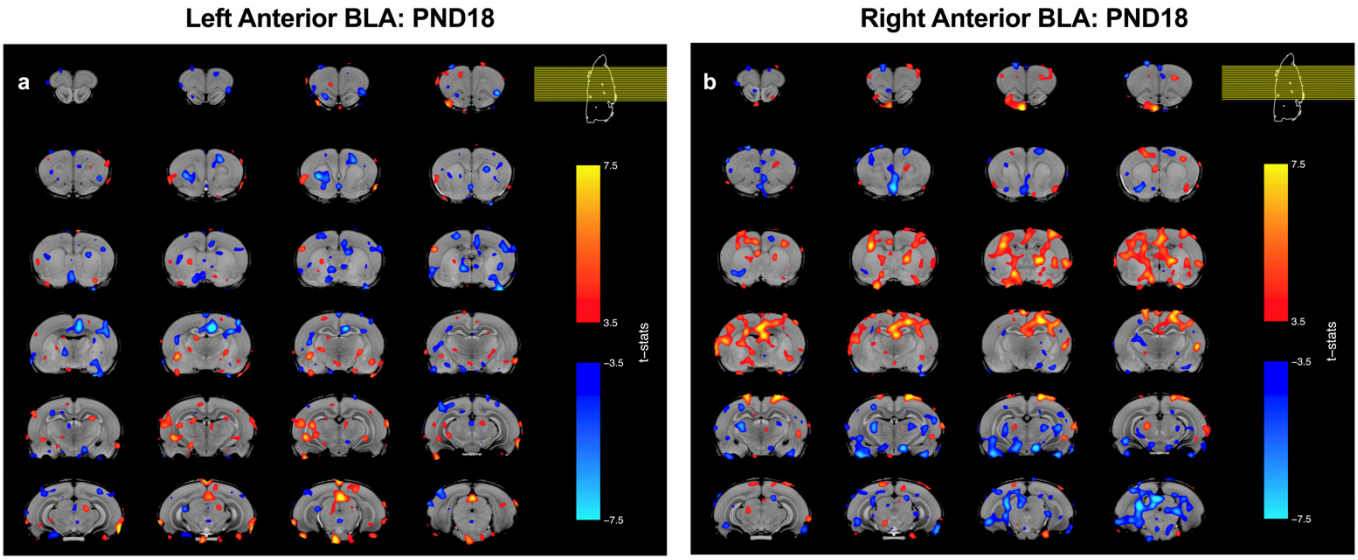

Figure III-2. T-statistical maps for the resting state functional connectivity of the anterior basolateral amygdala (aBLA) seeds (Bregma = -2.04) in PND18 preweaning rats, overlaid on structural template images. A representative template brain diagram illustrating the scan slices is included at the top, right-hand corner of the figure. Results were corrected for multiple comparison using Random Field Theory and statistical threshold value is set at p = 0.05. N=12 animals/group

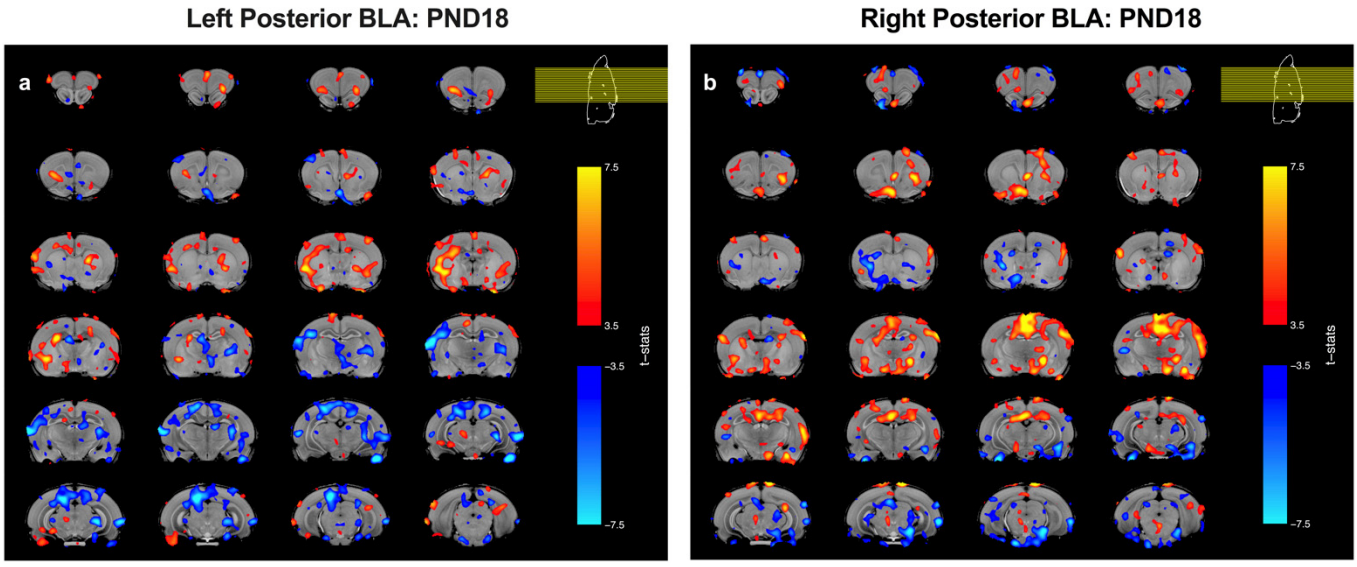

Figure III-3. T-statistical maps for the resting state functional connectivity of the posterior basolateral amygdala (pBLA) seeds (Bregma -3.12) in PND18 preweaning rats, overlaid on structural template images. A representative template brain diagram illustrating the scan slices is included at the top, right-hand corner of the figure. Results were corrected for multiple comparison using Random Field Theory and statistical threshold value is set at p = 0.05. N=12 animals/group

		Seed			
		Anterior BLA (aBLA)		Posterior BLA (pBLA)	
		Left	Right	Left	Right
PFC					
PL	Ipsi		↓	↓	
	Contra	↓	↓	↓	
IL	Ipsi		↓	↓	↑
	Contra		↓	↑	
Hippocampus					
Dorsal CA1/2/3	Ipsi	↑↓	↑	↓	↑*
	Contra	↓*	↑	↑↓*	↑*
Dorsal DG/Subiculum	Ipsi	↑	↑	↑↓	↑*↓
	Contra		↑↓*	↓*	↑*↓
Ventral CA1/3	Ipsi	↑*	↓	↑	↓*
	Contra	↑	↓*	↓	
Ventral DG/Subiculum	Ipsi		↓	↑	↓
	Contra	↑	↓	↓	↓
Preoptic Area, BNST, Hypothalamus					
PO	Ipsi	↓		↑	↓↑
	Contra	↑↓	↑	↑	↓*
BNST	Ipsi	↓	↑	↓	↑
	Contra	↓		↓	
Hypothalamus	Ipsi	↑	↑↓	↑	↑↓
	Contra	↓		↓	↑
Amygdala					
BLA, BMA	Ipsi	↑	↑↓	↓	↑↓
	Contra	↑		↓	↑

LA	Ipsi	↑↓			↑↓
	Contra			↓	↑
CeA	Ipsi				
	Contra				
MeA	Ipsi		↓		↑
	Contra				

Deep Gray

VTA	Ipsi		↓		↓
	Contra		↓		↓
SNR	Ipsi		↓		↓
	Contra	↑	↓		↓
NAcc	Ipsi			↓	
	Contra			↓	↑
PAG	Ipsi			↑	
	Contra			↓	

Habenula, Thalamus

Habenula	Ipsi	↓	↑↓	↓	
	Contra	↑		↓	
Thalamus	Ipsi	↓			
	Contra		↓	↓*	

Table III-1. Summary of basolateral amygdala resting state functional connectivity alterations induced by LB in PND18 rats. The connectivity maps in Figures 2 and 3 were analyzed in rostro-caudal sections between +5.64 mm to -7.44 mm from bregma. Black arrows depict decreases in connectivity in LB (limited bedding) compared to NB (normal bedding) animals, whereas red arrows depict increases in connectivity. Arrows with a "*" symbol beside them indicate large increases or decreases in connectivity (identified on 3 or more sections in Figures 2 or 3). Regions were categorized and identified by referring to the Paxinos atlas (Paxinos and Watson, 2005). PFC = prefrontal cortex, PL = prelimbic, IL = infralimbic, DG = dentate gyrus, PO = preotic area, BNST = bed nucleus of the stria terminalis, BLA = basolateral amygdala, BMA = basomedial amygdala, LA = lateral amygdala, CeA = central amygdala, MeA = medial amygdala, VTA = ventral tegmental area, SNR= substantia nigra region, NAcc = nucleus accumbens, PAG = periaqueductal gray

Functional connectivity in adult offspring

Resting state seed functional connectivity was analyzed in rostro-caudal sections from adult male NB and LB offspring between +4.68 mm to -7.2 mm from bregma. A summary of differences in adult connectivity between NB and LB offspring is presented in Table 2 and connectivity maps are illustrated in Figures 4 and 5. Similarly to preweaning pups, adult LB offspring exhibited greater connectivity changes in the right versus the left BLA with a significant reduction in connectivity to ipsi- and contralateral PL and IL mPFC when the seed was placed in the right aBLA (Fig. 4b). This result was consistent with reduced connectivity from the aBLA that we also observed in preweaning LB pups. When connectivity with the pBLA seed was considered, the ipsilateral component showed an increased connectivity, but the opposite was observed for the contralateral component of the mPFC (either IL or PL) (Fig. 5).

As in the preweaning pups, connectivity from the right aBLA to the ventral CA1/CA3 fields as well as the ventral DG/Subiculum was consistently reduced in LB adult offspring (Fig. 4b, Table 2). No changes in connectivity were observed from the left aBLA to the ventral HIPP regions in adult rodents (Fig. 4a). Both pBLA seeds showed increased ipsilateral connectivity to the ventral CA1/3 regions (Fig. 5), contrasting the results in preweaning rodents where a decreased right pBLA connectivity to the ipsilateral ventral HIPP was noted. When examining the dorsal HIPP regions, connectivity from the left BLA seeds was largely increased ipsilaterally in LB compared to NB adults, whereas connectivity from the right BLA seeds was both increased or decreased depending on the rostrocaudal distribution of the sections examined (Fig. 4 and 5). A mixed effect was seen with the hypothalamic regions, which showed mostly increased connectivity from the left

BLA seeds and both increased and decreased connectivity from the right seeds. The left BLA seeds generally showed reduced connectivity to the preoptic areas in LB rats (Fig. 4a and 5a).

LB-exposed adult males displayed changes in BLA connectivity to deep gray and midbrain regions, with the aBLA seeds showing increased connectivity to the NAcc, SNR and PAG regions (Fig. 4). This pattern was opposite to that of preweaning rats that exhibited mostly reduced connectivity in these sites. The pBLA seeds generally showed decreased connectivity to the bilateral NAcc and PAG (Fig. 5). Interestingly, the reduced connectivity of BLA (left aBLA: Fig. 4a and right pBLA: Fig. 5b) to VTA that we observed in LB preweaning rats was maintained here in LB adult offspring. Connectivity from the right BLA seeds was only increased to the contralateral SNR (Fig. 4b and 5b). Additionally, the pBLA seeds exhibited mostly enhanced connectivity to the bilateral median raphe nucleus. The habenula and thalamic nuclei mostly showed reduced connectivity in adults as in preweaning rats.

Unlike preweaning animals exposed to LB, adult connectivity from the BLA to the output CeA nucleus was affected by the LB exposure. Specifically, the aBLA exhibited a reduction in connectivity (Fig. 4), whereas the pBLA connectivity to the CeA was increased (Fig. 5).

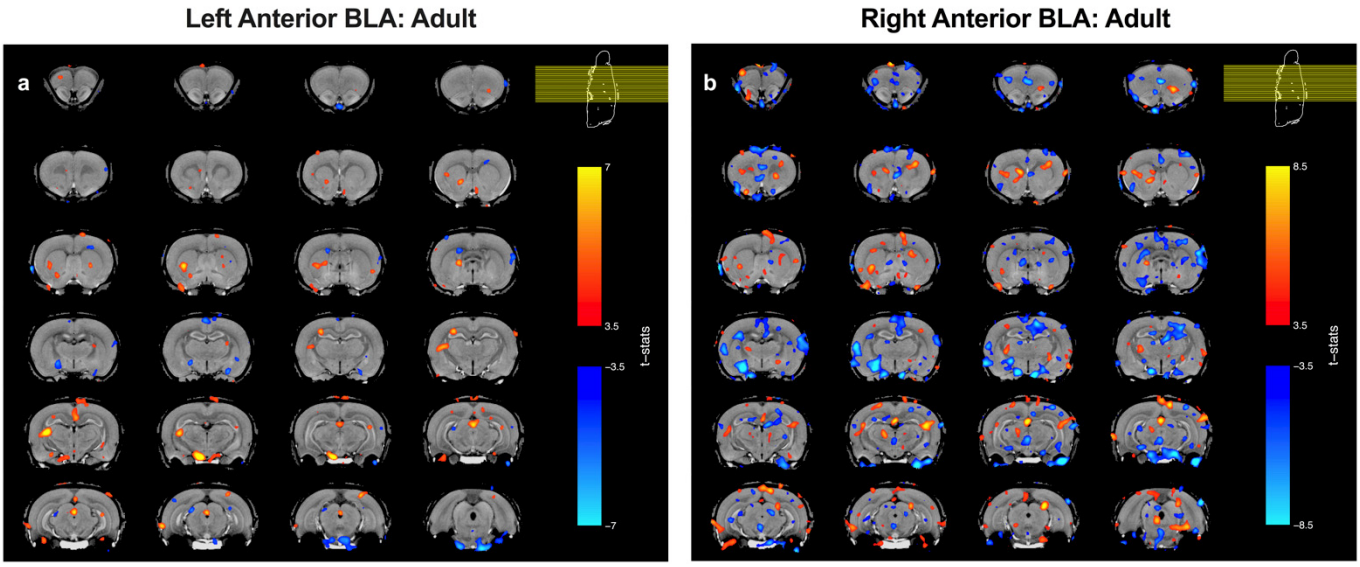

Figure III-4. T-statistical maps for the resting state functional connectivity of the anterior basolateral amygdala (aBLA) seeds (Bregma -2.04) in adult rats (PND74-76) overlaid on structural template images. A representative template brain diagram illustrating the scan slices is included at the top, right-hand corner of the figure. Results were corrected for multiple comparison using Random Field Theory and statistical threshold value is set at p = 0.05. N=6 animals/group

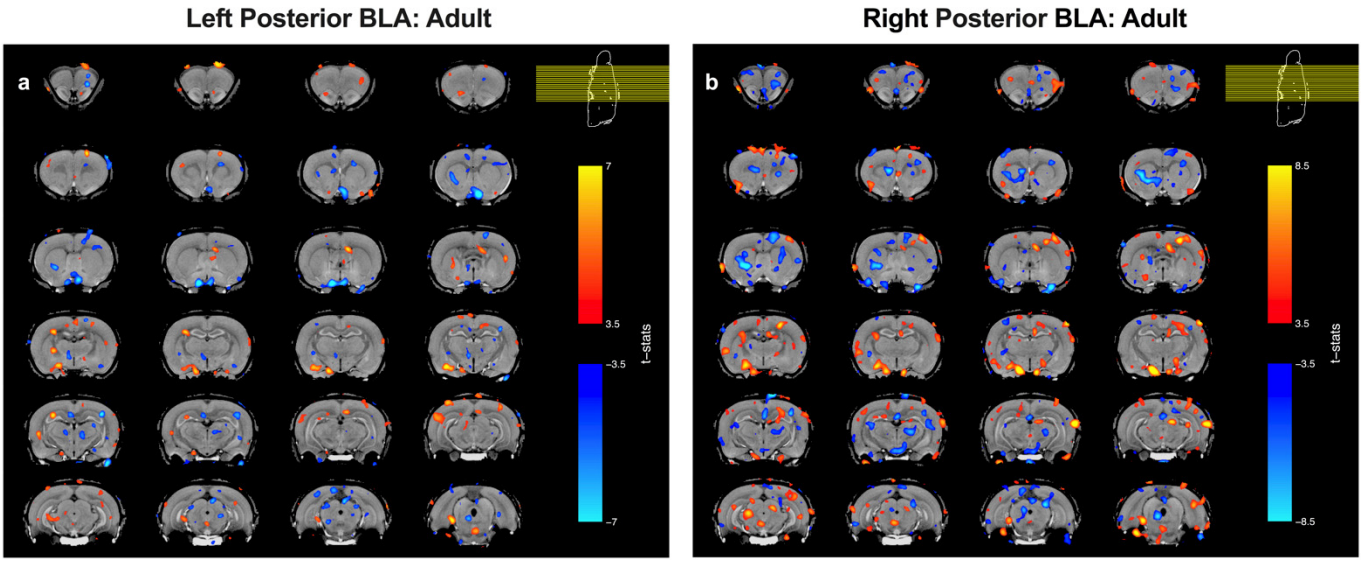

Figure III-5. T-statistical maps for the resting state functional connectivity of the left and right posterior basolateral amygdala (pBLA) seeds (Bregma -3.12) in adult rats (PND74-76) overlaid on structural template images. A representative template brain diagram illustrating the scan slices is included at the top, right-hand corner of the figure. Results were corrected for multiple comparison using Random Field Theory and statistical threshold value is set at p = 0.05. N=6 animals/group

		Seed			
		Anterior BLA (aBLA)		Posterior BLA (pBLA)	
		Left	Right	Left	Right
PFC					
PL	Ipsi	↑	↓		↑
	Contra		↓		↓
IL	Ipsi	↑	↓		↑
	Contra		↓		↓
Hippocampus					
Dorsal CA1/2/3	Ipsi	↑	↑↓*	↑*	↑*↓
	Contra	↑	↓	↓	↑↓
Dorsal DG/Subiculum	Ipsi		↓	↑	↑*↓
	Contra	↑↓	↓	↑	↑↓
Ventral CA1/3	Ipsi		↓	↑	↑
	Contra		↓		
Ventral DG/Subiculum	Ipsi		↓		
	Contra		↓		
Preoptic Area, BNST, Hypothalamus					
PO	Ipsi	↓	↑	↑↓*	
	Contra		↓	↓	
BNST	Ipsi				
	Contra				↓
Hypothalamus	Ipsi	↑↓	↑	↑	↑↓
	Contra	↑	↓	↑	↑↓
Amygdala					
BLA, BMA	Ipsi		↑	↑	
	Contra		↓	↓	↑

LA	Ipsi		↓		
	Contra				
CeA	Ipsi			↑	
	Contra		↓		
MeA	Ipsi			↑	
	Contra	↓	↓		↑

Deep Gray

VTA	Ipsi	↓			↓
	Contra	↓			
SNR	Ipsi				
	Contra		↑		↑
NAcc	Ipsi	↑	↑		↓
	Contra	↑	↑	↓	↓
PAG	Ipsi	↑	↑↓	↓	↓
	Contra	↑	↑↓		↓
Raphe	Ipsi			↑	↑
	Contra			↑	↑↓

Habenula, Thalamus

Habenula	Ipsi		↓		
	Contra				
Thalamus	Ipsi				↓
	Contra		↑	↓	

Table III-2. Summary of basolateral amygdala resting state functional connectivity alterations induced by LB in adult (PND74-76) rats. The connectivity maps in Figures 4 and 5 were analyzed in rostro-caudal sections between +4.68 mm to -7.2 mm from bregma. Black arrows depict decreases in connectivity in limited bedding (LB) compared to normal bedding (NB) animals, whereas red arrows depict increases in connectivity. Arrows with a "*" symbol beside them indicate large increases or decreases in connectivity (identified on 3 or more sections in Figures 4 and 5). Regions were categorized and identified by referring to the Paxinos atlas (Paxinos and Watson, 2005). PFC = prefrontal cortex, PL = prelimbic, IL = infralimbic, DG = dentate gyrus, PO = preotic area, BNST = bed nucleus of the stria terminalis, BLA = basolateral amygdala, BMA = basomedial amygdala, LA = lateral amygdala, CeA = central amygdala, MeA = medial amygdala, VTA = ventral tegmental area, SNR= substantia nigra region, NAcc = nucleus accumbens, PAG = periaqueductal gray

Structural volume changes

Early-stress induced alterations in structural volume of the BLA, PFC, and HIPP were determined due to the involvement of these regions in the corticolimbic emotion processing circuit (Godsil et al., 2013). We previously reported a modest enhancement of BLA volume in PND20 pups exposed to LB conditions using the cellular Cavalieri method (Guadagno et al., 2018a). Thus, we predicted that LB would result in similar volumetric changes in the current study. We compared both left and right side volumes in particular for lateralized structures such as the amygdala and the mPFC (Baker and Kim, 2004; Haritha et al., 2013). In preweaning pups, BLA volume was significantly higher in the left compared to the right side ($F(1,44) = 26.09$, $p < 0.001$), but there was no bedding or bedding x side interaction (Fig. 6A). A similar effect of side was observed for the hippocampus (Fig. 6b) ($F(1,44) = 6.35$, $p < 0.05$) and IL mPFC (Fig. 6d) ($F(1,44) = 3.55$, $p = 0.066$) although for these structures, the right volume was higher than the left. Significant effects of bedding were observed for the hippocampus (Fig. 6b) ($F(1,44) = 18.01$, $p < 0.001$), the PL mPFC (Fig. 6c) ($F(1,44) = 4.26$, $p < 0.05$) and were close to significant for the IL mPFC (Fig. 6d) ($F(1,44) = 3.32$, $p = 0.075$) with LB pups having a greater volume compared to NB pups. There was no bedding x side interaction in any of the structures examined. Total brain volume was significantly greater in LB compared to NB pups ($t(22) = -1.743$, $p < 0.05$) although NB and LB groups had comparable total body weights (Table 3).

When volumetric measures were taken in adulthood (Fig. 7), a significant effect of side was maintained for the BLA ($F(1,20) = 73.79$, $p < 0.001$, left>right) and hippocampus ($F(1,20) = 5.82$, $p < 0.05$, right>left) as observed in preweaning pups. There was no effect

of side in the mPFC, either for the PL or IL volume. Bedding condition did not modify the volume of any region examined and we found no bedding x side interaction for any of the regions. There were no significant effects of early stress on total brain volume or body weight in adulthood (Table 3).

In order to examine volumetric correlations between the various regions of interest, we built bilateral correlation matrices of left and right volumes for both preweaning and adult rats. These matrices revealed generally more positive inter and intra-hemispheric cross-correlations in LB compared to NB rats, in adults (Fig. 8). In preweaning pups, negative correlations between the right BLA and ipsilateral mPFC, both PL and IL, were observed in NB pups (PL: r = -0.32; IL: r = -0.7), but disappeared in LB pups (PL: r = -0.17; IL: r = -0.08) (Fig. 8a and 8b). In LB pups, the right BLA volume showed a strong positive correlation with the contralateral (left) PL (r = 0.47), which was not observed in NB rats. Data for the left BLA in pups showed that a negative correlation existed with the ipsilateral (left) IL in NB pups (r = -0.5) and was weaker in LB pups (r = -0.23). Compared to the correlations in preweaning pups, adult correlations were mostly positive in both NB and LB groups (Fig. 8c and 8d), however, much stronger in the LB adult group (Fig. 8d). Notably, the only large negative correlations in the adult NB matrix were found between the left IL and the left (r = -0.48) and right (r = -0.54) BLA (Fig. 8c). These negative correlations were not observed in adult LB offspring. Positive correlations specific to LB adult rodents included the right BLA with the left (r = 0.37) and right (r = 0.26) IL regions (Fig. 8d). There was a stronger positive correlation between the right BLA and right PL in LB (r = 0.88) compared to NB adults (r = 0.38).

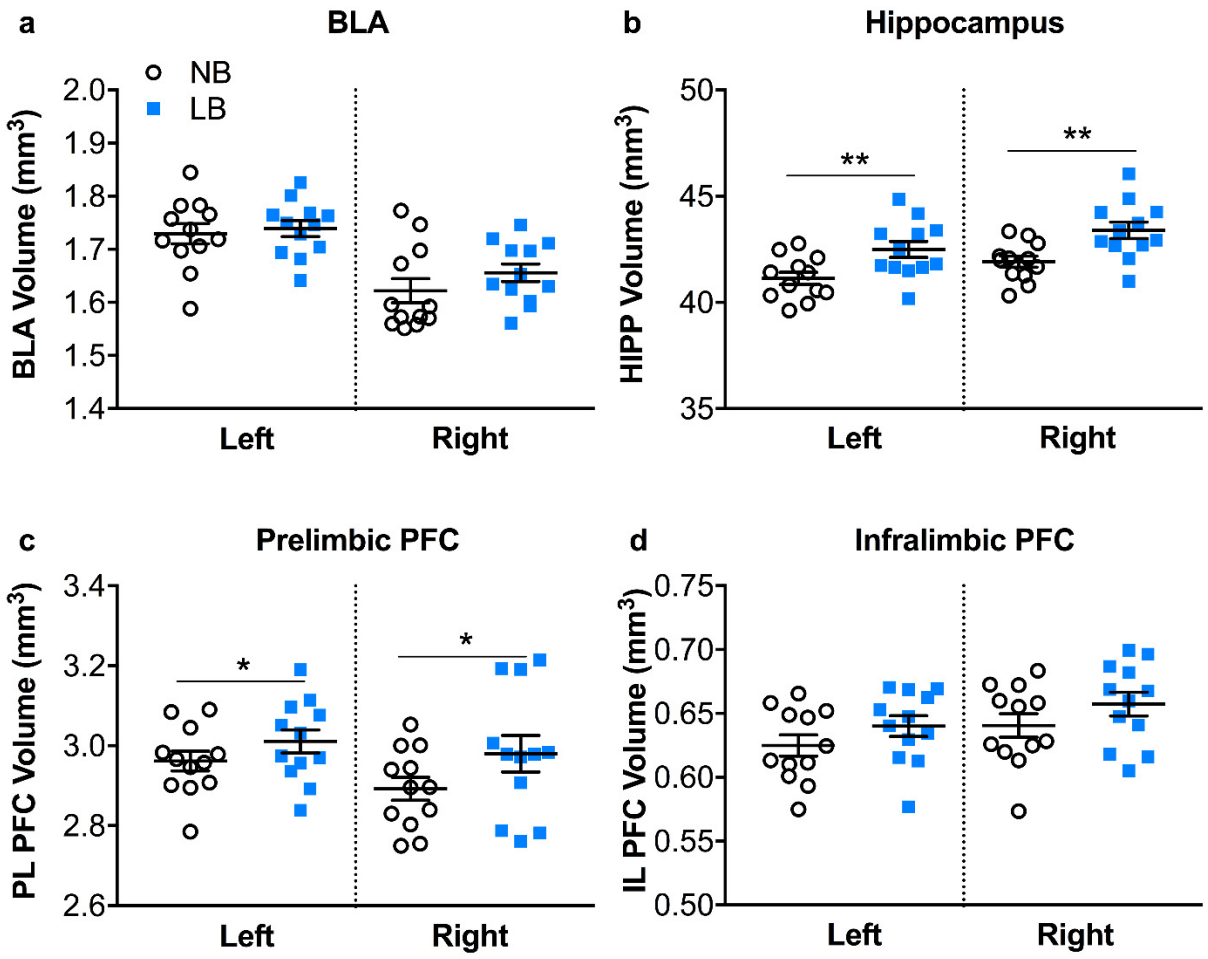

Figure III-6. Total volumes of the left and right basolateral amygdala (BLA, a), hippocampus (HIPP, b), prelimbic (PL, c) and infralimbic (IL, d) prefrontal cortex in normal (NB) and limited bedding (LB) PND18 rats. As shown in panels b and c, LB pups had significantly larger total hippocampal ($p<0.001$; ANOVA) and PL ($p<0.05$; ANOVA) volumes, compared to normal bedding (NB) pups. BLA volume was significantly greater in the left compared to the right hemisphere ($p<0.001$; ANOVA). Right compared to left hemisphere volume was higher for the HIPP ($p<0.05$; ANOVA). Values represent mean ± SEM of n = 12 animals/group. *$p<0.05$, **$p<0.001$ NB vs LB

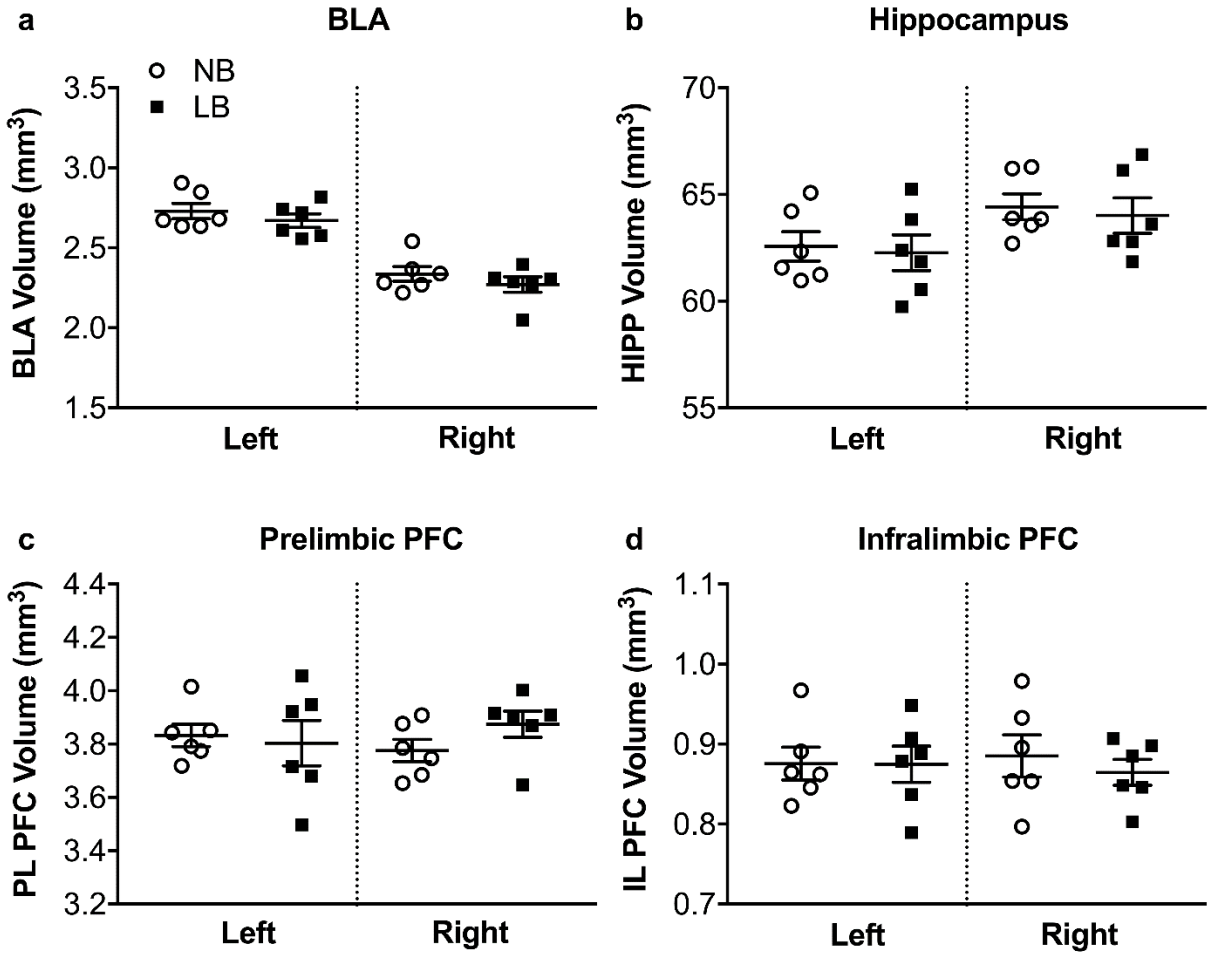

Figure III-7. Total volumes of the left and right basolateral amygdala (BLA, a), hippocampus (HIPP, b), prelimbic (PL, c) and infralimbic (IL, d) prefrontal cortex in normal (NB) and limited bedding (LB) adult rats. No effects of bedding were observed for any of the structural volumes. A significant effect of side was maintained in adulthood for the BLA (left>right, $p<0.001$; ANOVA) and HIPP (right>left, $p<0.05$; ANOVA). Values represent mean ± SEM of n = 6 animals/group.

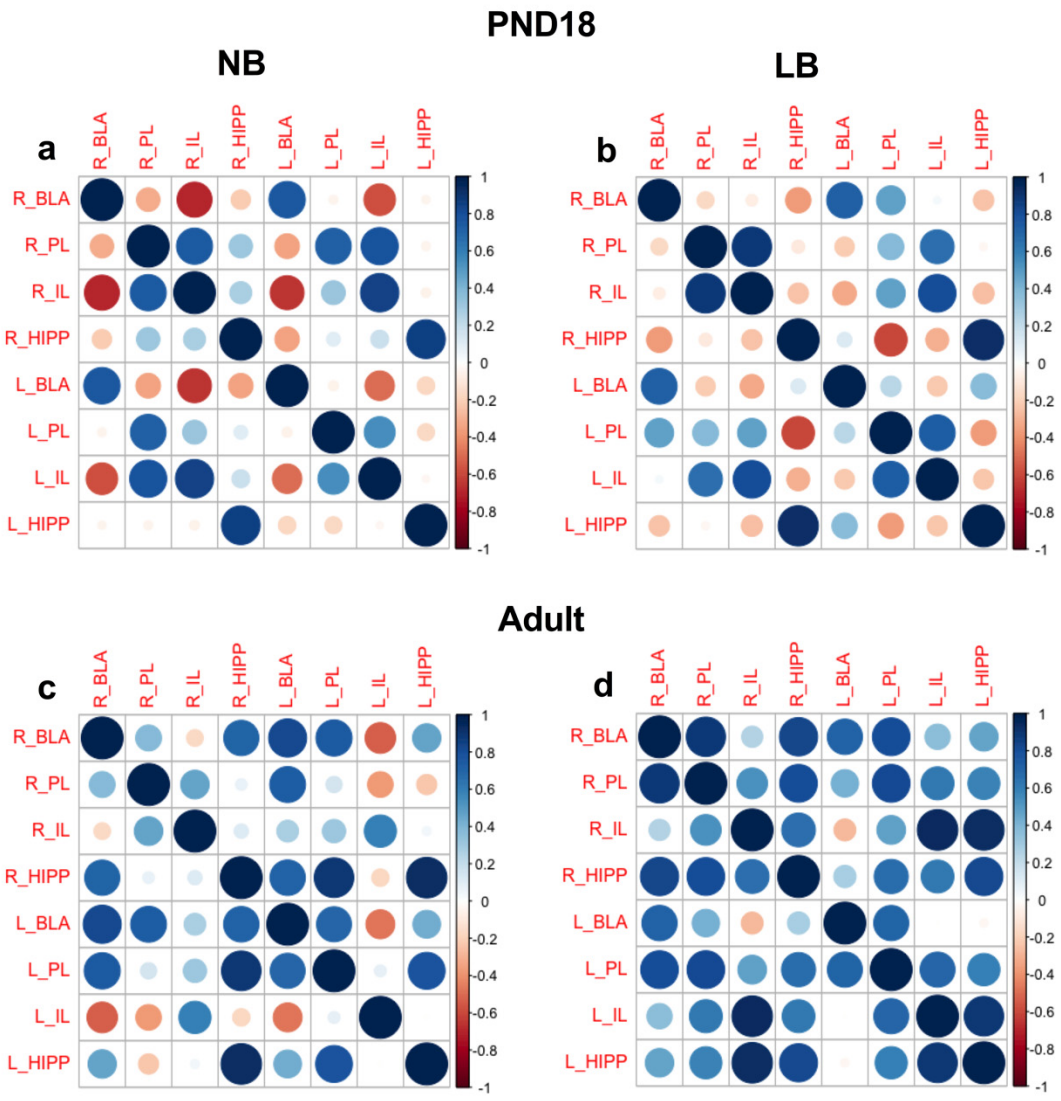

Figure III-8. Structural correlation matrices of left (L) and right (R) hemispheres of the basolateral amygdala (BLA), prelimbic (PL) and infralimbic (IL) medial prefrontal cortex and hippocampus (HIPP) in preweaning (panel a: NB, panel b: LB) and adult (panel c: NB, panel d: LB) rats. Volumes were residualized for effects of total brain volume. Scale depicts the degree of correlation (Pearson r value). NB: normal bedding, LB: limited bedding.

Total Brain Volume (mm^3)	*NB*	*LB*
PND18	2078.4 ± 20.3	2123.41 ± 15.95*
Adult (PND74-76)	3336.53 ± 41.77	3297.71 ± 44.06
Total Body Weight (g)	*NB*	*LB*
PND18	54.56 ± 1.24	54.31 ± 1.08
Adult (PND74-76)	566.67 ± 17.94	591.17 ± 9.2

Table III-3. Total brain volumes and body weights in limited (LB) and normal bedding (NB) animals. In preweaning pups (PND18), total brain volume was significantly greater in LB compared to NB pups ($p<0.05$; t-test), although total body weight was not significantly affected by early stress. There were no significant effects of LB on total brain or body weight in adulthood (PND74-76). Values represent mean ± SEM of n = 12 animals/group for preweaning pups and n= 6 animals/group for adults. *$p<0.05$ NB vs LB (t-test).

Effect of LB conditions on adult fear conditioning and fear extinction

Amygdala-dependent cued fear conditioning (day 1) was compared between NB and LB adult offspring before adult imaging, followed by fear extinction on the next day (day 2) (Fig 9). Fear conditioning (day 1) and extinction (day 2) data were first analyzed using separate two-way ANOVA, with bedding as a between-subjects factor, and tone as a within-subjects factor. During fear conditioning, there was no significant bedding effect, but a significant effect of tone ($F(5,110) = 32.36$, $p < 0.001$), indicating that conditioning was successful (Fig. 9a). On extinction day 2, there was a significant bedding effect, with LB rats displaying significantly greater freezing behavior during tones 1-8, compared to NB rats ($F(1,22) = 4.61$, $p < 0.05$) (Fig. 9b). There was also a significant main effect of tone ($F(7,182) = 5.14$, $p < 0.001$), but no bedding x tone interaction.

We next compared the difference in freezing between the initial and final tone during both fear conditioning (day 1) and fear extinction (day 2). For fear conditioning, we examined freezing during the presentation of the tone, as well as during the corresponding inter-trial interval, since we identified continued freezing behavior following the tone. The increase in freezing between trials 1 and 5 did not reach significance (Fig. 9a). For fear extinction on day 2, we focused specifically on freezing during tones to identify extinction to the auditory conditioned stimulus. The decrease in freezing from tones 1 to 8 was significantly lower in LB versus NB rats ($t(22) = 1.842$, $p < 0.05$) (Fig. 9b).

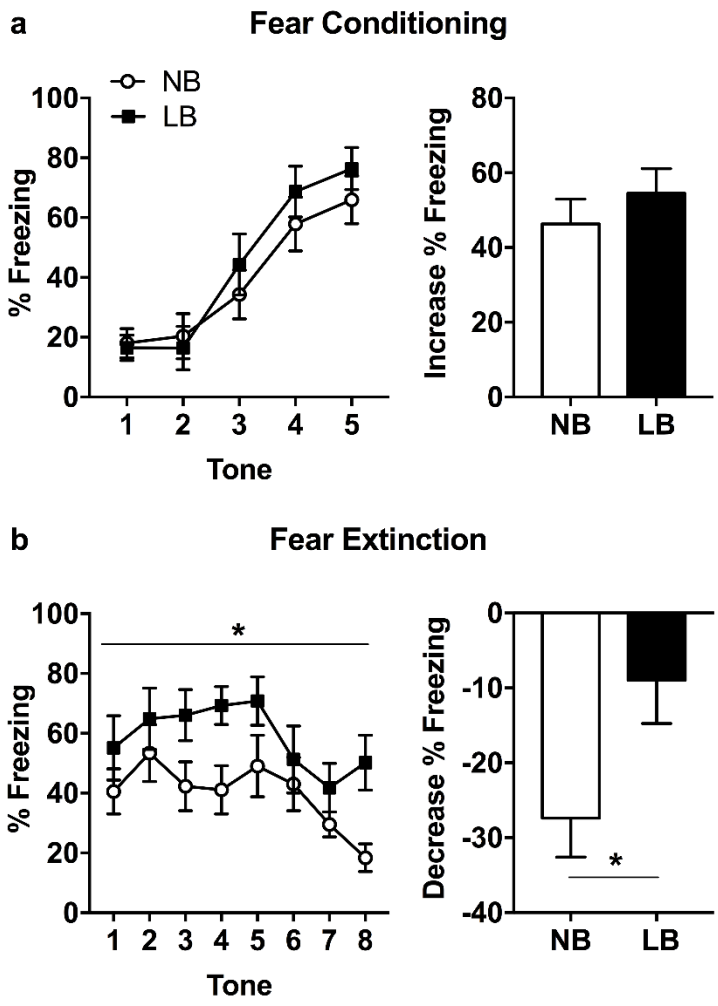

Figure III-9. Percentage of freezing behavior by normal bedding (NB) and limited bedding (LB) adult male rats during individual 30 sec tones for fear conditioning (a) and extinction (b). Increase in % freezing between the first and last trials (fear conditioning) is shown in panel a, and decrease in % freezing between tones 1 and 8 (fear extinction) is depicted in panel b. Two-way ANOVA did not reveal significant bedding effects during fear conditioning (a). During fear extinction, LB animals displayed significantly more freezing behavior compared to their NB counterparts (b, $p<0.05$; ANOVA). LB versus NB rats exhibited less decrease in freezing from tones 1 to 8 (b, $p<0.05$; t-test). Values represent mean ± SEM of n = 12 animals/group. *$p<0.05$ NB vs LB

Discussion

In this study, we used resting state functional MRI to investigate the effects of early life stress, in the form of LB exposure, on the functional connectivity of the preweaning and adult BLA. Our novel findings are that early chronic stress significantly reduced functional connectivity of the anterior BLA (aBLA) with the mPFC in preweaning and adult male rats and that hemispheric lateralization of the BLA and mPFC is already observed preweaning. Altered connectivity with other structures in the emotional and stress circuitry were also observed in LB offspring, some of these being maintained in adulthood, while others disappeared. Structural changes induced by LB were modest with weak negative volumetric correlations between the BLA and mPFC observed in LB animals. We propose that aberrant BLA-mPFC resting state connectivity might contribute to the enhanced fear conditioning and reduced extinction observed here and previously in LB adult offspring (Arp et al., 2016; Guadagno et al., 2018a).

LB-induced changes in BLA rs functional connectivity

In designing the analysis of resting state connectivity between the BLA and mPFC we used four different premises. First, the pattern of projections to the mPFC from anterior or posterior BLA differs (see Fig.1). The anterior BLA projects mainly to the PL, while the posterior BLA projections are to both PL and IL. This translates into a clear rostral-caudal dissociation in the role of the BLA in mediating fear behaviors in adulthood (Scicli et al., 2004; Stevenson, 2011). Second, the amygdala is a highly lateralized structure in adult rodents with the right amygdala being more active than the left (Baker and Kim, 2004)

and there is a possibility that lateralization is already observed preweaning. Third, the IL and PL portions of the mPFC exhibit very different functions in the regulation of stress responses (McKlveen et al., 2015) as well as fear conditioning and extinction (Wilber et al., 2011), emphasizing the need to consider these two regions separately. Fourth, while connections between the BLA and mPFC in adult rats are mostly observed ipsilaterally (Krettek and Price, 1977; McDonald, 1991), a small proportion of contralateral connections are observed and there is a possibility that ELS modifies the development of ipsi- vs contralateral BLA to mPFC projections.

Preweaning pups:

In our experiments, we chose to examine rs functional connectivity on PND18 because we previously found that LB induces significant morphological and functional changes in the BLA at this age (Guadagno et al., 2018a), one week after the return to normal bedding conditions and a few days after completion of the BLA-mPFC circuitry. The BLA-mPFC pathways mature during the neonatal period in rodents (Cunningham et al., 2002), with those from the BLA to the mPFC reaching an adult-like pattern of layer distribution from PND11 onwards (Bouwmeester et al., 2002a) encompassing the period during which many ELS manipulations have their effects. The amount of BLA to PFC projections continue to mature into late adolescence and adulthood (Cunningham et al., 2002). Reciprocal projections from the mPFC to BLA are maturing slightly later (Bouwmeester et al., 2002a; Arruda-Carvalho et al., 2017) from PND13 onwards suggesting that the ascending BLA to mPFC projections might represent a primary target

for the effects of the chronic LB stress (PND1-10) that we use. Thus, any early changes exerted on the BLA, including the increased spine density and reactivity that we demonstrated on PND10 and 20 (Guadagno et al., 2018a) could potentially participate in altering BLA-mPFC connectivity. Indeed, we found that in PND18 rats exposed to LB, the right aBLA seed showed significantly reduced functional connectivity to the ipsilateral PL subregion of the mPFC, whereas the left aBLA connectivity to the mPFC was unaffected. When connectivity from the pBLA was evaluated, the ipsilateral connectivity was reduced from the left pBLA to both the PL and IL, but increased from the right pBLA to the IL only. These results suggest that overall, the ipsilateral components from the BLA-mPFC circuit, which constitute the majority of BLA-mPFC afferents (Cunningham et al., 2002), exhibited a reduction in functional connectivity, consistent with most of the previous literature in adult rodents (Yan et al., 2017) and humans (Kim et al., 2013) exposed to ELS. The mechanisms responsible for decreased BLA-mPFC connectivity after exposure to early chronic stress are currently unclear. Others have suggested that ELS might also accelerate BLA maturity in rat pups via increased corticosterone action on the amygdala (Moriceau et al., 2006) or that there is a premature shift to an “adult-like” phenotype of BLA-mPFC connections in humans (VanTieghem and Tottenham, 2017). The increased resting state connectivity from the right pBLA to the ipsilateral IL on PND18 might reflect advanced maturation of BLA connectivity specifically to this mPFC subregion.

Comparing both left and right BLA seeds, we found that the right BLA connectivity was generally more affected than the left BLA in preweaning pups, suggesting that lateralization of the BLA is already present in developing pups. For instance, the right aBLA showed a reduction in connectivity to the ipsi- and contralateral PL mPFC regions,

whereas the left aBLA connectivity was only decreased to the contralateral PL mPFC. We also observed reduced right, but not left BLA connectivity to other brain areas such as the VTA and SNR from both the anterior and posterior BLA seeds. Connectivity to the NAcc was reduced only from the left posterior BLA. The BLA is tightly linked to the mesocorticolimbic dopaminergic pathway, by receiving dopaminergic afferents from the VTA/SNR and sending projections to the NAcc (Sharp, 2017). Our observation that LB reduced rs-fMRI BLA connectivity to these regions in preweaning pups suggests that activity in the mesocorticolimbic pathway might also be altered as a result of the early chronic stress of LB. Indeed, reduced neural activity in the NAcc and mPFC was shown previously in a variant of the LB paradigm in weaned rats (Rincon-Cortes and Sullivan, 2016) and our own data demonstrate a reduced connectivity with the mPFC in pups. Interestingly, both the VTA and SNR send major efferents to the PL and IL regions of the mPFC and thus, are likely to participate in the top-down mPFC control over the BLA (Hoover and Vertes, 2007).

The BLA and mPFC regulate memory and emotional processing with convergent information from the HIPP, together forming an interconnected corticolimbic network (Godsil et al., 2013). The BLA to ventral HIPP pathway in particular, is implicated in the consolidation of fear memories (Bast et al., 2001; Huff et al., 2016) and the mediation of social and anxiety behaviors (Felix-Ortiz et al., 2013; Felix-Ortiz and Tye, 2014). Exposure to LB in our study led to complex alterations in BLA-HIPP connectivity with both increased and reduced connectivity in dorsal and ventral hippocampus subfields. For instance, the aBLA generally showed increased connectivity to the ipsilateral CA1/2/3 and DG/Subiculum subfields of the dorsal HIPP and increased or decreased connectivity to

the ipsilateral CA1 and DG/Subiculum ventral HIPP regions depending on the brain side. The pBLA revealed mostly increased connectivity to the same ipsilateral dorsal HIPP subfields and reduced ipsilateral connectivity from the right, but not the left, pBLA to the CA1 and DG ventral HIPP regions, highlighting once again the functional differences segregating the left and right BLA. Interestingly, most of the increased connectivity in the BLA to ventral HIPP was observed with the left BLA and ipsilateral ventral CA1/CA3. Given the large documented effects of LB on HIPP-associated behaviors (Walker et al., 2017), a more complete study of the BLA-HIPP connectivity is required to better assess the effects of ELS on this pathway.

Thus far, we mostly considered LB-induced changes in ipsilateral connectivity, although we also observed contralateral alterations in BLA connectivity. In particular, all BLA seeds, except for the right pBLA, exhibited a reduction in connectivity to the contralateral PL. Connectivity to the contralateral IL was reduced from the right aBLA, but increased from the left pBLA. Using high resolution diffusion tensor imaging, a recent study performed in PND56 rats exposed to chronic ELS revealed an increased number of cross-midline tracts between the amygdala (BLA and CeA) and mPFC in both hemispheres of the brain (Bolton et al., 2018). Inter-hemispheric BLA-mPFC projections have been discovered in PND18-20 rats (Verwer et al., 1996), and our data suggest that LB might modify the functional integrity of such contralateral connections, possibly through a reduction in the developmentally programmed pruning of contralateral projections. This hypothesis is being actively investigated in our laboratory and supported by recent findings of an increase in fiber tracts crossing the midline in LB adult offspring (Bolton et al., 2018). It is also conceivable to indirectly modulate contralateral BLA-mPFC

connectivity via glutamatergic mPFC callosal neurons, which allow for the inter-hemispheric exchange of stress-sensitive information. Chronic stress in adult rodents induces mPFC callosal neuron spine loss and dendritic atrophy (Luczynski et al., 2015), potentially reducing interhemispheric communication, but the effects of ELS on callosal neurons remain to be investigated.

The integration of various sensory inputs and outputs in the amygdala relies heavily on intra-amygdalar connectivity, which we found to be modestly altered by our LB paradigm. The BLA connects primarily with the lateral (LA) and central (CeA) amygdala, the essential output subnucleus of the amygdala (Duvarci and Pare, 2014). We found both increases and decreases in connectivity of the BLA with the ipsilateral LA, but no significant changes in connectivity between the BLA and the CeA in LB vs. NB pups. In LB adults, the right aBLA displayed reduced connectivity with the LA and the left pBLA showed increased connectivity with the CeA. The differences in intra-amygdala connectivity between preweaning and adult rats suggest that some of these connections have not yet reached maturation before weaning.

Adult offspring:

Previous studies have identified aberrant functional (Yan et al., 2017) and structural (Bolton et al., 2018) connectivity of the adolescent and adult BLA-mPFC in rodents exposed to models of ELS comparable to ours. Here we confirm that LB exposure induces long lasting alterations in BLA rs-fMRI connectivity and extend the analysis to demonstrate that reduced resting state functional connectivity persisted in LB offspring

until adulthood, with almost the exact same connectivity distribution compared to the preweaning pups. Furthermore, as for the preweaning rats, lateralization of the BLA and the rostro-caudal BLA level critically determined the effects of LB in the long-term. Connectivity between the right aBLA to the ipsilateral IL and PL mPFC remained reduced in the adult LB offspring, although in the pBLA, there was an increase in connectivity with both mPFC regions. In the left BLA, only the anterior portion displayed increased connectivity to the PL and IL regions in adults. Interestingly, hemispheric asymmetry in connectivity also persisted in adults, with the right BLA connectivity alterations outnumbering those from the left hemisphere. One putative reason for enhanced sensitivity of the right BLA to the stress of LB could be due to a reduction in local inhibitory control over principal BLA neurons (Butler et al., 2018). This is supported by a recent study demonstrating a higher density of parvalbumin-positive interneurons in the left compared to the right BLA of adult rats (Butler et al., 2018).

Although LB-induced changes in connectivity in adults were not as widespread as in pups, nearly all the BLA seeds in adults exhibited changes in connectivity to the bilateral NAcc with increased connectivity from the aBLA and reduced connectivity from the pBLA irrespective of side. Projections from the BLA (which encode affective information) and the mPFC are known to converge within the NAcc, and the BLA may thus activate specific sets of NAcc neurons either through direct or indirect projections via the recruitment of the mPFC (McGinty and Grace, 2008). It would be important to determine whether anterior and posterior BLA neurons provide segregated inputs to either the mPFC or NAcc because the effect of LB on BLA-NAcc connectivity was clearly segregated as well (McGinty and Grace, 2008).

It is important to highlight the caveats of rs-fMRI in our study. First, as reported in recently published studies (Yan et al., 2017), the rs-fMRI was conducted with isoflurane anesthesia to minimize stress to the animals and help improve image quality by reducing potential motion artifacts (Leslie and James, 2000). As the rs-fMRI analysis uses the measures dependent on neurovascular coupling, the use of anesthesia can introduce a confounding factor to the BOLD (Blood-oxygen-level dependent) signals (Franceschini et al., 2010). However, to minimize such confounding factor, we used a low dose of isoflurane (<2-3%) and the effects of isoflurane were monitored such that all animal physiological parameters (temperature, heart rate and breathing rate) were stable and similar to those when at rest. Furthermore, both groups underwent the same rs-fMRI imaging methodology under isoflurane anesthesia. Therefore, the effect of isoflurane on group contrast would be normalized. In addition, rs-fMRI BOLD signals are reported to be detectable at reduced level in the presence of anesthesia, effectively leading to diminished yet similar network connectivity at seed-based approach (Wu et al., 2016; Venkatraghavan et al., 2017).

Lastly, it is also worth mentioning that while preweaning rats were scanned during the light phase of the 12 hr light: 12 hr dark cycle, adult animals were subsequently switched to a reverse light:dark cycle for behavioral testing and scanned during the active or dark phase of their light:dark cycle. As feeding is a major component determining diurnal activity in pups and is performed mostly during the light phase of the cycle (i.e. when the mother is resting) (Stern and Levin, 1976; Tallett et al., 2009), we considered that scanning in the light phase for pups was close to equivalent to the normal nocturnal

feeding activity of adult rats. Thus, we adapted our scanning protocol to the phase of the diurnal cycle most relevant to feeding and overall activity in both groups.

Structural changes in preweaning and adult corticolimbic structures induced by LB

The volume correlations between the segmented corticolimbic structures (mPFC, HIPP and BLA) revealed mostly positive correlations, especially in adults and a greater mix of positive and negative correlations in preweaning pups. Notably, there were negative correlations between the right BLA and the ipsilateral IL and PL in NB preweaning pups that disappeared in LB pups. A similar trend was observed in adults, with volumetric correlations between the right BLA and the ipsilateral IL and PL becoming more positive in LB than in NB adult offspring. The reason for these volumetric correlation changes are presently unclear, but might be related to activity-dependent changes in dendritic morphology occurring in both BLA and mPFC under conditions of chronic stress (Vyas et al., 2006; Eiland et al., 2012).

The stress of LB transiently increased total volume of the hippocampus and PL mPFC in preweaning pups, but this was not observed in adults (Bolton et al., 2018), despite reports of altered cognitive functions and behaviors associated with these structures (Walker et al., 2017). The significant increase in hippocampal volume might have contributed to the enhanced total brain volume observed in LB pups. The increased volume could result from a deficit in normal programming of synaptic pruning that is occurring during this developmental period and continuing into adolescence (Johnson and Kaffman, 2018). An interesting hypothesis that we are currently testing is that reduced microglia activity in LB pups would lead to a significant impairment in synaptic

pruning (Paolicelli et al., 2011; Wu et al., 2015). Indeed, we have preliminary data showing that the number of Iba-1 positive cells, as markers of microglia, is reduced in LB compared to NB pups (Walker et al. unpublished). As indicated by our present results, this effect of LB on synaptic pruning might be region-specific and is transient because we did not observe region or total brain volume changes in adults. Despite this, we still observed behavioral changes in adulthood that we speculate might be linked more to the activity and connectivity of neural projections rather than the amount or volume of those projections.

Numerous studies have investigated the consequences of ELS on cognitive and memory functions and examined more specifically the effects of LB on key structures involved in these processes, such as the hippocampus and mPFC (Walker et al., 2017). Interestingly, LB initially increased neurogenesis in the hippocampus of male mice on PND9, but later induced a reduction in hippocampal volume and neurogenesis on PND150 (Naninck et al., 2015). Not surprisingly, selective loss of hippocampal volume in adulthood was associated with impaired performance in spatial learning and cognitive tasks (Naninck et al., 2015). The chronic stress-induced increase in neurogenesis described above might help to explain the transient increase in hippocampal volume we documented in preweaning, but not adult, LB male rats. The purpose of stress-induced early neurogenesis is unclear, but we hypothesize that it might serve as a compensatory mechanism that is activated in males to prevent stress-induced atrophy in the preweaning period. It has been suggested that the newborn population of hippocampal cells depletes or becomes less functional after LB exposure to ultimately result in impaired hippocampal function in adulthood (Walker et al., 2017).

In the present study, BLA volume was not modified by LB, in contrast with an enhancement reported in our previous cellular BLA volume determination (Guadagno et al., 2018a). The discrepancy between the two results may arise from differences in method sensitivity as the resolution of MRI is inferior to cellular volumetric estimates. Interestingly, in both preweaning pups and adults, BLA volume in all groups was consistently greater in the left compared to the right hemisphere and the opposite was seen for the HIPP volume, which was greater in the right hemisphere. It is unclear how to interpret these lateralized data in view of functional connectivity data that tends to favor the right BLA as being more connected than the right, for instance. Since we did not parcellate HIPP subfields in our segmentation analysis, we cannot comment on the extent to which dorsal versus ventral HIPP volumes were affected.

Consequences of LB exposure on adult fear conditioning and extinction

Our study has revealed significant and persistent alterations in BLA-mPFC functional connectivity, with some parallel effects on structural volumetric correlations. Since this pathway is critical for fear conditioning and extinction (Sotres-Bayon and Quirk, 2010), we wanted to confirm the behavioral effects of LB on fear conditioning and extinction in our adult cohort. LB animals exhibited a trend towards more freezing during fear conditioning compared to NB animals and greater freezing behavior during fear extinction, showing that extinction is impaired by LB. The activity of the IL mPFC is critical for fear extinction (Sotres-Bayon and Quirk, 2010; Duvarci and Pare, 2014), as IL inactivation reduces extinction learning in rats (Sierra-Mercado et al., 2011). In adults exposed to LB, functional connectivity from the BLA to the IL mPFC was largely

decreased, likely resulting in the silencing of this pathway and a weakening of fear extinction (Bukalo et al., 2015). The BLA-mPFC pathway also mediates other relevant behaviors, such as pain-induced cognitive dysfunctions (Thompson and Neugebauer, 2017), anxiety and social behaviors (Felix-Ortiz et al., 2016). In fact, optogenetic activation of this pathway reduces social interaction and increases anxiety-like behaviors (Felix-Ortiz et al., 2016). While we observed mostly reduced connectivity from the BLA to the mPFC in adults, we consistently found increased functional connectivity of the right posterior BLA to the ipsilateral mPFC. Increased ipsilateral connectivity between the BLA and mPFC might predict the enhanced anxiety-like behavior and reduced social contact previously reported in LB adult offspring (Guadagno et al., 2018a), although this possibility has not yet been directly tested.

Conclusions

Our results demonstrate that the early stress of LB significantly reduced resting state functional connectivity between the BLA and mPFC, both in preweaning pups and adult offspring, suggesting a mechanism that could contribute to increased fear conditioning and reduced fear extinction after ELS. In addition, because the BLA-mPFC pathway is also critical for many cognitive and pain behaviors, changes in its formation and functional integrity/capacity might have consequences for a large spectrum of behavioral regulation. Our studies have revealed important mechanistic aspects that might be critical in mediating the consequences of ELS. However, to gain a better understanding of the potential consequences in humans, these preclinical studies should be extended to

include fMRI analyses in awake animals. Our studies also highlight the important effect of hemispheric lateralization of the BLA and mPFC as determining different patterns of connectivity and structural effects. This aspect of regulation is often overlooked in many studies and our results suggest that changes in the formation of ipsi- and contralateral BLA-mPFC projections after ELS might significantly contribute to behavioral outcomes. Thus, we propose that the increased amygdala activity observed previously in LB neonates would either accelerate the maturation of the BLA-mPFC circuit and/or weaken BLA projections to the mPFC, leading to reduced connectivity. These possibilities should be examined in the context of interhemispheric differences in both BLA and mPFC activity that appear to be already present in neonatal life.

Acknowledgements and Disclosures

We thank Dr Jürgen Germann and Ms Elisa Guma (Douglas Institute, Imaging Center) for their assistance with the Display software package for manual segmentation analyses and preliminary co-registration analyses, respectively. This study was funded by an NSERC grant to CDW (Grant #138199) and an internal McGill-Faculty of Medicine studentship award to AG.

Conflict of Interest: The authors declare that they have no conflict of interest.

Research involving animals and ethical approval: All experimental procedures carried out on Sprague-Dawley rats were approved by the University Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care.

Connecting Statement to Chapter IV

In Chapter II, we demonstrated that LB exposure induced hypertrophy of excitatory BLA neurons in preweaning male, but not female rats, in parallel with enhanced evoked synaptic responses. These early morphological and functional impairments in the BLA associated with increased fear and anxiety-related behaviors in adult males exclusively. Amygdala hypertrophy (Tottenham et al., 2010) and hyperactivity (Tottenham et al., 2011; van Harmelen et al., 2013) in humans having experienced ELS are believed to accelerate the development of a more "mature" BLA-mPFC circuit and weaken the resting-state functional MRI connectivity between these regions, ultimately leading to lasting emotional dysfunctions (Gee et al., 2013a). In Chapter III, we addressed whether ELS could similarly disrupt BLA function on a larger scale and affect functional connectivity of the BLA with the mPFC, as well as with other areas, in preweaning male rats. Consistent with the human (Burghy et al., 2012; Gee et al., 2013a) and adult rodent literature (Yan et al., 2017), we reported that decreased BLA-mPFC functional connectivity in PND18 male rats subjected to LB conditions persisted until adulthood and possibly contributed to heightened fear behaviors. We are also the first to show that connectivity of the BLA was already lateralized in preweaning life, as the right BLA displayed significantly more connectivity changes compared to the left hemisphere, notably with the mPFC and vHIPP, two key regions in the emotion regulation circuitry.

Given our previous findings that were mostly acquired in males, and the fact that we did not differentiate left from right BLA slices during our electrophysiological recordings in Chapter II, in the upcoming chapter we aimed to unravel the potential mechanisms by which ELS exerts sex and hemispheric-dependent alterations in the developing BLA. We first examined synaptic plasticity, neuron excitability and NMDAR subunit expression and proposed that increased synaptic plasticity and neuron excitability in LB juvenile males would be linked with modified indices of GABA functioning. Parvalbumin (PV) cells comprise half of the interneurons in the BLA and directly modulate glutamatergic synaptic output (Woodruff and Sah, 2007; Wolff et al., 2014). Many PV-positive and excitatory cells in the BLA are surrounded by perineuronal nets (PNNs) (Morikawa et al., 2017; Santiago et al., 2018), specialized extracellular matrix structures that significantly influence neuron firing and synaptic plasticity (Gogolla et al., 2009; Balmer, 2016; Reichelt et al., 2019). In another series of experiments, we hypothesized that LB exposure would suppress the activity of PV-positive cells and increase the formation of PNNs around GABAergic neurons, specifically those expressing PV.

Chapter IV

It is all in the right amygdala: increased synaptic plasticity in male, but not female juvenile rat pups after exposure to early-life stress

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In Preparation

Abstract

Early-life stress (ELS) is associated with increased vulnerability to mental disorders. The basolateral amygdala (BLA) plays a critical role in fear conditioning and is extremely sensitive to ELS. Using a naturalistic rodent model of ELS, the limited bedding paradigm (LB) between postnatal days (PND) 1-10, we previously documented that LB male, but not female preweaning rat pups display increased BLA neuron spine density paralleled with enhanced evoked synaptic responses, and altered BLA functional connectivity. Since ELS effects are often sexually dimorphic and amygdala processes exhibit hemispheric asymmetry, we investigated changes in synaptic plasticity and neuronal excitability of BLA neurons *in vitro* in the left and right amygdala of PND22-28 male and female offspring from normal bedding (NB) or LB mothers. We report that LB conditions enhanced synaptic plasticity in the right, but not the left BLA of males exclusively. LB males also showed increased perineuronal net (PNN) density, particularly around parvalbumin (PV) cells, and impaired fear-induced activity of PV interneurons only in the right BLA. Action potentials fired from BLA neurons in the right amygdala of LB females displayed slower maximal depolarization rates and decreased amplitudes compared to NB females, concomitant with reduced NMDAR GluN1 subunit expression in the right BLA. These findings suggest that LB differentially programs synaptic plasticity and PV/PNN development in the left and right BLA. Furthermore, our study elucidates how sex moderates the effects of LB in the developing BLA and points to an organizational role of sex hormones on BLA synaptic function after ELS exposure.

Introduction

Chronic early-life stress (ELS) exposure during critical periods of brain development has long-term consequences on cognitive and emotional health in both humans and rodents (Pechtel and Pizzagalli, 2011; Krugers et al., 2017). The basolateral amygdala (BLA) is perhaps one of the most sensitive brain regions undergoing changes in response to ELS (Malter Cohen et al., 2013; Tottenham and Gabard-Durnam, 2017). It is also crucial in the acquisition and expression of conditioned fear (Teicher et al., 2003; Stevenson et al., 2009; Marek et al., 2013). We previously reported that ELS in the form of altered maternal care between PND1-10 increases spine density in excitatory BLA neurons in male neonatal and preweaning rats exclusively (Guadagno et al., 2018a). These morphological changes were paralleled with enhanced evoked synaptic responses in preweaning male offspring and heightened anxiety-like and fear behaviors in adult males, but not females (Guadagno et al., 2018a). Our findings demonstrate that sex impacts of ELS are observed in the developing BLA and associate with future behavioral impairments (Guadagno et al., 2018a). Sex-specific effects of ELS have also been documented in the prefrontal cortex and hippocampus (Walker et al., 2017; Goodwill et al., 2019; White and Kaffman, 2019). These effects can be age-dependent, possibly reflecting a differential time course of brain development between males and females (Lenroot et al., 2007) and thus, different windows of vulnerability between sexes (Derks et al., 2016).

Precisely how ELS leads to sex-dependent functional and cellular alterations remains elusive, especially with respect to the BLA (Walker et al., 2017; White and

Kaffman, 2019). In adult males, adolescent social isolation induces hyperexcitability in BLA pyramidal neurons, concomitant with enhanced anxiety-like behavior (Rau et al., 2015). This is also observed in adolescent (PND28-32) male rats exposed to chronic restraint stress (Hetzel and Rosenkranz, 2014). In adulthood, chronic stress increases LTP formation in the amygdala, which elicits the long-term consolidation of fear memories observed in male rats (Dalton et al., 2012; Suvrathan et al., 2014). Importantly, changes in synaptic plasticity in males are dependent upon the activation of glutamatergic NMDA (N-methyl-d-aspartate) receptors (NMDARs) (Dalton et al., 2012; Suvrathan et al., 2014), which are developmentally regulated (Lopez de Armentia and Sah, 2003) and critically contribute to fear learning (LeDoux, 2003; Miller et al., 2019). All three major NMDAR subunits, GluN1, GluN2A and GluN2B are expressed in the juvenile and adult BLA (Delaney et al., 2013), but only expression of GluN1 was elevated in the BLA of adult male mice after chronic stress (Yi et al., 2017). In the developing BLA, the effect of ELS on the expression of these receptors and their contribution to synaptic plasticity are still unclear.

In addition, the firing activity and glutamatergic synaptic output of BLA pyramidal cells are directly modulated by a class of inhibitory GABAergic interneurons that are implicated in fear conditioning (Wolff et al., 2014) and express the calcium-binding protein parvalbumin (PV) (Berdel and Morys, 2000; McDonald and Betette, 2001; Woodruff and Sah, 2007). PV cells form approximately half of the interneurons in the adult BLA (McDonald and Betette, 2001; Woodruff and Sah, 2007) and only mature between PND14-30 (Berdel and Morys, 2000; Davila et al., 2008). PV interneurons in the BLA are encapsulated by proteoglycan-rich specializations of the extracellular matrix called

perineuronal nets (PNN) (Kwok et al., 2011; Santiago et al., 2018) that reach adult levels by PND28 (Gogolla et al., 2009). The formation of PNN around PV neurons coincides with and likely contributes to the closure of critical periods of synaptic plasticity (Pizzorusso et al., 2002; Hensch, 2005). In a restricted model of early life adversity, the intensity of PNN staining in the BLA of PND23 male and female rats was found to be reduced (Santiago et al., 2018), suggesting that PNN in the developing BLA can be modified by early environmental influences.

Investigating cellular changes induced by ELS in the BLA requires an in depth understanding of how the amygdala processes information. Hemispheric asymmetry of the BLA has been demonstrated in humans and rodents, with the right amygdala being more heavily involved in fear and pain processing than the left (Baker and Kim, 2004; Ji and Neugebauer, 2009). Remarkably, we observed that lateralization of the amygdala is already observed pre-weaning, with more resting-state functional MRI connectivity changes being documented in the right versus the left BLA in males exposed to ELS (Guadagno et al., 2018b). In addition, more PV-positive cells are found in the left compared to the right BLA in adult male rats, supporting the idea that the amygdala not only displays functional, but also anatomical laterality (Butler et al., 2018).

Given that the BLA is a lateralized structure and ELS effects are often sexually dimorphic, we tested for asymmetrical effects and sex differences of ELS by investigating synaptic plasticity, neuron excitability and NMDAR subunit expression in the juvenile BLA. The well-established limited bedding (LB) paradigm, a preclinical model of ELS, was used between PND1-10 to elicit fragmented maternal care and chronic stress among pups (Walker et al., 2017). We hypothesized that increased BLA synaptic plasticity and neuron

excitability in early stressed juvenile males would be associated with reduced activity of PV cells and accelerated development of PNN around these PV interneurons.

Methods

Animals

Untimed-pregnant (received on gestation day 16-17) Sprague-Dawley female rats (Charles River, St-Constant, QC, Canada) were individually housed under controlled conditions of light (reverse 12 hr light:12 hr dark, lights off at 08:00 h), temperature (22-24°C), and humidity (70–80%) and provided *ad libitum* access to rat chow and water. The day of parturition was considered PND0 and litters were culled to 10 pups on PND1 with 5 males and 5 females if possible. Animals were weaned on PND22 and same-sex group housed with environmental enrichment until sacrifice on PND22-29. No more than 1-3 pups per sex and litter were used for each experimental data point. All experimental procedures were approved by the University Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care.

Limited bedding paradigm

Early chronic stress in the offspring was induced using the limited bedding (LB) and nesting protocol adapted from Baram and colleagues (Molet et al., 2014; Walker et al., 2017), which causes disruptions of maternal care as previously validated in our laboratory (McLaughlin et al., 2016; Guadagno et al., 2018a). On PND1, mothers and their litters were randomly assigned to the LB or normal bedding (NB) condition. LB

mothers and their litters were placed on a wire mesh platform elevated 2.5 cm above the cage floor, with approximately 1.5 cm of bedding underneath. Nesting material consisted of one-half of one paper towel. NB cages were given a 2.5 cm layer of woodchips and one-half of one paper towel. Cages were changed on PND4 and on PND10, all LB mothers/litters were returned to the normal bedding conditions. Dams and litters were weighed on PND1, 4, 10 and 22. All litters remained with their biological mother until weaning on PND22. Pups were also weighed on PND28 when possible.

Slice preparation for field and whole cell recordings

NB and LB animals aged PND22-28 (n=5-8 pups/group and sex from n=2-3 mothers/group) were anesthetized with isofluorane before being decapitated. We and others have successfully recorded field excitatory postsynaptic potential (fEPSP) responses in the BLA at these early ages (Aroniadou-Anderjaska et al., 2001; Guadagno et al., 2018a). The brains were rapidly removed, marked with a blade on the top-right side, and coronal slices (250 μm) containing the amygdala were cut using a Vibratome (Leica, Concord, ON) in hyperosmotic, ice-cold carbogenated (95% O_2 and 5% CO_2) sucrose-substituted artificial cerebrospinal fluid (S-aCSF) containing (mM) 252 sucrose, 2.5 KCl, 0.1 $CaCl_2$, 4 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, and 1.25 NaH_2PO_4 (pH 7.35, 360–370 mOsm). Slices were then incubated in carbogenated normal aCSF (125 mM NaCl in place of sucrose; 310–320 mOsm) at 32°C for 1 hr and subsequently kept at room temperature for a minimum of 1 hr prior to recording onset. All recordings were amplified by MultiClamp 700B and stored in a PC for offline analysis using Clampfit (Axon, Molecular Devices, Sunnyvale, CA).

Field and whole cell recordings

The protocols for slice recording were described previously (Guadagno et al., 2018a). Bicuculline (5 μM), a $GABA_A$ receptor antagonist, was added in the perfusing solution during fEPSP recordings. Evoked fEPSPs were induced by a bipolar stimulating electrode that was placed in the LA, adjacent to the BLA and recorded by an aCSF-filled glass electrode. Long-term potentiation (LTP) was induced by two 1 sec. applications of high frequency stimulation (HFS; 100 Hz, 100 pulses), separated by 20 sec. The stimulus intensity for LTP experiments was adjusted to evoke a field potential of an amplitude 40% of the maximum fEPSP response. LTP of fEPSP slope was estimated at 25-30 min after HFS. Input-ouput recordings were performed on separate slices from those used for LTP induction.

Evoked excitatory postsynaptic currents (EPSCs) were recorded in the whole-cell patch-clamp mode in BLA neurons with pipettes containing (in mM): 120 K-Gluconate, 17.5 KCl, 2 $MgCl_2$, 0.5 ethylene glycol tetraacetic acid (EGTA), 10 HEPES, 4 Na_2-ATP, and with the pH adjusted to 7.2 with KOH (~290 mOsm). Neurons recorded in current clamp mode were injected with 1000 ms long depolarizing current pulses in increasing steps of 10 pA to evoke action potentials (20 sweeps total, 10 sec per sweep). The average action potential amplitude and total number of action potentials were calculated from all spikes obtained over 20 sweeps. The action potential threshold and maximal depolarization rate were determined from the first spike induced. The voltage at which point the *dV/dt* trace first passed 10 mV/ms was considered to be the threshold. Only recordings with stable and low access resistance <15 MΩ were used. Only recordings from neurons showing clear spike adaptation and after hyperpolarization patterns similar

to BLA pyramidal neurons were kept for analyses, since those fast-spiking neurons that show little spike adaptation are normally resembling the properties of inhibitory interneurons (Woodruff and Sah, 2007; Ehrlich et al., 2012).

NMDAR subunit protein expression by Western blotting

NMDAR are developmentally regulated and critical for synaptic plasticity in the BLA (Muller et al., 2009; Suvrathan et al., 2014). Thus, we examined BLA expression of the three major NMDAR subunits, GluN1, GluN2A and GluN2B (Delaney et al., 2013). Tissue punches (1 mm diameter per side) from PND28 male and female animals (n=5-6 pups/group, from 3 mothers/group) were collected from fresh tissue, frozen on dry ice and later sonicated for 30 sec and homogenized in 1X RIPA buffer (#9806, Cell Signaling) with 2 mM phenylmethanesulfonyl fluoride (PMSF, P7626, Sigma-Aldrich). Samples were centrifuged at 13,000 rpm for 30 min at 4°C. The pellet was discarded and protein concentration in the supernatant was determined by the Bradford Protein Assay. A quantity of 30 µg (GluN1 and GluN2B) and 40 µg (GluN2A) of lysate was diluted in 2x Laemmli sample buffer (BioRad), heated at 95°C for 5 min to denature proteins and then separated on a 8% Tris-glycine gel at 110 V for 12 min and 160 V for 50 min in Tris–glycine running buffer (pH 8.3, 25 mM Tris base, 192 mM glycine, 0.1% SDS), and transferred onto nitrocellulose membranes (0.45 micron, Hybond-C Extra, RPN203E Amersham Biosciences) at 0.22 A (2 gels) for 2 hr on ice with Tris-glycine transfer buffer (pH 8.3, 25 mM Tris base, 192 mM glycine, 20% methanol). The membranes were left to air dry for 15 min, cut into two pieces according to the protein band size and washed 3 x 5 min in 1X TBST (1X Tris buffered saline with 0.1% Tween-20). The cut blots were

incubated in a blocking solution for 90 min (1X TBST and 5% nonfat dry milk) then incubated separately overnight at 4°C with the appropriate primary antibody (all produced in rabbit, 1:1000 dilution): GluN1 (G8913, Sigma-Aldrich), GluN2A (#4205, Cell Signaling), GluN2B (NB300-106, Novus Biologicals) and actin (A2066, Sigma-Aldrich). The next day, the blots were washed in 1X TBST buffer, then incubated for 60 min at room temperature with the secondary antibody (1:1000 goat anti-rabbit IgG Horseradish Peroxidase (HAF008, Novus Biologicals). The blots were developed using Clarity Western ECL Substrate (BioRad) and visualized with the ChemiDoc™ XRS+ System (BioRad). Band intensity was analyzed using Image J (NIH Bethesda, USA). Optical density (OD) measurements were normalized using actin to control for the amount of protein loaded on the gel.

Fear conditioning

All fear tests were performed in the dark under red light between 09:30 h and 15:30 h and rats were given at least 30 min to acclimatize to the experimental room prior to the onset of testing. The fear chamber was cleaned with Peroxyguard between trials. Male and female offspring from NB and LB mothers were separated into fear testing or control groups (n= 5 pups/condition and sex from n= 2 mothers/group) on PND28-29. Fear group animals were placed in an operant chamber with floor metal rods for shock delivery. Rats were habituated to this environment for 5 min, then exposed to two habituation 80 dB tones alone, followed by six tone-shock pairings (1 sec 0.6 mA shock, co-terminating with 30 sec 80 dB tone, average of 2 min variable inter-trial interval). Control animals were placed in the boxes for the same total duration as the fear animals, but were not exposed

to the tones or shock. At the end of testing, animals were weighed then individually housed and perfused 60 min after the onset of testing. Freezing behaviour was manually scored by an experimenter blind to treatment conditions and converted to percentage of freezing time during the 30 sec tone. Freezing was defined as the absence of movement, except for respiration, providing the animal was awake (Stevenson et al., 2009).

Brain collection for immunohistochemistry

Following fear conditioning, animals were anesthetized with a cocktail of ketamine/xylazine (0.1 mL/100g body weight, subcutaneous injection) and transcardially perfused with 0.9% ice-cold saline for 5 min, followed by a 15 min perfusion with 4% paraformaldehyde (PFA). The brains were extracted and stored in 4% PFA overnight, then transferred to a 30% sucrose solution in 1X PBS for 48 hr at 4^{o}C. The right side of the brains was marked using a blade, and brains were stored at -80^{o}C until sectioning. Free-floating coronal sections of 50 µm were stored at -20^{o}C until processed for staining.

Triple fluorescence immunohistochemistry: Parvalbumin, perineuronal nets and Fos

To examine the activation of parvalbumin (PV) neurons ensheathed by perineuronal nets (PNN), we first performed triple immunohistochemistry on brain sections from PND28-29 NB or LB pups after control or fear conditioning experiments. Fos is a commonly used marker for recent neuronal activity (Bullitt, 1990). On day one, free-floating sections were brought to room temperature for 30 min, then washed 3 x 5 min in 1X PBS. They were incubated for 20 min in 0.3% H_2O_2 (30%, H1009, Sigma) in 1X PBS, then washed 3 x 5 min in 1X PBS. After a 1 hr incubation in blocking solution

(0.02% Normal Horse Serum, S-2000, Vector Laboratories Inc., 0.004% Triton X-100 Sigma, 1X PBS), sections were incubated with the primary anti-PV antibody (1:500, Polyclonal Guinea Pig antiserum, #195 004, Synaptic systems) for 45 min at room temperature followed by overnight incubation at 4°C on a rotating platform. The next day, sections were washed 3 x 5 min in 1X PBS and incubated for 2 hr at room temperature in the dark with the Secondary Goat Anti-Guinea Pig antibody Alexa 568 (1:500, A-11075, Invitrogen by ThermoFisher Scientific). Next, sections were washed 3 x 5 min in 1X PBS and incubated with the primary anti-Fos antibody (1:500, Rabbit anti-Rat, #Sc-52, Santa Cruz Biotechnology) as well as the lectin *Wisteria floribunda* agglutinin (WFA) conjugated with Fluorescein (1:500, FL-1355, Vector Laboratories) for 45 min at room temperature, then overnight at 4°C. WFA binds to N-acetylgalactosamine residues and is widely used to detect PNNs (Seeger et al., 1996). On the third day, sections were washed 3 x 5 min in 1X PBS, then incubated for 2 hr with Secondary Donkey Anti-Rabbit antibody Alexa 647 (1:500 dilution in blocking solution, #711-605-152, Jackson Immunoresearch Laboratories, Inc.). Sections were washed 3 x 5 min 1X PBS and mounted onto charged glass slides using DAPI Hardset mounting medium (H-1500, Vector Laboratories Inc.).

Microscopy imaging

Pictures of triple immunostained sections were taken with an Olympus BX63 fluorescence microscope and the Olympus F1200 confocal microscope in that order to avoid bleaching. Eight pictures were taken per brain on the BX63, four of the left and four of the right amygdala spanning Bregma levels -1.92 to -3.00 µm based on the Paxinos and Watson atlas of the rat brain (Paxinos and Watson, 2005). The pictures on the BX63

were taken at 20x magnification, with exposure times of 13 ms for DAPI, 20 ms for GFP (PNN), 50ms for RFP (PV), 70 ms for FR (Fos). Three Z-stack images (20 slices at 9 mm depth) per brain side between Bregmas -2.04 and -2.52 μm were captured on the confocal microscope for WFA intensity measurements at 20x magnification with 0.1% FITC 488nm laser intensity.

Cell quantification and PNN intensity measurements

Counting was manually performed using the QuPath software. The BLA was outlined on four sections per brain side with micrometers (μm) as unit of length. Pixel width, pixel height, and voxel depth all equaled 0.5119. Total counts were converted to cell density measurements expressed as mm^2. The categories counted were the following: PV+, PNN+, PV+/PNN+, Fos+, PV+/Fos+, PV+/Fos+/PNN+. To examine the relative maturity of PNN, WFA fluorescence intensity was measured on three sections per brain side as previously described by Slaker et al. with Image J software (NIH Bethesda, USA) (Slaker et al., 2016). All immunohistochemical analyses were performed by experimenters blind to the treatment conditions.

Statistical analysis

Data were analyzed using two- and/or three-factor ANOVA with applicable within and between subject factors. Simple effect tests were used to analyze any significant interactions. Data are presented as means ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$. Electrophysiology trace drawings were created with CorelDraw X7. Graphs were created with Prism 7 (GraphPad Software).

Results

LB-reared preweaning animals display a transient reduction in body weight

Mothers and litters were weighed on PND1, 4, 10 and 22. Pup weight was calculated from the litter weight irrespective of sex. After weaning, individual animals were weighed on PND28. Consistent with our previous findings and others (Walker et al., 2017; Guadagno et al., 2018a), LB reduced pup body weight on PND10 ($F(1,107) = 17.83$, $p = 0.0005$) and PND22 ($F(1,107) = 15.3$, $p = 0.00016$) (n=14-15 litters/group) (Table 1). There was no significant effect of bedding on body weight of mothers ($F(1,27) = 0.79 = 0.38$, n=14-15). On PND28, there was no significant effect of bedding ($F(1,50)=3.47$, $p = 0.068$), sex ($F(1,50) = 1.9$, $p = 0.17$), and no bedding x sex interaction on pup body weight ($F(1,50) = 0.00074$, $p = 0.97$, n=13-14 animals/group and sex).

LB exposure enhances LTP formation and evoked fEPSP responses in the right BLA of juvenile males exclusively

The effects of LB on synaptic plasticity in the left and right BLA were measured using *in vitro* brain slices (n=9-12/group, side and sex) from PND22-28 male and female rats (n=5-8 animals/group). The stimulating electrode for all field recordings was placed in the LA, adjacent to the recording electrode in the BLA. We induced LTP of fEPSPs in the BLA by HFS (two 1 sec trains of 100 pulses delivered at 100 Hz, separated by 20 sec) in the LA.

	Bedding	PND1	PND4	PND10	PND22	PND28
Mother Body Weight (g)	NB	331.5 ± 8.8	361.9 ± 7.3	388.9 ± 5.9	380.6 ± 5.4	-
	LB	328.2 ± 8.7	350.7 ± 8.6	388.3 ± 8.8	362.5 ± 12.1	-
Pup Body Weight (g)	NB	7.86 ± 0.19	14.54 ± 0.39	32.29 ± 0.91	81.17 ± 1.06	Males: 122.65 ± 2.34 Females: 119.8 ± 2.09
	LB	7.69 ± 0.15	12.78 ± 0.5	27.91 ± 0.89 ***	77.11 ± 1.01 ***	Males: 118.8 ± 1.98 Females: 116.1 ± 1.62

Table IV-1. Mother and pup body weights. Mothers and litters were weighed on postnatal-days (PND) 1, 4, 10 and 22 and pup weight was calculated from the litter weight irrespective of sex. Animals were individually weighed on PND28. Limited bedding (LB) transiently decreased pup body weight on PND10 and PND22 relative to normal bedding (NB) animals. Values represent mean ± SEM of n=14-15 litters/group (PND1, 4, 10, 22) and 13-14 animals/group and sex (PND28). ***p<0.001; Two-way ANOVA

Successful LTP in the BLA with similar placement of electrodes has been documented as early as PND25 (Aroniadou-Anderjaska et al., 2001). fEPSP responses 25-30 min after HFS (post-tetanic LTP period) were calculated relative to the last 2 min of baseline recording (time 13-15 min, pre-tetanic LTP period) (see Fig 1A, B, C, D for slope graphs and representative traces). In the left BLA, there was no significant main effect of bedding ($F(1,35) = 0.89$, $p = 0.76$) or sex ($F(1,35) = 1.23$, $p = 0.27$), and no bedding x sex interaction ($F(1,35) = 1.97$, $p = 0.16$) for LTP as a function of percent change from baseline (Fig. 1E). In contrast, in the right BLA, LB exposure in males significantly enhanced LTP compared to NB animals ($F(1,38) = 18.18$, $p = 0.00013$), whereas this effect was not observed in females ($F(1,38) = 0.11$, $p = 0.74$) (Fig. 1F). Additionally, sex differences emerged in the LB group, with males displaying greater LTP formation than females ($F(1,38) = 7.67$, $p = 0.0086$) (Fig. 1F). Two-way ANOVA with sex and side as between-subjects factors revealed a significant effect of side for both NB ($F(1,37) = 4.10$, $p = 0.05$) and LB animals ($F(1,36) = 30.05$, $p = 0.0001$), with LTP responses being enhanced in the right versus the left BLA.

No significant interactions were observed (NB: $F(1,37) = 1.67$, $p = 0.2$; LB: $F(1,36) = 3.74$, $p = 0.061$). To further analyze the side effect, a three-way ANOVA was performed and gave a significant bedding x sex x side interaction ($F(1,73) = 5.43$, $p = 0.022$). LTP in the right BLA (569% change from baseline) was significantly increased compared to the left side (183% change from baseline) in LB males: $F(1,73) = 34.18$, $p = 0.00001$), as well as in NB ($F(1,73) = 4.10$, $p = 0.046$) and LB ($F(1,73) = 7.83$, $p = 0.0065$) females, but not in NB males ($F(1,73) = 0.21$, $p = 0.64$).

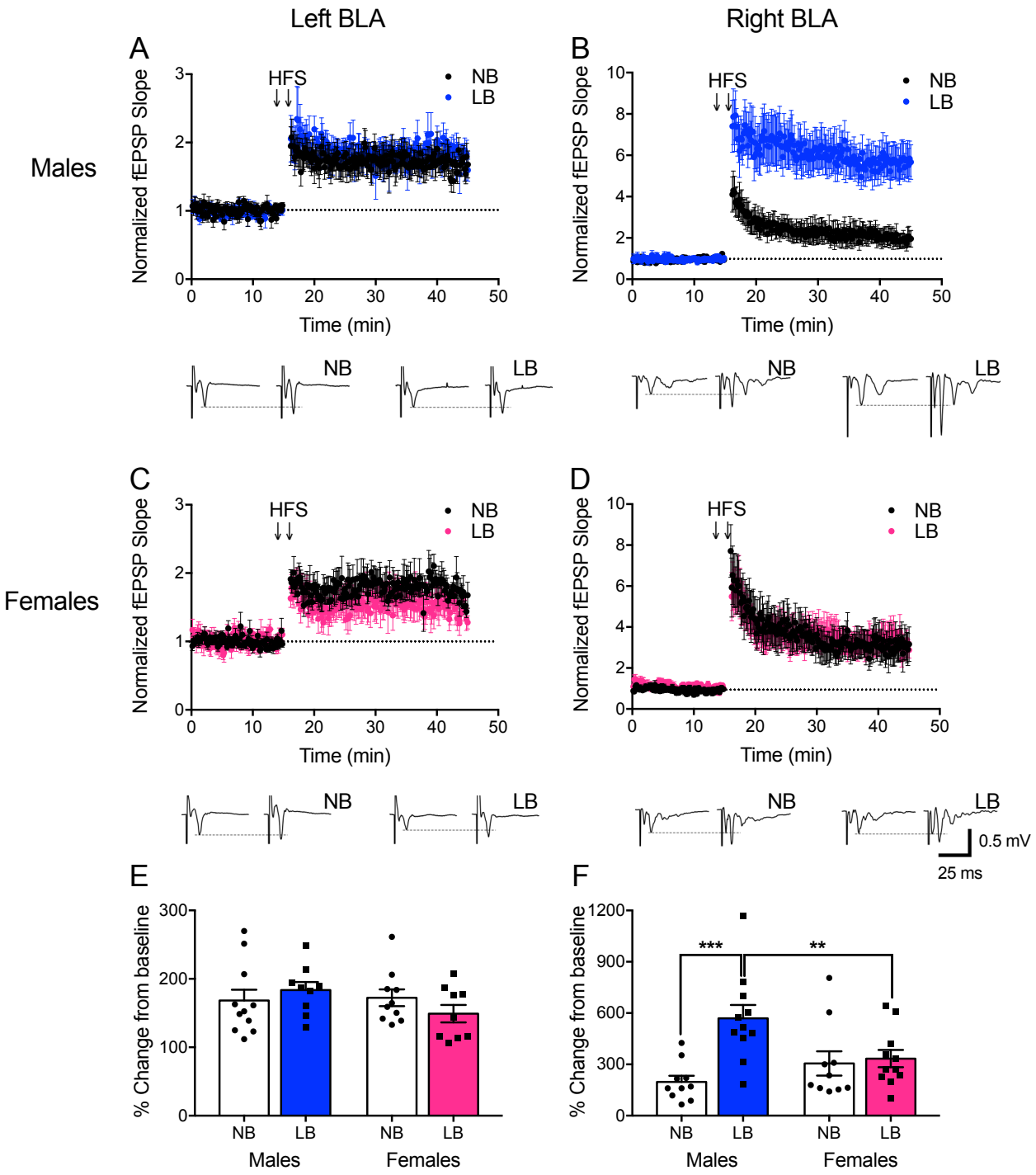
Left BLA
Right BLA
Males
Females
A
B
C
D
E
F
HFS
NB
LB
Normalized fEPSP Slope
Time (min)
0.5 mV
25 ms
% Change from baseline
***
**
Males
Females

Figure IV-1. Long-term potentiation (LTP) in the left and right basolateral amygdala (BLA) of PND22-28 animals. Plots of fEPSP slope against time are normalized to baseline for each recording. Representative traces below each plot were obtained 2 min before (left traces) and 25 min after high frequency stimulation (HFS) (right traces). **A, B,** In males, limited-bedding (LB) enhanced LTP in the right BLA only. **C, D,** There was no effect of LB-rearing on LTP formation in females in either the left or right BLA. **E, F** Percentage change of fEPSP slope from baseline in normal bedding (NB) or LB male and female groups in the right and left BLA 25-30 min after HFS. LB male pups displayed significantly enhanced LTP compared to NB males and LB females in the right BLA only. Values represent mean ± SEM of n=9-11 slices/group, 5-8 animals/group, **p<0.01, ***p<0.001; Two-way ANOVA

We previously showed that LB enhances evoked synaptic responses in the BLA of males on PND18-22 (Guadagno et al., 2018a). However, we did not differentiate left from right BLA slices, nor did we perform electrophysiological recordings in female pups. To probe for potential hemispheric and sex-dependent effects on input-output BLA functioning, in the current study we evoked fEPSPs at fiber volley sizes ranging from 0.1 to 0.4 mV by adjusting the stimulus intensity accordingly. Two-way ANOVAs were computed with bedding and fiber volley amplitude as between- and within-subjects factors, respectively. As shown in Figure 2, LB-reared males displayed increased fEPSP responses at fiber volley size 0.4, compared to NB-reared males, in the right BLA only ($F(1,67) = 11.15$, $p = 0.001$, Left BLA bedding effect: $F(1,19) = 0.34$, $p = 0.56$). In females, there was no effect of bedding (Left: $F(1,17) = 1.46$, $p = 0.24$; Right: $F(1,17) = 0.013$, $p = 0.91$) or bedding x fiber volley amplitude interaction in either BLA side (Left: $F(3,51) = 0.75$, $p = 0.52$; Right: $F(3,51) = 1.04$, $p = 0.38$). To further evaluate sex and side differences in the BLA, three-factor ANOVAs were computed. In males, the right BLA produced larger fEPSP responses at fiber volley sizes 0.3 ($F(1,101) = 5.81$, $p = 0.017$) and 0.4 ($F(1,101) = 14.59$, $p = 0.00023$), compared to the left BLA. This lateralized effect was not seen in females (main effect of side: $F(1,34) = 2.09$, $p = 0.15$). Early chronic stress potentiated lateralization of BLA fEPSP responses, as there was only an effect of side at fiber volley sizes 0.3 ($F(1,108) = 6.25$, $p = 0.013$) and 0.4 ($F1,108) = 14.83$, $p = 0.0002$) in LB animals (side x fiber volley amplitude interaction: $F(3,111) = 6.46$, $p = 0.00045$), and no main effect of side in NB animals ($F(1,34) = 2.87$, $p = 0.099$).

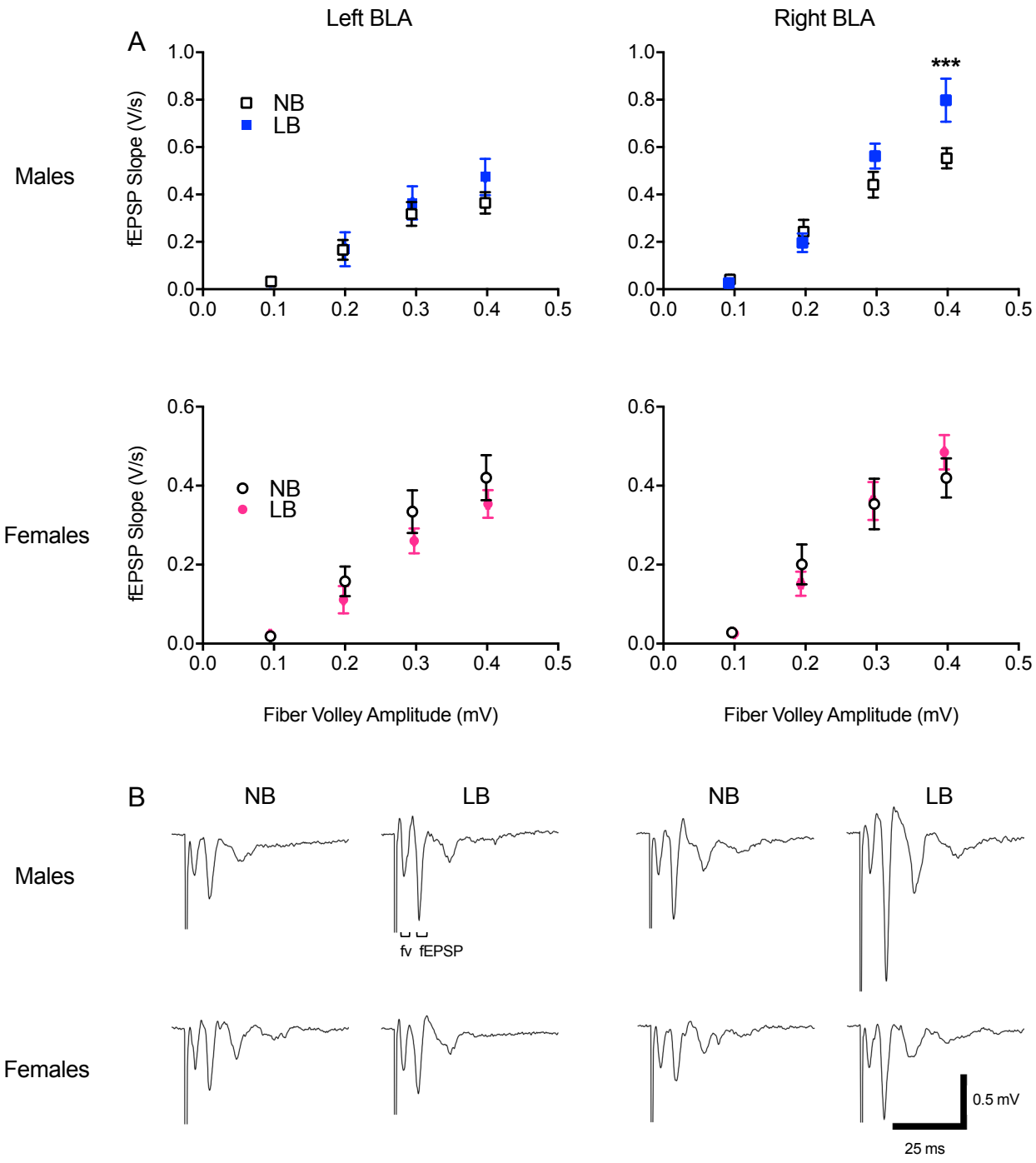
A
Left BLA
Right BLA
Males
Females
fEPSP Slope (V/s)
NB
LB
***
Fiber Volley Amplitude (mV)
B
fv
fEPSP
0.5 mV
25 ms

Figure IV-2. Input-output evoked synaptic responses in the left and right basolateral amygdala of PND22-28 animals. **A,** Limited bedding (LB) exposure significantly increased evoked fEPSP slope in the right, but not the left, BLA of males at fiber volley size 0.4. **B,** Representative traces are of fEPSP at fiber volley (fv) size 0.4. Values represent mean ± SEM of n = 9-12 slices/group, 5-8 animals/group. ***p = 0.001**;** Two-way ANOVA

Sex-dependent effects of LB on action potential properties in right BLA neurons of juvenile animals

Changes in synaptic plasticity could be linked to alterations in intrinsic neuron excitability (Xu et al., 2005). We previously reported that miniature EPSC properties remain unchanged under basal conditions in LB versus NB preweaning male animals (Guadagno et al., 2018a). Here, we examined the effect of LB on BLA neuron activity under stimulated conditions (Fig. 3). Adolescent social isolation, a form of ELS, has been associated with increased BLA pyramidal neuron excitability in adult male animals (Rau et al., 2015). We proposed that similar changes would occur in BLA neurons of juvenile males following LB exposure. Whole cell patch-clamp recordings were restricted to the right BLA because the effects of ELS on synaptic plasticity were only observed on that side. We characterized properties of action-potential firing in the BLA (n = 8-9 cells/group) in both sexes. Representative traces and corresponding phase plane plots for the first action potential evoked by current step injection are illustrated in Figure 3A-C. LB females displayed slower maximal depolarization rates compared to NB females ($F(1,30) = 5.75$, $p = 0.022$) (Fig. 3D), but a trend for the opposite effect was seen in males ($F(1,30) = 3.22$, $p = 0.082$). NB males displayed slower maximal depolarization rates compared to NB females ($F(1,30) = 7.31$, $p = 0.011$). There was no effect of bedding ($F(1,30) = 0.028$, $p = 0.86$), sex ($F(1,30) = 0.042$, $p = 0.83$) and no bedding x sex interaction ($F(1,30) = 0.047$, $p = 0.82$) for action potential threshold (Fig. 3E). LB animals displayed significant hyperpolarization of resting membrane potentials compared to NB animals ($F(1,30) = 4.34$, $p = 0.045$) (Fig. 3F). The average action potential amplitude of BLA neurons was decreased in LB versus NB females ($F(1,30) = 5.9$, $p = 0.021$) and a trend towards the

opposite effect was seen in males ($F(1,30) = 3.15$, $p = 0.085$). NB males had lower action potential amplitude compared to NB females ($F(1,30) = 8.95$, $p = 0.0055$) (Fig. 3G). Finally, the total number of action potentials fired did not differ between bedding groups ($F(1,30) = 1.62$, $p = 0.21$), sexes ($F(1,30) = 1.98$, $p = 0.16$), and yielded no bedding x sex interaction ($F(1,30) = 0.097$, $p = 0.75$) (Fig. 3H).

LB reduces GluN1 expression in the right BLA in juvenile females

We evaluated the hemispheric effect of LB on protein expression of the GluN1, GluN2A and GluN2B subunits using Western Blot (Fig. 4). For GluN1 expression, there was no effect of LB or side in the male amygdala, although LB significantly reduced GluN1 expression, specifically in the right BLA in females ($F(1,40) = 9.31$, $p = 0.004$) (Fig. 4A). On the same amygdala side, LB females also had significantly lower levels of GluN1 compared to LB males ($F(1,40) = 7.54$, $p = 0.009$). GluN1 expression was lateralized only in LB females, with decreased expression in the right compared to the left BLA ($F(1,40) = 8.95$, $p = 0.0047$). For both GluN2A (Fig. 4B) and GluN2B expression (Fig. 4C), there were no main effects of bedding (GluN2A: $F(1,39) = 0.67$, $p = 0.41$; GluN2B: $F(1,40) = 0.19$, $p = 0.66$), sex (GluN2A: $F(1,39) = 0.44$, $p = 0.5$; GluN2B: $F(1,40) = 2.63$, $p = 0.11$), side (GluN2A: $F(1,39) = 0.74$, $p = 0.39$; GluN2B: $F(1,40) = 0.55$, $p = 0.46$) and no significant interactions.

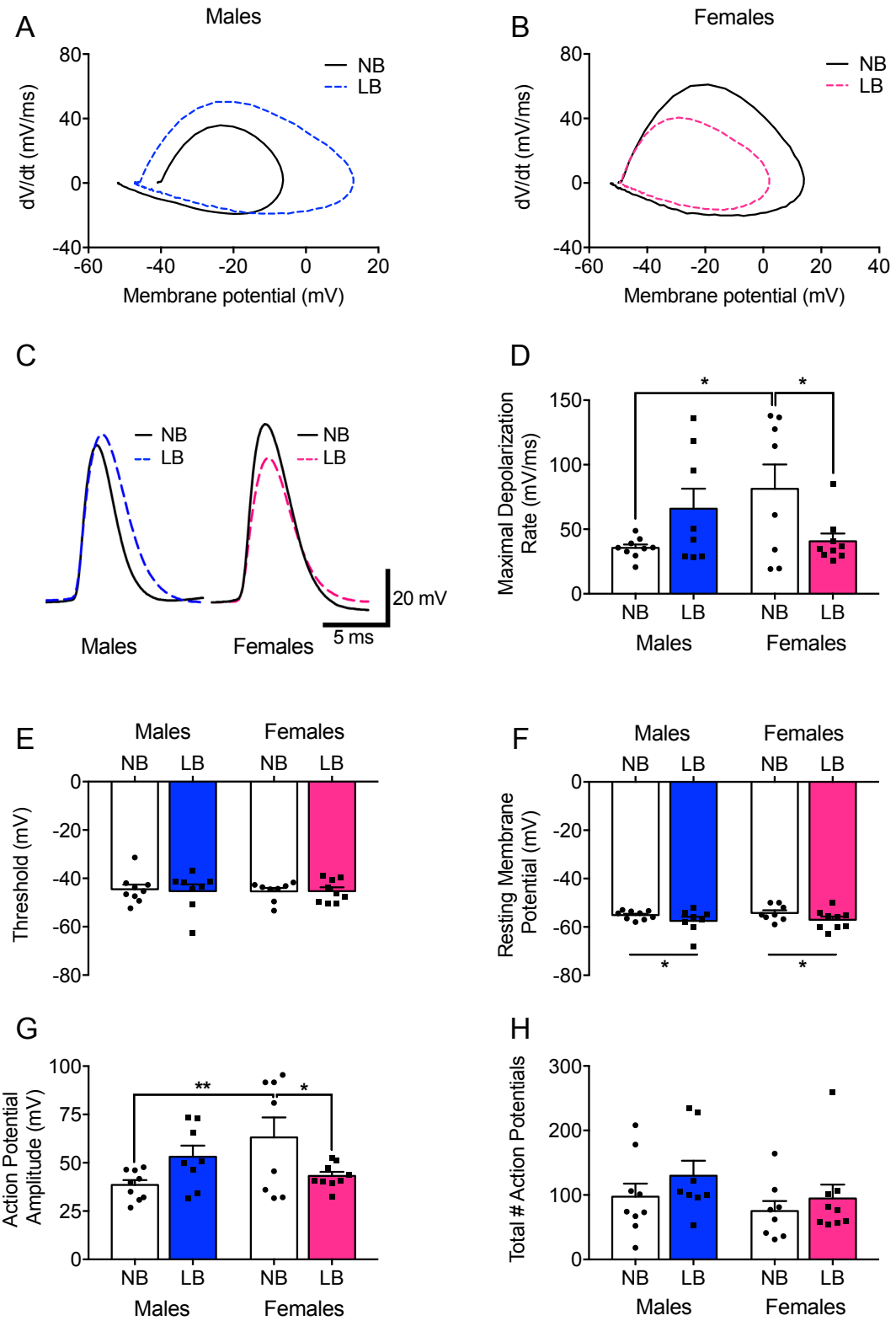
A
Males
NB
LB
dV/dt (mV/ms)
Membrane potential (mV)
B
Females
C
20 mV
5 ms
Males
Females
D
Maximal Depolarization Rate (mV/ms)
E
Threshold (mV)
F
Resting Membrane Potential (mV)
G
Action Potential Amplitude (mV)
H
Total # Action Potentials

Figure IV-*3.* Action potential properties of neurons in the right basolateral amygdala (BLA) of PND22-28 normal (NB) and limited bedding (LB) offspring. **A, B, C,** Representative traces and corresponding phase plane plots for the first spike elicited by a 10 pA current step injection. **D,** Maximal depolarization rate was decreased in LB versus NB females, and in NB males compared to NB females. **E,** Conversely, action potential threshold was not significantly affected by bedding condition. **F,** Resting membrane potential was hyperpolarized in animals exposed to LB conditions. **G,** Action potentials fired from BLA neurons were of a lower amplitude in LB compared to NB females, as well as in NB males compared to NB females. **H,** The total number of action potentials fired did not differ between groups. Values represent mean ± SEM of n=8-9 neurons/group, 5-6 animals/group. *p<0.05, **p<0.01; Two-way ANOVA

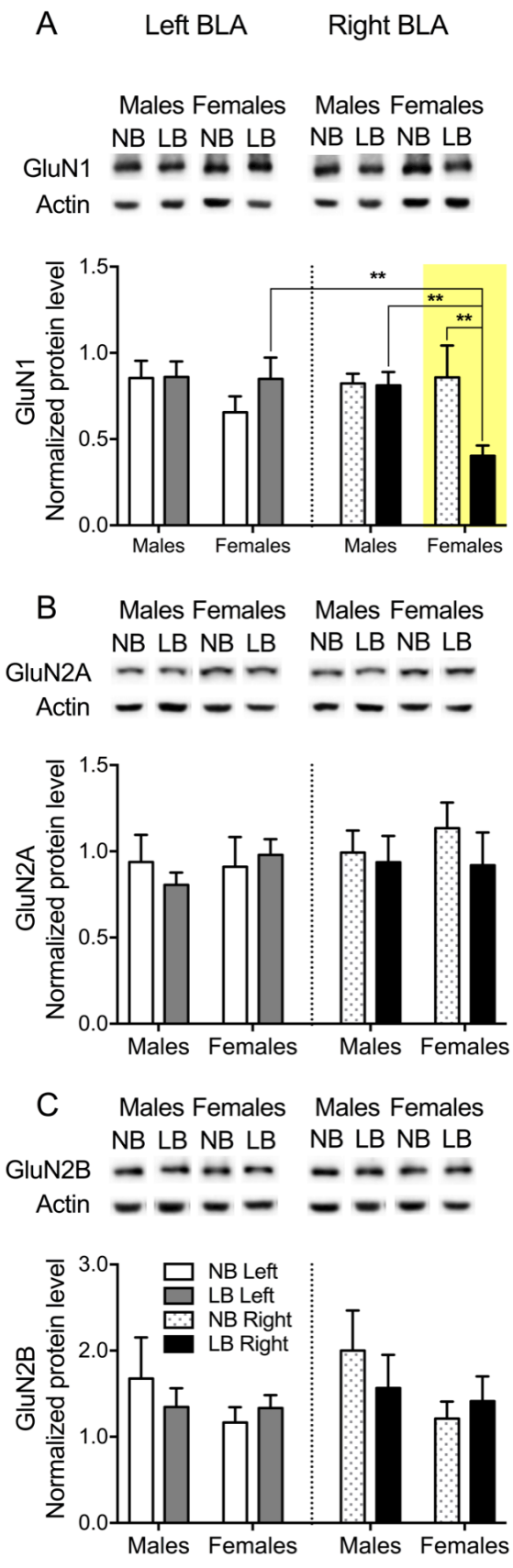
A
Left BLA
Right BLA
Males Females Males Females
NB LB NB LB NB LB NB LB
GluN1
Actin
GluN1 Normalized protein level
1.5
1.0
0.5
0.0
**
**
**
Males Females Males Females
B
Males Females Males Females
NB LB NB LB NB LB NB LB
GluN2A
Actin
GluN2A Normalized protein level
1.5
1.0
0.5
0.0
Males Females Males Females
C
Males Females Males Females
NB LB NB LB NB LB NB LB
GluN2B
Actin
GluN2B Normalized protein level
3.0
2.0
1.0
0.0
NB Left
LB Left
NB Right
LB Right
Males Females Males Females

Figure IV-4. Expression of NMDA receptor subunits GluN1, GluN2A and GluN2B in the left and right basolateral amygdala (BLA) of PND28-29 animals. Protein levels were normalized to actin and representative Western Blot bands are displayed above each graph. **A,** In the right BLA, GluN1 expression was significantly reduced in limited bedding (LB) relative to normal bedding (NB) females. LB females also displayed lower levels of GluN1 compared to LB males. Asymmetrical GluN1 expression was only observed in LB females, with decreased expression in the right compared to the left BLA. **B, C,** GluN2A as well as GluN2B expression were unaffected by early chronic stress. Values represent mean ± SEM of n=5-6 animals/group. **p<0.01; Three-way ANOVA

Behavioral responses to fear conditioning in juvenile animals

As illustrated in Figure 5, fear conditioning was successful to increase freezing in both male and female on PND28-29 juvenile animals. A three-way ANOVA gave a significant treatment by tone interaction for both males ($F(5,80) = 10.56$, $p = 0.00001$) and females ($F(5,80) = 19.29$, $p = 0.00001$). Males and females that were fear conditioned displayed more freezing behavior during the 30 sec tone compared to controls at tones 2 (Males: $F(1,85) = 8.23$, $p = 0.0052$; Females: $F(1,60) = 13.18$, $p = 0.00056$) through 6 (Males: $F(1,85 = 22.29$, $p = 0.00001$); Females: $F(1,60) = 35.48$, $p = 0.00001$). There were no sex differences in freezing for fear ($F(1,16) = 0.16$, $p = 0.69$) or in control animals ($F(1,16) = 0.17$, $p = 0.68$). In contrast to what we previously documented in adult male rats (Guadagno et al., 2018a), we did not find a significant effect of bedding on freezing behavior in male ($F(1,8) = 0.000$, $p = 0.99$) or female ($F(1,8) = 1.75$, $p = 0.22$) juvenile (PND28-29) rats.

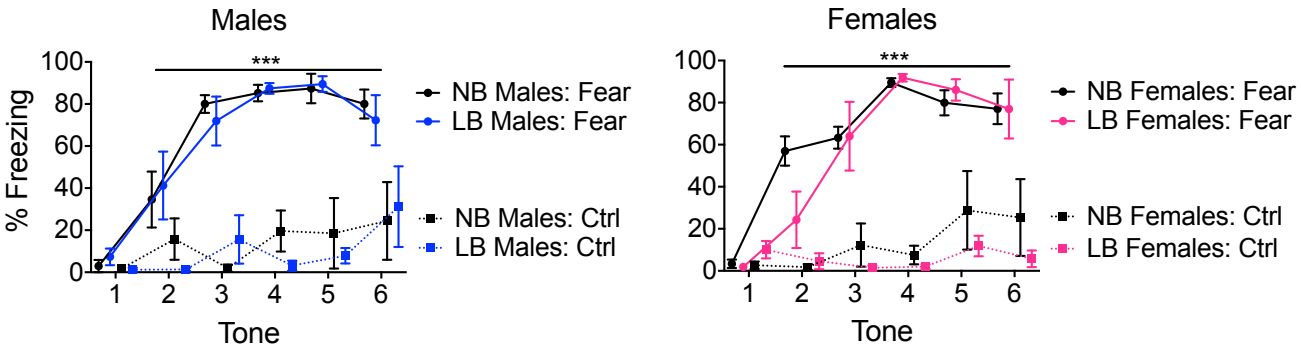

Figure IV-5. Percentage of freezing time during exposure to a 30 sec tone paired with shock in male and female PND28-29 pups from either normal (NB) or limited bedding conditions (LB). Control (Ctrl) animals were placed in the same context as fear animals, but not exposed to tone or shock. Although fear conditioning was successful in both males and females, we did not find significant effects of early rearing conditions on freezing in either fear or control group animals. Values represent mean ± SEM of n=5 animals/group. ***p<0.001 Fear vs Control tones 2-6; Two-way ANOVA

Effect of LB, sex and side on PV- and PNN-positive cells in the BLA

For the determinations of total cell numbers (PV and PNN positive), we pooled both control and fear groups (n = 10 pups/group and sex) from the fear conditioning experiment that is displayed in Figure 8. We assumed that the total number of PV-positive or PNN-positive cells would not be affected by acute exposure to fear conditioning. This was confirmed by the lack of significant differences in a three-way ANOVA with bedding (NB or LB), treatment (control, fear) and sex analysis for both the left (PV density: $F(1,32) = 0.97$, $p = 0.33$; PNN density: $F(1,32) = 0.49$, $p = 0.49$) and right amygdala (PV density: ($F(1,32) = 0.00$, $p = 0.95$; PNN density: $F(1,32) = 0.4$, $p = 0.53$).

As depicted in Figure 6A and 6B, the density of PV positive cells was not changed by either sex or bedding, in either side of the BLA (Left BLA: sex: $F(1,36) = 1.26$, $p = 0.26$; bedding: $F(1,36) = 2.68$, $p = 0.11$; interaction: $F(1,36) = 0.00017$, $p = 0.98$); Right BLA: sex: $F(1,36) = 1.56$, $p = 0.21$; bedding: $F(1,36) = 8.29$, $p = 0.63$; interaction: $F(1,36) = 1.05$, $p = 0.31$). In the left BLA, we found an overall bedding and sex effect on PNN density, whereby PNN density was increased in males relative to females ($F(1,36) = 6.99$, $p = 0.012$), and in LB versus NB animals ($F(1,36) = 5.5$, $p = 0.024$) (Fig. 6C). For PNN density in the right BLA, there were significant effects of sex ($F(1,36) = 18.75$, $p = 0.0001$) and bedding ($F(1,36) = 9.78$, $p = 0.0034$), and a sex x bedding interaction ($F(1,36) = 10.18$, $p = 0.0029$). As for the left BLA, LB males had significantly more PNN compared to NB males ($F(1,36) = 19.96$, $p = 0.00008$), whereas no bedding effect was found in females ($F(1,36) = 0.002$, $p = 0.96$) (Fig. 6D). As a result, we discovered a significant effect of sex only in the LB condition, with males having more PNN than females ($F(1,36) = 28.28$, $p = 0.0001$). Most strikingly, LB males had more PV+ cells surrounded by PNN

compared to NB males in the right ($F(1,36) = 21.22$, $p = 0.00005$), but not the left BLA (bedding effect: $F(1,36) = 2.88$, $p = 0.098$) (Fig. 6E and 6F). LB males also had more PV+ cells expressing PNN compared to LB females, again only in the right BLA ($F(1,36) = 37.9$, $p = 0.00001$).

In order to examine how many PV-positive cells harbor PNN, we calculated the percentages of PV-positive cells expressing PNN over the total PV cell population. In the left BLA, there were no significant effects of sex ($F(1,36) = 1.41$, $p = 0.24$) or bedding ($F(1,36) = 0.77$, $p = 0.38$) and no sex x bedding interaction ($F(1,36) = 0.46$, $p = 0.5$) for percentages of PV cells with PNN (Males: NB: 33%, LB: 37%; Females: NB: 31%, LB: 32%). However, in the right BLA, there was a significant bedding x sex interaction ($F(1,36) = 18.48$, $p = 0.0001$), whereby LB increased the percentage of PV cells with PNN in males ($F(1,36) = 22.88$, $p = 0.00003$, NB: 36%, LB: 57%), but not females ($F(1,36) = 1.68$, $p = 0.2$, NB: 37%, LB: 31%).

In addition, we estimated which cell type was associated with PNN by calculating the percentage of PV+PNN cells over the total PNN cell population. Two-way ANOVA revealed no effect or sex ($F(1,36) = 0.00$, $p = 1$) or bedding $F(1,36) = 0.66$, $p = 0.42$) and no sex x bedding interaction ($F(1,36) = 0.3$, $p = 0.58$) in the left BLA (Males: NB: 27%, LB: 30%; Females; NB: 28%, LB: 29%). In the right BLA, there was an effect of sex ($F(1,36) = 4.74$, $p = 0.036$), with males displaying greater percentages of PNN on PV cells relative to females, but no effect of bedding ($F(1,36) = 3.41$, $p = 0.94$) or sex x bedding interaction ($F(1,36) = 3.41$, $p = 0.07$, Males: NB: 31%, LB: 35%; Females; NB: 30%, LB: 26%).

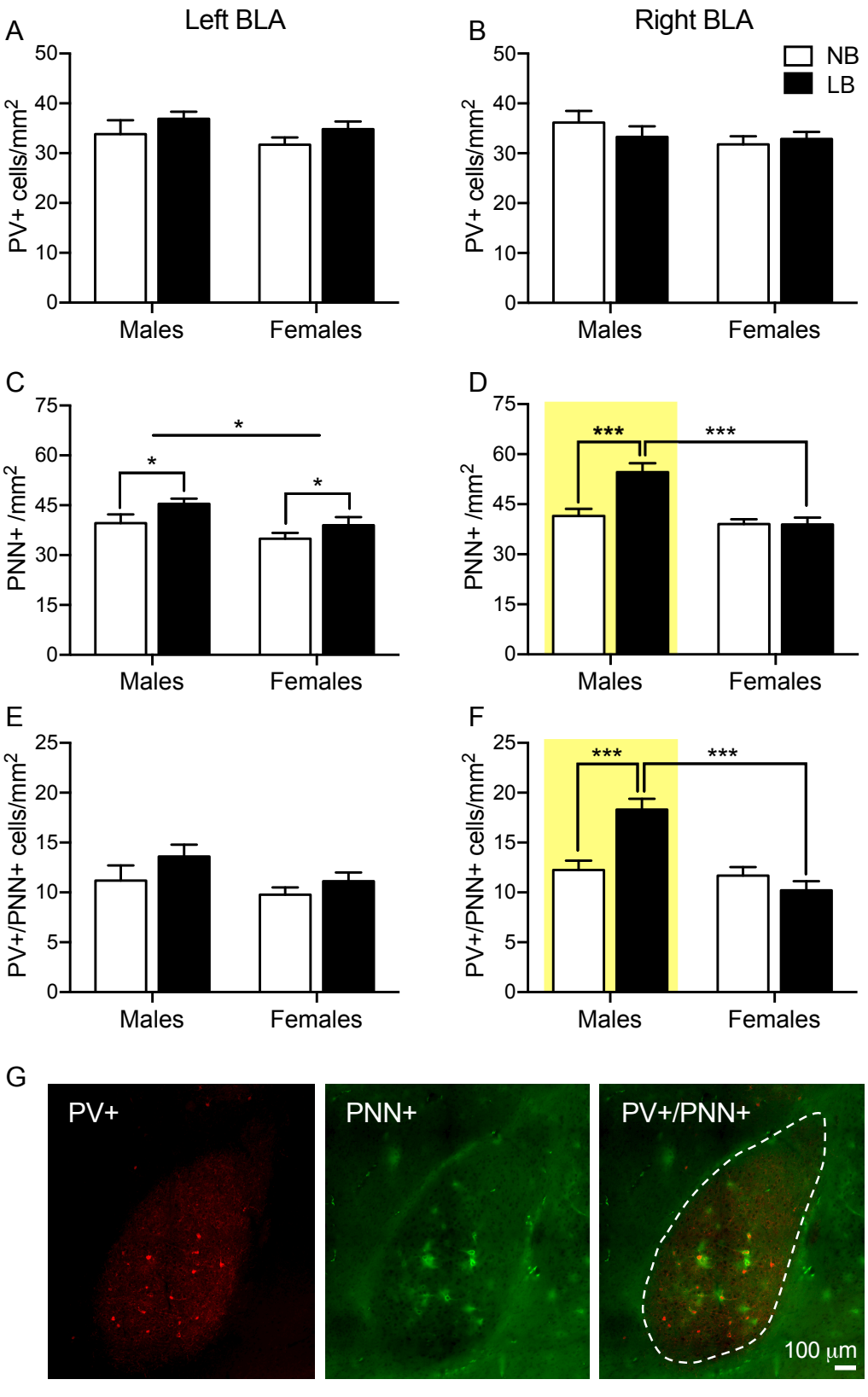
A
Left BLA
PV+ cells/mm2
50
40
30
20
10
0
Males
Females
B
Right BLA
NB
LB
C
PNN+ /mm2
75
60
45
30
15
*
D
***
E
PV+/PNN+ cells/mm2
25
20
15
10
5
F
G
PV+
PNN+
PV+/PNN+
100 µm

Figure IV-6. Parvalbumin+ (PV) interneuron and perineuronal net (PNN) density in the left and right basolateral amygdala (BLA) of male and female rats on PND28-29. **A,B,** Early bedding conditions did not affect PV+ cell density in the left or right BLA in either sex. **C,** In the left BLA, PNN density was heightened in males compared to females, and in LB versus NB animals. **D,** LB males had significantly increased PNN density in the right BLA compared to NB males and LB females. **E,F,** In the right, but not the left BLA, LB-exposed male rats expressed more PV+ cells ensheathed with PNN than NB males and LB females**. G,** Representative images of the LB male right BLA with labeled parvalbumin inhibitory interneurons (PV+) and perineuronal nets (PNN+) taken at 20x magnification. Values represent mean ± SEM of n=10 animals/group (Control and fear group animals combined). *p<0.05, ***p<0.001; Two-way ANOVA

We next asked whether PNN maturity stage, as determined by the intensity of the WFA staining (Slaker et al., 2016), would be altered by LB in either side of the BLA. There was no significant effect of fear treatment on PNN intensity and therefore, both control and fear groups were pooled (Three way ANOVA: left BLA: $F(1,32) = 1.7$, $p = 0.2$; right BLA: $F(1,32) = 3.52$, $p = 0.069$). WFA intensity measures showed that there was a significant effect of BLA side as PNN in the right BLA displayed significantly more intense staining compared to the left BLA in males ($F(1,36) = 5.85$, $p = 0.02$), but not females ($F(1,36) = 0.25$, $p = 0.62$) (Fig. 7). There was no significant effect of bedding in either sex (Males: $F(1,36) = 0.83$, $p = 0.36$; Females: $F(1,36) = 1.47$, $p = 0.23$),

Lateralized and sex-dependent cellular responses to fear conditioning after LB

To investigate the effects of LB on cellular activation in the BLA, we measured the density of Fos-expressing cells 60 min after the onset of fear conditioning or control testing. Three-way ANOVA revealed asymmetrical effects of sex and treatment on expression of Fos+ cells (Fig. 8A and 8B). Notably, only in the right BLA did males have higher Fos+ cell density compared to females ($F(1,32) = 7.08$, $p = 0.012$). Animals in the fear treatment group displayed enhanced Fos+ cell density compared to control animals exclusively in the right amygdala as well ($F(1,32) = 6.27$, $p = 0.017$). Fear conditioning significantly enhanced the activation of PV+ cells in the left compared to the right BLA ($F(1,32) = 7.63$, $p = 0.0094$) (Fig. 8C). In the right BLA, PV+ cells of NB, but not LB males were significantly activated in response to fear treatment ($F(1,32) = 22.32$, $p = 0.00004$) (Fig. 8D). Control LB males already displayed significantly more activated PV+ cells compared to NB males ($F(1,32) = 8.2$, $p = 0.0073$). In line with these findings, we found

blunted PV+ cell activation in the LB compared to NB fear group ($F(1,32) = 9.6$, $p = 0.004$). In NB animals that were fear treated, activation of PV+ cells was higher in males compared to females ($F(1,32) = 5.4$, $p = 0.026$). Lastly, we measured the density of activated PV+ also expressing PNN (Fig. 8E and 8F). In the left BLA, there was no main effect of bedding, ($F(1,32) = 1.4$, $p = 0.24$), sex ($F(1,32) = 1.04$, $p = 0.31$) or treatment ($F(1,32) = 3.18$, $p = 0.084$) and no significant interactions. Fear conditioning only increased the density of PNN around activated PV+ cells in the right BLA ($F(1,32) = 5.15$, $p = 0.03$).

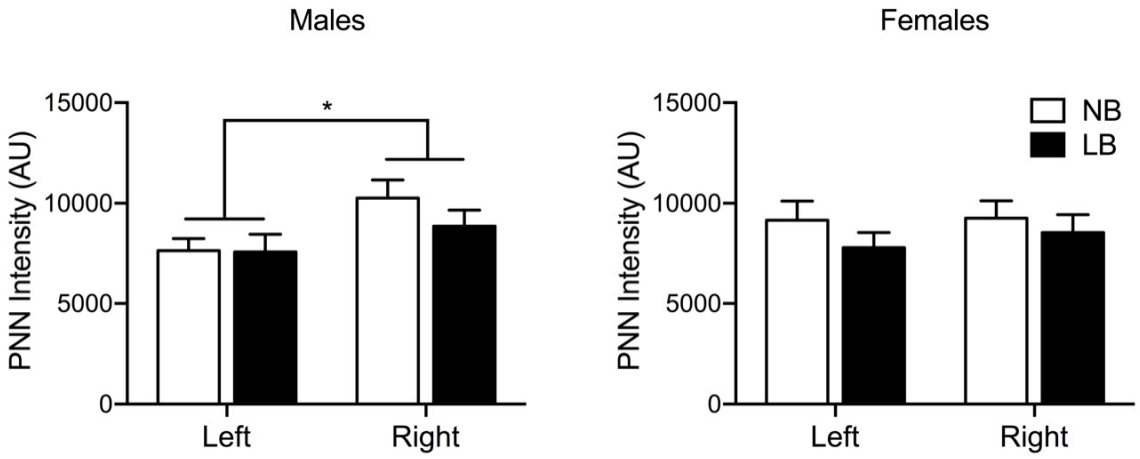

Figure IV-7. PNN intensity in the left and right basolateral amygdala of male and female PND28-29 rats. Intensity is expressed in arbitrary units (AU). The right BLA displayed increased PNN intensity compared to the left BLA in males only. Values represent mean ± SEM of n=10 animals/group (Control and fear group animals combined). *p<0.05; Two-way ANOVA

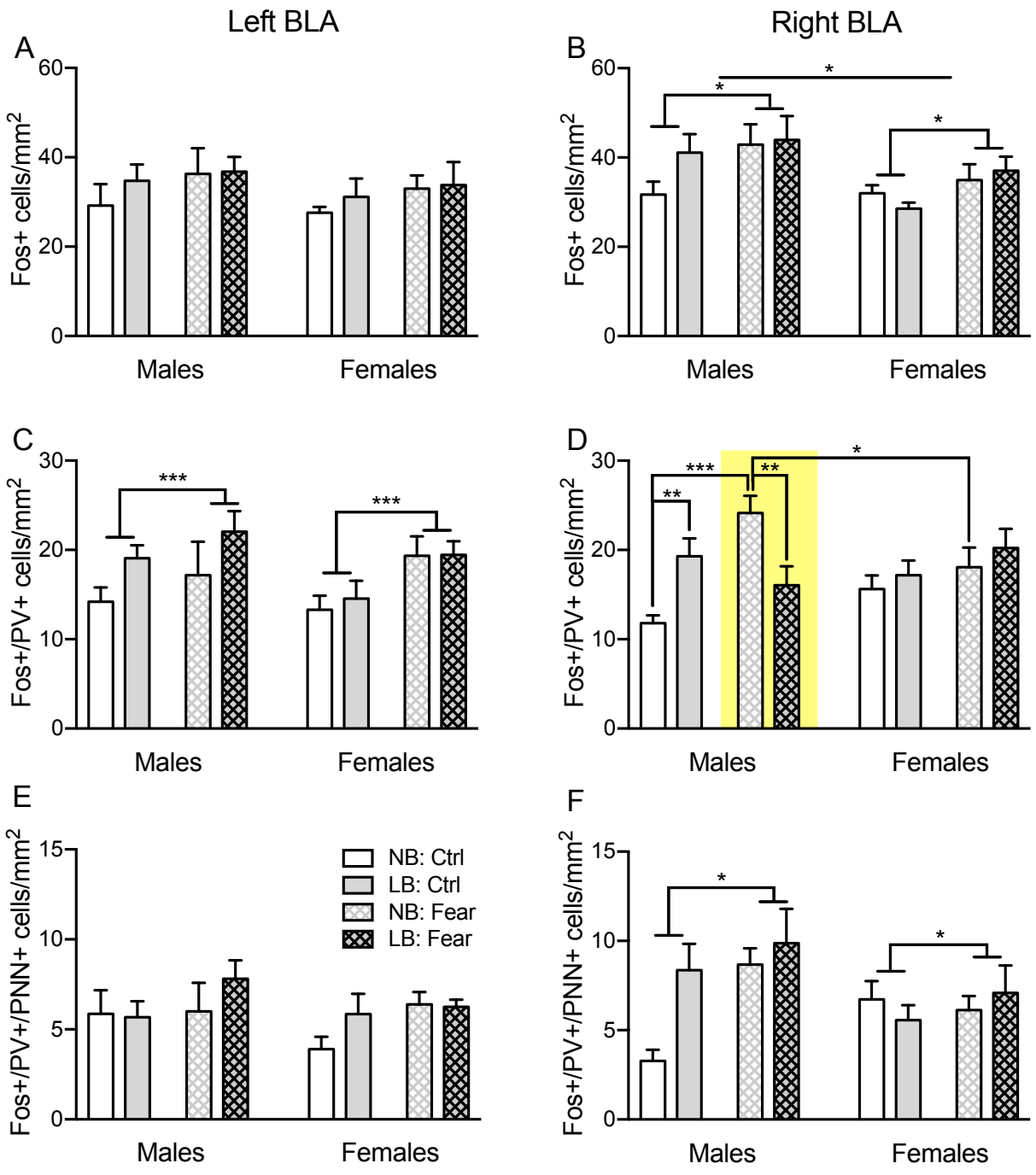
Left BLA
Right BLA
A
B
C
D
E
F
Fos+ cells/mm2
Fos+/PV+ cells/mm2
Fos+/PV+/PNN+ cells/mm2
Males
Females
NB: Ctrl
LB: Ctrl
NB: Fear
LB: Fear

Figure IV-8. Fos+, PV+ and PNN+ cell density after fear conditioning in PND28-29 animals. **A,B,** Fos+ cell density was elevated only in the right BLA of males compared to females and fear conditioned versus control animals. **C,** Fear treatment increased the density of Fos-activated PV+ cells in the left BLA in all animals. **D,** In the right BLA, fear conditioning activated PV+ cells of NB males relative to controls, whereas this was not the case in LB males. Control LB males had significantly increased activation of PV+ cells compared to NB males. Thus, LB-reared males in the fear group displayed reduced Fos+/PV+ cell density compared to their NB counterparts. In NB fear conditioned animals, males displayed increased PV+ cell activity compared to females. **E,** The density of activated PV+ cells expressing PNN was not affected by bedding, sex, or treatment in the left BLA. **F,** However, in the right BLA, fear conditioning increased the density of PNN around activated PV+ cells. Values represent mean ± SEM of n=5 animals/group. *p<0.05, **p<0.01, ***p<0.001**;** Three-way ANOVA

Discussion

In this study, we examined whether early life stress through exposure to suboptimal rearing conditions (limited bedding) would affect BLA synaptic plasticity and neuron excitability in juvenile animals in a sex- and hemispheric-dependent manner. Because projecting pyramidal neurons of the BLA are influenced by inhibitory interneurons, we also investigated LB-induced changes in parvalbumin (PV) interneuron activation after fear conditioning and the expression of PV associated perineuronal nets (PNNs) that might contribute to regulate overall BLA excitability. Our novel findings are that LB exposure enhanced BLA synaptic plasticity exclusively on the right side in males, with no changes being detected in either side in females. Right BLA neuronal excitability was marginally increased in LB males, but fear-induced activity of PV interneurons was reduced in the right, but not the left BLA compared to controls. Parvalbumin interneurons in LB males also displayed increased density of PNNs compared to control NB males, suggesting higher stabilization of synaptic inputs on these inhibitory interneurons. In contrast, the only effects of early life stress noted in LB females were reduced neuron excitability and NMDAR subunit expression in the right BLA.

Synaptic plasticity changes in the BLA of juvenile rats exposed to LB

We previously reported that LB exposure induces synaptic remodeling in the pre-weaning BLA, with male, but not female, excitatory neurons displaying enhanced spine densities and dendritic lengths (Guadagno et al., 2018a). Increased spine density suggests that those neurons receive stronger excitatory inputs, as evidenced by the

heightened input-output relationship we observed in the BLA of PND18-22 male pups (Guadagno et al., 2018a). Here, we extended these initial *in vitro* electrophysiological studies by showing that LB enhanced LTP formation and input-output function in the BLA of juvenile male, but not female pups. Strikingly, this effect was only observed in the right, but not in the left BLA, which was unaltered by LB conditions in either sex. We believe that this constitutes the first demonstration that synaptic function in the BLA is already lateralized in juvenile PND28 pups and is sensitive to early-life stress. The amygdala is known to be a lateralized structure that generally exhibits greater involvement of the right hemisphere in fear conditioning (Baker and Kim, 2004) and pain processing (Ji and Neugebauer, 2009) in adult rodents and in humans (Baker and Kim, 2004; Vasa et al., 2011). Using resting-state functional MRI, we also observed that connectivity of the BLA was lateralized in preweaning and adult male LB offspring, with more connectivity changes being documented in the right versus the left hemisphere (Guadagno et al., 2018b). We now provide further electrophysiological evidence for the lateralization of synaptic plasticity in juvenile males that is revealed after early-life stress exposure. Interestingly, laterality of LTP was observed in LB, but not NB males, suggesting that early stress might accelerate the development of LTP lateralization in juvenile males. In NB females, LTP was lower in the left compared to the right BLA, but LB exposure eliminated lateralization of LTP. This supports the idea that negative emotional information encoding in LB male offspring is biased towards the right hemisphere as shown previously in adult rats (Adamec et al., 2005b; Young and Williams, 2013).

Enhanced LTP formation in the adult male BLA after chronic stress participates in the potentiation of fear learning (Suvrathan et al., 2014) and in neonates (PND10-19),

changes in synaptic plasticity are thought to promote the encoding of fearful stimuli (Thompson et al., 2008). Thus, the early synaptic strengthening in juvenile males exposed to LB might lead to increased fear conditioning as observed previously in adult male, but not female, LB offspring (Guadagno et al., 2018a). The lack of sex-difference in the magnitude of LTP in control (NB) animals agrees with a previous study showing no sex effect on LTP in the BLA of naïve PND20-27 male and female rats and no sex differences in the baseline expression of estrogen receptor α, androgen receptor or aromatase at this age (Bender et al., 2017), However, our study reveals a large sexual dimorphism for juvenile LTP in response to early LB conditions that could be caused by early stress-induced mechanisms overlapping with the organizational period of brain sexual differentiation during the first week of life (Naninck et al., 2011), a period coincident with the time of exposure to the early stressor. There is growing evidence that sex differences in microglial cells and their level of activation actively contribute to brain masculinization in neonatal rodents (Lenz et al., 2013) and more generally in sex differences in the maturation and development of neuronal circuits (Johnson and Kaffman, 2018). Microglia also regulate experience-dependent synaptic plasticity in the cortex of juvenile mice (Tremblay et al., 2010) and are sensitive to chronic stress in adult male rats (Tynan et al., 2010; Hinwood et al., 2013) as well as ELS exposure (Delpech et al., 2016; Johnson and Kaffman, 2018) in a number of stress-responsive regions, including the amygdala (Tynan et al., 2010). These data support the idea that microglia function in the BLA may be modified following LB exposure to participate in sexually dimorphic synaptic changes.

Microglia also mediate experience-dependent synaptic plasticity in developing mice for instance (Tremblay et al., 2010), by engulfing synapses (Paolicelli et al., 2011)

and modifying spine number and size. In adult rats, chronic stress enhances microglial activation in the CeA, but not BLA (Tynan et al., 2010), highlighting cellular differences between the two subnuclei. The extent to which this occurs in development is unknown, but it is possible that reduced microglial activation in the preweaning BLA following LB might contribute to decrease synaptic pruning and thus, allow for increased spine numbers in males.

Sex-dependent alterations in right BLA neuron action potential properties

Enhanced synaptic plasticity in stressed males might stem from increased intrinsic BLA pyramidal neuron excitability (Xu et al., 2005) as shown previously in adult males subjected to social isolation in adolescence (Rau et al., 2015). This was confirmed in our study because LB induced a trend for enhanced maximal depolarization rate and action potential amplitude in males, while the opposite effect was significant in females. The results in males are in a direction that is consistent with differences in LTP formation in the right amygdala. Increased right BLA neuron excitability in LB males may result from increased expression of voltage-gated sodium-channels, as this was shown in the ventral hippocampus for male offspring of low maternal licking and grooming (LG) mothers (Nguyen et al., 2015). Conversely, the opposite effect on sodium-channel expression may be seen in females with reduced right BLA excitability. This state in females would be adaptive in face of early adversity and occlude LB-dependent facilitation of synaptic plasticity. Changes in sodium-channel expression might indirectly modify calcium conductance through NMDAR (Nguyen et al., 2015). Although NMDAR are more classically understood to modulate synaptic plasticity, recent evidence suggests they also

regulate the intrinsic excitability of neurons during postnatal development (Hou and Zhang, 2017).

NMDAR subunit expression in the post-weaning BLA after LB exposure

In the neonatal brain, glutamatergic synapses contain few AMPAR and consist primarily of NMDAR (Hou and Zhang, 2017), which are developmentally regulated (Sheng et al., 1994) and critically involved in modulating LTP formation in the amygdala (Maren, 1999a; Muller et al., 2009). Variations in maternal care have been found to influence NMDAR expression (Bagot et al., 2012) and subunit composition (Bath et al., 2016) in the hippocampus, although it is unknown whether changes in maternal behavior induced by LB would alter NMDAR subunits in the juvenile amygdala. We found a significant reduction in GluN1 protein levels in the right BLA exclusively of females, but not males, which might have contributed to reduced neuron excitability in female LB offspring. There were no other effects of LB on GluN1, GluN2A or GluN2B expression in the BLA that could associate with changes in neuronal excitability found in LB males. Consistent with our findings, GluN1 levels in the amygdala of adult females exposed to stress in utero were reduced compared to controls and no such differences were observed in male offspring (Wang et al., 2015). It is unclear why none of the NMDAR subunits were modified in male offspring, given the role of GluN2A/2B in BLA LTP formation (Muller et al., 2009) and the large effect of LB to enhance LTP in the right BLA of males. While NMDARs are crucial for the induction of LTP, other factors might also contribute to enhanced LTP amplitude, such as the stronger dV/dt and heightened neuron excitability we observed in LB males. A decrease in protein kinase beta II activity, a downstream

cellular signaling molecule coupled to NMDARs (Orman and Stewart, 2007), increased spine density and/or a reduction of inhibitory control over pyramidal BLA neurons (Butler et al., 2018) could also be prevailing over NMDAR expression for regulating stress-related synaptic plasticity in the male BLA.

Inhibitory PV activity in the BLA is altered by LB conditions

Parvalbumin (PV) positive neurons constitute approximatively 50% of the total population of inhibitory GABAergic interneurons in the BLA, although other interneuron populations expressing somatostatin, cholecystokinin (CCK) or vasoactive intestinal peptide also modulate BLA pyramidal neurons (Spampanato et al., 2011). In the BLA, PV interneurons emerge around PND14 and are morphologically mature by PND30 (Berdel and Morys, 2000). In the current study and in agreement with others using a modified ELS procedure (Santiago et al., 2018), we found no changes in the density of PV cells in the BLA as a function of early stress, sex or laterality (Fig 6). However, when juveniles were exposed to acute fear conditioning, the number of activated PV neurons (Fos+/PV+) in the right BLA was significantly reduced in male rats exposed to early-life stress (LB) compared to their control NB counterparts (Fig 8). This effect was not observed in the male left BLA where fear conditioning increased PV neuron activation in both NB and LB offspring. Thus, rather than affecting PV+ cell density, early stress might change intracellular properties of these neurons, making them resistant to activation under emotional fear conditioning conditions. A reduction of inhibitory tone in the right BLA would be associated with enhanced synaptic activity and neuronal excitability as we observed in male LB pups in the right, but not left BLA. In females, fear conditioning significantly activated PV+ neurons in both NB and LB juveniles, but only in the left BLA.

The relationship between inhibitory interneuron activity and synaptic plasticity is complex and depends on several other components modulating neurotransmission, including perineuronal nets (PNNs). These specialized lattice structures containing chondroitin sulfate proteoglycans and extracellular matrix components ensheath mainly PV interneurons and excitatory cells in the BLA (Kwok et al., 2011; Morikawa et al., 2017). They are believed to serve various roles in neurotransmission, including the stabilization of synaptic inputs and to participate in the opening/closure of critical periods of plasticity in the brain (Pizzorusso et al., 2002; Hensch, 2003; Takesian and Hensch, 2013). Interestingly, PNN also develop postnatally in the rat BLA and the number of PNN-positive cells increase until PND28 (Gogolla et al., 2009). Our results demonstrate that the density of PNNs in the BLA of juveniles is significantly increased by ELS on both hemispheres for males and only on the left side in females. This is similar to chronic stress in adults, which increases PNN density in the hippocampal CA1 (Riga et al., 2017) and mPFC (Pesarico et al., 2019) specifically. The significance of stress-induced increases in PNNs is not well understood.

Behaviorally, a greater number of neurons harboring PNNs might facilitate the storage of fearful memories (Banerjee et al., 2017) and it is proposed that PNNs serve as a physical barrier to protect neurons from ions and molecules that can cause oxidative damage (Cabungcal et al., 2013; Reichelt et al., 2019). The exacerbated neuronal activity in the right BLA of juvenile males suggests that those synapses require increased PNNs to buffer cations involved in neurotransmission (van 't Spijker and Kwok, 2017). Given that PNNs limit synaptic plasticity in the adult BLA (Gogolla et al., 2009), why does LB exposure simultaneously enhance male synaptic plasticity and PNN expression? The first

assumption can be that PNNs are mostly restricting the plasticity of inhibitory PV+ interneurons. However, LB did not influence the percentage of PNN on PV+ cells, which ranged between 26-35%, in line with what is observed in the medial and posterior regions of the adult BLA (Morikawa et al., 2017). Therefore, the majority of PNNs in the BLA of post-weaning rats (PND28-29) may be on excitatory neurons and other subtypes of inhibitory interneurons (Morikawa et al., 2017). Alternatively, since spines on excitatory BLA neurons are increased in LB males as early as PND10 (Guadagno et al., 2018a), many synaptic contacts are already established in the developing BLA before PNNs appear (Reichelt et al., 2019). Upon being integrated into the circuitry, PNNs may then selectively encapsulate highly active neurons and prevent the formation of additional synaptic contacts (Pesarico et al., 2019). Future work is required to test these hypotheses.

In addition to PNN cell density, the maturation stage of these lattice-like structures is important to establish their function in neurotransmission. The intensity of WFA staining represents a good proxy for PNN maturity (Slaker et al., 2016) and was found to be decreased in the BLA of weaning age rats by early life trauma (PND8-12) (Santiago et al., 2018). In our study, PNN intensity was not significantly affected by earlier stress exposure (PND1-10), although staining intensity was elevated in the right compared to the left BLA in males only. This suggests that PNN maturation is accelerated in the right amygdala to possibly accommodate lateralized BLA functions.

The density and percentage of PV+ cells ensheathed by PNNs was exclusively elevated in the right BLA in males, but not females. In the cortex, PNNs enhance the excitability of PV+ interneurons (Balmer, 2016), but it is unknown how they affect

physiology of these cells in the amygdala. The increased right BLA excitability we found in LB males suggests that, at least under stimulated conditions, inhibitory control on glutamatergic pyramidal neurons is impaired.

Lateralized and sex-dependent cellular responses to fear conditioning after LB

To compare our electrophysiological findings to *in vivo* cellular and behavioral responses, we tested whether LB conditions would influence fear-induced Fos+ expression and PV+ cell activity in the BLA. While fear conditioning significantly increased Fos responses in both males and females, it only did so in the right BLA. This is consistent with the lateralization of fear responsiveness in adult BLA neurons (Scicli et al., 2004). In male juveniles, fear conditioning stimulated PV+ interneurons in the left BLA, in line with previous work demonstrating excitation of these cells during the acquisition phase of fear learning (Wolff et al., 2014). In the right BLA, there was no further activation of PV+ neurons by fear conditioning in LB males, possibly because the number of activated PV+ neurons under “basal” conditions was considerably higher in LB compared to NB males. We speculate that the increased PNN ensheating of PV+ interneurons induced by LB conditions in males might enhance their firing activity (Balmer, 2016) in order to oppose BLA hyperexcitability and silence overexcited circuits (Czeh et al., 2005). Accelerated PNN development around PV+ cells after LB may therefore serve as a protective mechanism (Cabungcal et al., 2013), but could also be causing more subtle changes later on in the physiology of interneurons that cannot be detected using these methods. In females, LB conditions did not significantly influence BLA inhibitory neuron activity in either the left or right hemisphere, suggesting that functional effects of early stress on

BLA neuronal circuitry in females might predominantly be on glutamatergic cells, as reported in our study, or on other inhibitory neuron subtypes. Fos+ expression in the right BLA was also decreased in females compared to males and provides evidence that cellular activity under baseline and stimulated conditions is generally sexually dimorphic in this region. This was also supported by our finding in NB fear conditioned animals, where females displayed blunted PV+ cell activity relative to males. Although this has yet to be confirmed, it is possible that PV neurons in the right BLA of females require a much stronger stimulus during fear conditioning (i.e., shock intensity) to become activated to the same level as in males.

Behavioral responses to fear conditioning

Changes in BLA synaptic plasticity are essential for fear learning in both early development (Thompson et al., 2008) and adulthood (Suvrathan et al., 2014). As demonstrated in our study, and in particular in males, ELS exposure can negatively impact these synaptic learning processes to enhance anxiety and fear behaviors in adulthood (Guadagno et al., 2018a). In the current study, we examined whether enhanced synaptic plasticity in LB males would associate with increased fear conditioning in the juvenile period. At this age (PND28-29), and in accordance with our findings, rats can be conditioned to express adult-like levels of fear (Jovanovic et al., 2013). In contrast to what we observed in adult males (Guadagno et al., 2018a), there was no effect of LB on fear conditioning in male or female juveniles. A relatively mild shock intensity was used (0.6 mA), yet freezing time often reached ~90% of the trial duration. It is conceivable that freezing responses hit ceiling levels and hindered the effects of bedding and/or sex on

fear behavior. Alternatively, it is possible that the magnitude of the effects we report on synaptic plasticity and neuronal excitability after exposure to LB in males is insufficient to significantly alter behavioral outcomes in this developing circuit. After puberty and later in life, stabilization of adult fear circuits might allow to fully reveal the behavioral effects of early stress exposure as we documented earlier in our model and in adult rats (Guadagno et al., 2018b; Guadagno et al., 2018a).

Conclusions

In summary, our results demonstrate that exposure to LB conditions leads to male-specific enhancements in synaptic plasticity and neuron excitability that are exclusively displayed in the right BLA. These functional changes in post-weaning males were associated with accelerated development of PNNs, notably around PV cells, and impaired fear-induced inhibitory activity in the BLA. Females instead compensated for early adversity by reducing neuron excitability, in parallel with right amygdala NMDAR subunit expression (Fig. 9). These data offer mechanistic insight into how ELS affects the post-weaning BLA in a sex- and hemispheric-selective manner. Our study highlights the right amygdala and early steroid milieu as promising sites for the development of interventions aimed at reducing consequences of ELS in disease states.

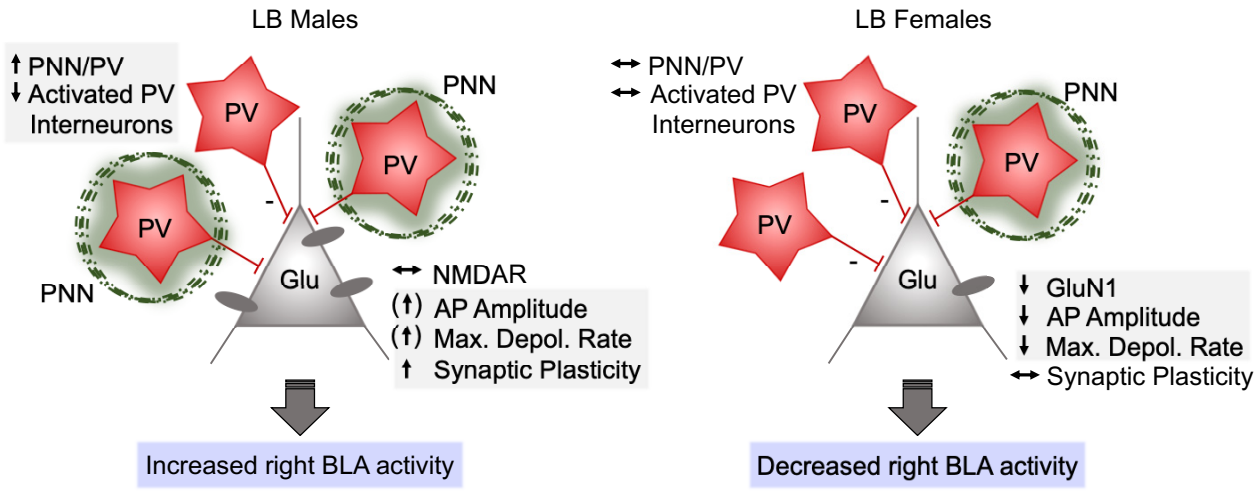

Figure IV-9. Working model of how limited bedding (LB) exposure induces sexually dimorphic changes in right basolateral amygdala (BLA) activity in juvenile (PND22-29) rats. In LB males, we found an increased density of parvalbumin-positive (PV) interneurons harboring perineuronal nets (PNNs) in the right BLA, which might have contributed to decreased fear-induced activity of PV interneurons. LB males also showed modest enhancements in action potential amplitude and maximal depolarization rate in excitatory right BLA neurons, which associated with enhanced LTP formation and evoked synaptic responses. Conversely in LB females, GluN1 NMDAR subunit expression was decreased only in the right BLA, along with action potential amplitude and maximal depolarization rate. In summary, LB exerts sex-dependent effects on different components of the neuronal circuitry in the right BLA to likely increase activity in males, and decrease activity in females.

Acknowledgements and Disclosures

The authors wish to thank Alyssa Guerra and Alice Wong for helping with the analysis of fear behavior and the Western Blot experiments, respectively. The present study used the services of the Molecular and Cellular Microscopy Platform at the Douglas Institute Research Center. This work was supported by a grant from the Canadian Institutes for Health Research (CIHR) grant to CDW #PJT162376 and NSERC PGSD award to AG.

Conflict of Interest: The authors have no financial interest in, or conflict of interest with the subject and materials discussed in the present manuscript.

Chapter V: General Discussion and Conclusions

Summary of main findings

Exposure to early-life stress, for instance by receiving suboptimal maternal care induces long-lasting consequences on cognitive and emotional health, increasing vulnerability to the development of psychopathologies (Essex et al., 2011; Pechtel and Pizzagalli, 2011; Krugers et al., 2017). While structural and functional changes in the amygdala across species have been implicated in the long-term effects of ELS (Burghy et al., 2012; Malter Cohen et al., 2013; Rau et al., 2015; VanTieghem and Tottenham, 2017), the proximal consequences and cellular mechanisms operating during and immediately after the exposure to early stress in neonatal life are still unclear. In addition, laterality of the amygdala and sex are critical variables for understanding the enduring effects of ELS and vulnerability to early maltreatment (Walker et al., 2017; Roth et al., 2018; White and Kaffman, 2019), yet they are often overlooked in neurodevelopmental studies. Using a well characterized preclinical model of ELS, the limited bedding (LB) paradigm during the first 10 postnatal days (Walker et al., 2017), we examined the effects of chronic ELS on the developing BLA and elaborated tentative mechanisms underlying early sexually dimorphic and asymmetrical stress-induced changes.

Consistent with previous findings in adult rodents (Vyas et al., 2002; Rau et al., 2015), in **Chapter II** we demonstrated that exposure to LB induced sex-specific outcomes on BLA morphology and function in neonatal and preweaning rats. We are the first to show that the BLA of male, but not female rats, undergoes significant hypertrophy and structural remodeling following early adversity on PND10 and until PND20, in parallel with enhanced evoked synaptic responses. The increased spine numbers observed on BLA excitatory neurons in young males suggests that those neurons receive stronger synaptic

inputs, which in turn facilitates heightened electrophysiological responses under stimulated conditions. Interestingly, we found that LB leads to blunted stress-induced CORT release on PND10 without significantly affecting basal CORT levels, in line with results from another study conducted in our laboratory (McLaughlin et al., 2016). This implies that the LB model, at least in our hands, suppresses HPA activity under stimulated conditions. In contrast to the adult literature demonstrating that glucocorticoids critically mediate effects of chronic stress on BLA neuronal structure (McEwen et al., 2016), the findings above suggest that elevated basal CORT levels may not be required for dendritic remodeling in the neonatal BLA and that other CORT-independent processes may be involved at this developmental stage. Importantly, structural and functional alterations in the developing BLA were associated with lasting sexually dimorphic deficits in emotional regulation, as evidenced by increased fear and anxiety-like behaviors in male, but not female adult LB offspring.

On a larger scale, we hypothesized that enhanced amygdala activity in preweaning males would negatively affect BLA functional connectivity with the mPFC and other brain regions, as documented in humans and adult rodents (Burghy et al., 2012; Gee et al., 2013b; Pagliaccio et al., 2015; Yan et al., 2017). Indeed, in **Chapter III**, we reported reduced ipsilateral BLA-mPFC connectivity in PND18 males that persisted until adulthood and likely contributed to increased fear behaviors in male LB animals. Another novel finding of this study is that lateralization of BLA connectivity was already observed in preweaning life, with connectivity changes from the right BLA outnumbering those from the left. This asymmetry determined different patterns of connectivity in the long-term, notably with the mPFC and vHIPP, two regions heavily implicated in emotional processing

and emotional memories (Fanselow and Dong, 2010; Godsil et al., 2013; Eden et al., 2015). Lastly, LB exposure also affected connectivity of the BLA with the contralateral mPFC, which is surprising given the relatively small numbers of inter-hemispheric BLA-mPFC projections documented at this age (Verwer et al., 1996). This finding is the first evidence that in addition to modifying the functional integrity of such contralateral connections, LB might also influence the interhemispheric crossing of anatomical connections, since adult LB offspring display higher midline crossings than NB controls (Bolton et al., 2018). This could be a result of reduced pruning of inter-hemispheric BLA-mPFC projections at the time of projection development during the first 2-3 weeks of life. We are currently investigating this hypothesis in developing animals using a combination of anterograde and retrograde tracer injections in the BLA and mPFC, respectively.

Given our previous findings that were acquired for the most part in males, we next probed for asymmetrical effects and sex differences of ELS by examining synaptic plasticity, neuron excitability and NMDAR subunit expression in the juvenile BLA. As described in **Chapter IV**, we found that LB exposure increased synaptic plasticity in the BLA of juvenile male, but not female pups. Remarkably, this effect was only detected in the right, but not the left BLA, which was unaffected by LB exposure in either sex. We believe this represents the first demonstration that synaptic function in the juvenile BLA is lateralized and sexually dimorphic in its sensitivity to ELS. This study also provides additional electrophysiological evidence supporting our right-lateralized functional connectivity data obtained in the BLA of preweaning and adult males. It is worth noting that LB female pups exhibited a reduction in NMDAR subunit expression in the right BLA, which suggests a mechanism through which right BLA neuron excitability was decreased

in these animals. Since NMDAR subunit expression was unaltered by LB exposure in the BLA of males, this implies that early stress-induced changes in male synaptic plasticity may be largely mediated by other factors, such as modified indices of GABA functioning and expression of PNNs. This possibility was examined under the fourth aim, in which we proposed that increased BLA synaptic plasticity and neuron excitability in juvenile males reared under LB conditions would be associated with reduced activity of PV cells and accelerated development of PNNs around these PV interneurons. As expected, we showed that functional alterations in LB juvenile males coincided with increased density of PNNs, especially around PV cells, suggesting greater stabilization of synaptic inputs onto these inhibitory interneurons. Fear-induced activity of PV interneurons was also suppressed in the right, but not the left BLA of LB males and perhaps contributed to the enhanced excitability of BLA pyramidal neurons on the right side.

Potential mediators of LB-induced structural alterations in the developing male BLA

Questions do remain about how LB exposure drives dendritic hypertrophy and increased spine formation in the neonatal and preweaning male BLA to ultimately enhance synaptic plasticity. Several factors might be responsible for this structural remodeling, in particular reduced BDNF gene methylation, which has been reported in the BLA of adult male LB offspring (Bath et al., 2013; Hill et al., 2014; Roth et al., 2014). Interestingly, adult male rats exposed to chronic restraint stress in adulthood also show significant and persistent increases in BDNF levels in the BLA that correlate with enhanced growth of dendrites and spines (Lakshminarasimhan and Chattarji, 2012). Again, the situation of the BLA is quite unique because chronic stress causes a reduction

in BDNF in other brain regions such as the mPFC and hippocampus (Bath et al., 2013) where reduced spine density is observed after chronic stress (Vyas et al., 2002; McEwen et al., 2016).

Glutamate release in the physiological range has also been posited to stimulate the outgrowth of mature spines (Sigler et al., 2017) in neonatal rat organotypic hippocampal slice cultures (Mattison et al., 2014) and neonatal mouse cortical (layers 2/3) slices (Kwon and Sabatini, 2011). The extent to which this occurs in the developing BLA is unknown, but our results suggest that glutamatergic activity is increased in preweaning and juvenile LB males and might initiate and/or potentiate morphological changes. Alternatively, apart from promoting the addition of new spines, ELS might impair the developmental process of spine pruning in the BLA. In the mouse cortex, the majority (65%) of all spines at PND21 are “transient”, that is, they are highly susceptible to removal and have an average lifetime of a few days (Berry and Nedivi, 2017). By adulthood, only one third of spines are transient, as they are replaced by “persistent” spines that remain for months or years (Berry and Nedivi, 2017). In the visual cortex of adult mice, chondroitin sulfate proteoglycans, which constitute the main component of PNNs (Reichelt et al., 2019), inhibit dendritic spine motility and stabilize structural plasticity (de Vivo et al., 2013). Although the cortex is quite different from the BLA, it is possible that ELS accelerates the transition of transient to permanent spines to mediate lasting changes in synaptic plasticity. This developmental shift may be achieved via the premature appearance of PNNs, which we observed primarily in the right BLA of juvenile males exclusively. Some preliminary data in our laboratory suggests that PNN density is similarly increased in the right BLA of preweaning LB males even prior to weaning on

PND20. Thus, as PNNs are prematurely integrated into the male BLA after ELS, they may stabilize spines that were otherwise destined for removal. This hypothesis will be discussed below in the context of our findings on synaptic plasticity.

A third possibility to explain morphological changes after ELS is the role of microglia to regulate spine density during development. Indeed, microglia have been shown to engulf synapses (Paolicelli et al., 2011) and actively participate in spine remodeling in an experience-dependent manner in developing mice (Tremblay et al., 2010). The large diversity of markers, receptors and secreted cytokines and chemokines expressed by microglia vary as a function of sex, microglia activation state, brain region and maturity, conferring unique temporal microglial characteristics that can influence neighboring neurons (Schwarz et al., 2012; Zhan et al., 2014; Pierre et al., 2017; Weinhard et al., 2018). Among these CX3CR1, a receptor expressed exclusively by microglia, has an important role in synaptic pruning, particularly during the first 2 weeks of life when CX3CR1-KO mice exhibit an excess of weak synapses in the hippocampus (Zhan et al., 2014). Interestingly, microglia express a high concentration of glucocorticoid receptors (GR), making them extremely sensitive to the effects of stress, notably in corticolimbic brain regions (Hinwood et al., 2013; Delpech et al., 2016; Johnson and Kaffman, 2018). For instance, increased immunoreactivity of ionized calcium-binding adaptor molecule-1 (Iba1), a marker of microglial activation, was reported in the central amygdala of chronically stressed adult rats (Tynan et al., 2010). The effects of LB on microglia in the developing BLA are still unclear, but it is conceivable that reduced microglial activation in neonatal LB males might decrease synaptic pruning and thereby increase spine numbers in the BLA. Sex differences in microglial brain colonization,

proliferation, activation, phagocytic activity and expression of cytokines and receptors are also observed in early development (Schwarz et al., 2012; Pierre et al., 2017; Weinhard et al., 2018) and might support sex-dependent changes in morphology, neuronal activity and connectivity in several brain regions, including the BLA. Studies observing these sex differences will later be discussed in the context of our findings.

The role of perineuronal nets on synaptic plasticity in juvenile males after LB exposure

An important question still remains: how can increased PNN numbers, observed mostly on the right side of the developing LB male BLA, potentially stabilize structural plasticity while enhancing LTP formation and neuronal excitability? We first assumed that PNNs were primarily limiting the structural and functional plasticity of inhibitory interneurons, such as PV cells, to diminish GABAergic input onto pyramidal BLA neurons. Indeed, we showed that the fear-induced activity of PV cells in the right BLA was significantly decreased in LB males. While we did find that PNN density was increased especially on PV cells in the right BLA of LB males, we also found that an overwhelming percentage of PNNs in the BLA of juvenile rats were likely on excitatory neurons and other subtypes of inhibitory interneurons, in line with other findings in the adult BLA (Morikawa et al., 2017). We are currently investigating this possibility and propose that LB male animals will have more PNNs on GABAergic inhibitory interneurons relative to glutamatergic neurons in the right BLA. If this is not the case and PNN numbers are generally enhanced in the right BLA of LB males, it is plausible that PNNs may serve a very different function in the amygdala than they do in the cortex, such that they are able to stabilize spines while permitting synaptic strengthening. To elaborate, loss of PNNs in

the adult mouse visual cortex enhances LTP formation (de Vivo et al., 2013), whereas similar manipulations in the adult mouse lateral amygdala reduces LTP (Gogolla et al., 2009). This suggests that PNNs can restrict and enhance synaptic plasticity in the cortex and amygdala, respectively. In addition to regional differences in the action of PNNs, changes in the structural composition of PNNs may also lead to different effects on synaptic transmission. In the cortex, varying structural compositions and combinations of chondroitin sulfate proteoglycans are found depending on the cell type they surround, which might lead to mixed functional consequences (Galtrey and Fawcett, 2007). Thus, additional work is needed to more precisely define the role that PNNs play in the developing amygdala and in particular whether early appearance and/or maturation of these structures have a net effect to enhance or reduce synaptic plasticity.

LB exposure and BLA development in males

Several findings from my thesis point to an effect of ELS exposure to accelerate the maturation of the BLA in male rat pups, through modified neuron morphology, PNN development, synaptic plasticity, and functional connectivity. This is consistent with the human literature, whereby ELS induces precocious amygdala development via amygdala hypertrophy (Tottenham et al., 2010), hyperactivity (Tottenham et al., 2011; van Harmelen et al., 2013) and adult-like amygdala-PFC functional connectivity (Gee et al., 2013a). Our studies reveal important cellular mechanisms that might be responsible for such premature development. For instance, we found that the appearance of PNNs especially in the right BLA of juvenile males was increased in LB offspring, which might have enabled the enhanced and adult-like synaptic plasticity we found in the right male BLA at a juvenile

stage. In the rodent visual cortex, PNN formation around PV interneurons has been implicated in the functional maturation of inhibitory connections and the closure of critical periods of synaptic plasticity at the end of the first postnatal month (Hensch, 2003). Our results suggest that in the right BLA, accelerated development of PNNs on PV cells after ELS exposure may prematurely stabilize immature GABAergic circuits and induce an adult network configuration. In our experiments, enhanced PNN formation in the juvenile amygdala did not associate with the closure of critical periods of synaptic plasticity specifically, since we observed enhanced, rather than restricted, LTP and evoked synaptic responses in LB male animals. As previously suggested, this discrepancy may be due, at least in part, to the different structural composition and functions of PNNs in the cortex compared to the amygdala.

Exaggerated synaptic BLA responses at earlier developmental time points may favor the development of a more mature BLA-mPFC circuit as suggested in humans (Gee et al., 2013a), and impair the resting-state functional MRI connectivity between these regions in the preweaning male rat. The amygdala displays early functionality and structural maturity relative to the mPFC (Tottenham and Gabard-Durnam, 2017). Thus, ascending BLA-mPFC projections, which mature by PND13 in the rat (Bouwmeester et al., 2002a), are presumably able to instruct mPFC development until the descending mPFC-BLA connections reach maturity around PND21 (Bouwmeester et al., 2002b). If ELS exposure accelerates the maturity of the developing male BLA and its projections to the mPFC, does this suggest that the top-down mPFC-BLA projections are also maturing earlier in LB pups? The top-down projections important for fear inhibition have been demonstrated to emerge after the termination of LB stress exposure, by PND13 in rats

(Bouwmeester et al., 2002b) and between PND10-15 in mice (Arruda-Carvalho et al., 2017). The delayed appearance and proliferation of glutamatergic mPFC inputs to the BLA that occurs until PND30 in the mouse associates with a significant increase in spontaneous BLA activity, as well as synaptic strengthening (Arruda-Carvalho et al., 2017). These findings provide convincing evidence that the descending mPFC projections critically influence the functionality of the developing BLA (Arruda-Carvalho et al., 2017). Although future work is required to answer these important questions, a potential mismatch between the maturation of the bottom-up (BLA to mPFC) circuits involved in fear conditioning and the top-down (mPFC to BLA) ones responsible for fear inhibition would prevent adaptive responses to aversive stimuli as observed after ELS. Converging evidence in the rodent and human literature indicates that precocious development of the BLA and BLA-PFC circuit, triggered by early adversity, has persistent consequences on emotional regulation (Burghy et al., 2012; VanTieghem and Tottenham, 2017).

How early BLA alterations might program lasting behavioral consequences in male LB offspring

To address the fifth aim of my thesis, we examined whether structural and functional alterations in the developing BLA would lead to impairments in behavioral regulation in adulthood. Indeed, we showed that male, but not female, adult LB offspring displayed increased anxiety-like and fear behaviors that associated with potentially accelerated BLA development and long-term reductions in BLA-mPFC functional connectivity. Several studies have shown that LB exposure causes precocious engagement of the amygdala in fear learning, resulting in avoidance instead of approach

behavior to a threat a few days earlier than normal (i.e., on PND7 instead of PND10) (Moriceau et al., 2009b; Junod et al., 2019; Opendak et al., 2019). This premature aversion learning may have also occurred in our neonatal LB animals and contributed to lasting impairments in the fear circuitry, as well as exaggerated adult behavioral responses to fearful stimuli.

Enhanced BLA reactivity is a hallmark symptom of ELS exposure and leads to an anxiogenic phenotype (Burghy et al., 2012; VanTieghem and Tottenham, 2017), as well as exaggerated emotional responses to fearful stimuli in both humans and rodents (Raineki et al., 2012; Gee et al., 2013b; Malter Cohen et al., 2013; Silvers et al., 2017). Therefore, BLA hyperactivity in our study likely contributed to increased anxiety-like and fear behaviors in adult LB males. Importantly, precocious BLA hyperactivity induced by ELS accelerates the development of more “mature” BLA-PFC connectivity and decreases the resting-state functional MRI connectivity between these regions in humans (Gee et al., 2013a), consistent with our findings in preweaning and adult male rats. Connectivity data do not allow us to determine the direction of functional impairment, only that the connectivity and relationships between these two structures are weakened after ELS. BLA-mPFC circuitry critically mediates fear conditioning and extinction. Thus, any disruptions in the functional integrity of this pathway can affect conditioned fear responses. Specifically, reciprocal connections between the BLA and PL mPFC are involved in fear conditioning (Sotres-Bayon et al., 2012; Arruda-Carvalho and Clem, 2014), while projections with the IL region mainly modulate extinction processes (Bloodgood et al., 2018; Ganella et al., 2018). In adult males exposed to LB, we found that BLA functional connectivity with the PL and IL regions of the mPFC was largely

decreased, and decreased functional connectivity between the BLA and IL mPFC likely resulted in the silencing of this pathway and impaired fear extinction (Bukalo et al., 2015; Ganella et al., 2018). It remains unclear how decreased BLA-PL functional connectivity would result in increased freezing behavior during fear conditioning, since reciprocal activation of this pathway elicits fear responses (Burgos-Robles et al., 2009). However, descending PL to BLA projections are also required for fear retrieval 24 hours after fear acquisition (Arruda-Carvalho and Clem, 2015). Although we cannot ascertain whether the ascending or descending circuit exhibits functional connectivity changes from our imaging study, we suspect that reduced descending connectivity would impair fear extinction and retrieval and thus maintain fear responses through unaltered connectivity in the ascending BLA to PL circuit.

Lastly, we observed elevated PNN density, particularly in the right BLA, of juvenile males exposed to LB conditions. A series of elegant experiments by Gogolla et al. demonstrated that the appearance of PNNs on PND23 in the mouse BLA corresponds with a developmental switch in fear learning, such that fear memories are protected from erasure following extinction training (Gogolla et al., 2009). Conversely, degradation of PNNs on all BLA cell types in adulthood reenables the permanent erasure of fear memories by extinction, a process normally only observed in juveniles (Gogolla et al., 2009). Thus, we propose that increased PNN numbers in the BLA of juvenile LB males facilitated freezing behavior during fear extinction in adulthood. Interestingly, we also observed that LB adult males displayed increased freezing responses during fear retrieval in our first study (see Chapter II), indicating that fear memories were not properly extinguished. Together, these results suggest that enhanced PNN formation in the

developing male BLA after ELS might persist until adulthood to program lasting changes in fear memory resilience.

LB exposure induces a lateralized BLA phenotype in developing males

To our knowledge, we are the first to reveal right-lateralized effects of LB exposure on BLA function and connectivity in developing male rats. It is worth noting that these asymmetries were associated with increased fear behaviors in adulthood, suggesting that ELS-induced dysfunctions in the right, but not the left BLA, are primarily responsible for mediating enduring behavioral consequences. Consistent with our findings, children and adolescents that were previously institutionalized showed increased right amygdala reactivity to fearful faces, however these effects were not sex-specific (Gee et al., 2013b). Another study documented increased right amygdala volume in adolescent boys reporting childhood neglect (Roth et al., 2018). Even though we did not measure hemispheric effects of LB on BLA neuron structure and cellular volumetric estimates in our first study (see Chapter II), it would certainly be interesting to do so to determine if early alterations in right BLA function and connectivity coincide with asymmetrical morphological changes.

During its normal developmental trajectory, the rat BLA does not appear to exhibit asymmetry in volume and total neuron and glial cell numbers on PND20, 35 or 90 (Rubinow and Juraska, 2009). Functionally, we found that LTP formation was greater in the right compared to the left BLA in LB, but not NB juvenile males. Collectively, these findings indicate that BLA asymmetry in developing male rats might be a direct consequence of ELS exposure and that the female BLA does not demonstrate such asymmetry with ELS. The functional significance of BLA asymmetry in response to ELS

remains elusive and is an essential question to address in future research. Based on human neuroimaging data, the right amygdala is preferentially activated during the evaluation and encoding of negative emotionally arousing memories, while the left amygdala is more involved in cognitive emotional processing (Baas et al., 2004; Butler et al., 2018). The inherently different properties of each amygdala likely bias their responses to ELS exposure, which is undoubtedly filled with emotionally charged negative experiences.

Sexually dimorphic consequences of LB on the developing BLA and related behaviors in adulthood

The studies presented in this thesis have unveiled sexually dimorphic consequences of LB on BLA morphology and function that emerge in neonatal life and associate with increased anxiety-like and fear behaviors in adulthood. Developing male rats were consistently more vulnerable to the effects of LB conditions, whereas females were either not affected or showed “protective” changes in the opposite direction compared to male pups. In the few studies that have examined sex as an experimental variable on amygdala outcomes after early adversity, it is often males, but not females, that are affected (White and Kaffman, 2019). For example, only adolescent male mice subjected to early weaning exhibit premature myelination in the BLA, suggesting that females are more resilient to this form of early stress (Ono et al., 2008). Similarly, a recent human study showed increased right amygdala volume and anxiety symptoms in adolescent boys, but not girls, subjected to self-reported childhood neglect (Roth et al., 2018). The mechanisms underlying the susceptibility of the male BLA to LB rearing are

not clear, but could overlap with the organizational role of sex steroids during development. Indeed, brain masculinization in rodents is characterized by a peak in testosterone production at the end of gestation (GD17-18) in males, which defeminize the brain mainly through transformation of testosterone into estradiol by the aromatase enzyme (Naninck et al., 2011). Testosterone levels remain high during the first few days of life, coincident with the period of LB exposure. During the same period of brain sexual differentiation in rodent females, estrogen levels are comparably low (Naninck et al., 2011). Interestingly, adult female rats that were injected with testosterone propionate on PND1 display marked increases in total synapse number in the medial amygdala to levels of adult males, suggesting that neonatal testosterone mediates sexually dimorphic synaptogenesis in the amygdala (Nishizuka and Arai, 1981).

As eluded to earlier, there is a strong sexual dimorphism in microglia colonization of the brain and their activation in several brain areas, which might be important for the conservation or pruning of spines and synapses (Schwarz et al., 2012; Lenz et al., 2013; Weinhard et al., 2018). In fact, neonatal microglia are twice as abundant and exhibit a more activated morphological profile in males relative to females and are required for developmental brain masculinization (Lenz et al., 2013). In the few studies that have examined microglia in the BLA, some failed to observe sex differences in neuron or total glia number (Rubinow and Juraska, 2009), while others document a large increase in microglia proliferation in the first 4 days postnatally in females and a larger number of microglia in females compared to males post-weaning (Schwarz et al., 2012). A proinflammatory molecule produced by microglia, PGE_2, is implicated in sex-specific regulation of spines in the preoptic area (POA), a sexually dimorphic nucleus,

differentiated during the early postnatal period (VanRyzin et al., 2018). In the POA, increases in microglial PGE_2 production leads to a higher trafficking of AMPAR to the membrane and both spine induction and stabilization, but only in males (Lenz et al., 2013). Disruption of microglia during short periods in early development reduces social play in juvenile rats, and has a negative effect on sex behavior of male, but not female adults (VanRyzin et al., 2016; VanRyzin et al., 2018), suggesting critical consequences for long-term behavioral regulation. Together these data suggest that ELS-induced alterations in microglia function might participate in the sexually dimorphic effects on the BLA that we have observed.

Although LB exposure did not significantly impair adult female behavior, this should not be interpreted to indicate that females are entirely resilient to early adversity. In fact, many human studies have shown that females exposed to childhood maltreatment are more sensitive (White and Kaffman, 2019) and likely to develop stress-related mental disorders, such as anxiety and depression, compared to males (McCarthy, 2016; McCarthy et al., 2017). Other reports have specified that sex differences in the behavioral consequences of early adversity strongly depend upon genetic susceptibility and the type and developmental timing of maltreatment (Walker et al., 2017; White and Kaffman, 2019). For instance, males appear to be more vulnerable to caregiving deprivation and early psychosocial neglect (Roth et al., 2018), whereas females may be more sensitive to severe trauma, such as physical and sexual abuse (White and Kaffman, 2019). It is also worth mentioning that the brains of males and females across species do not develop simultaneously (Lenroot et al., 2007; Lenz et al., 2013; Hammerslag and Gulley, 2016; McCarthy, 2016; Qiu et al., 2018), and differences in the trajectory of development could

also contribute to our sex-specific results, since we examined the same time point for both sexes. Thus, the male-specific effects we reported might reflect the type and time period of the chosen early stress paradigm. Future studies using variations of the LB procedure at different ages will help determine if a critical window of vulnerability similarly exists in females.

Conclusions

The aims and key findings of this dissertation are illustrated in Figure V-1 and our working model is depicted in Figure V-2. In summary, our experiments demonstrate that LB exposure induces sex and hemispheric-dependent alterations in the morphology, function and connectivity of the developing BLA.

Collectively, the data presented here suggest novel mechanisms through which early-life stress induced disruptions can program lasting impairments in anxiety-like and fear behaviors in adulthood. These mechanisms might also allow identifying critical periods of vulnerability and inform the guiding of future therapeutic interventions.

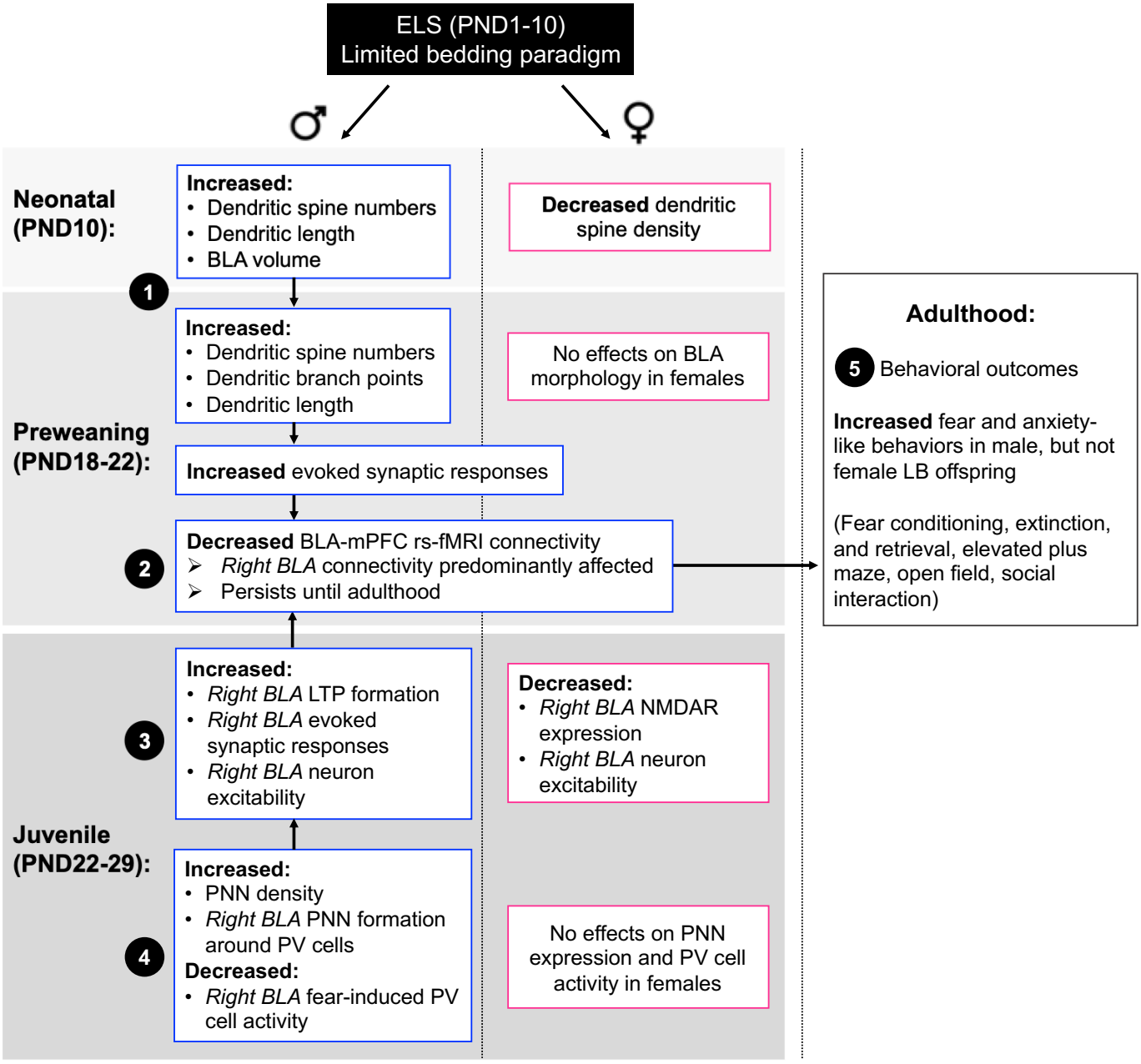

Figure V-1. A summary of the main findings and aims (numbers 1-5) of this PhD thesis. Effects of early-life stress (ELS) reported during the developmental period are in the basolateral amygdala (BLA).

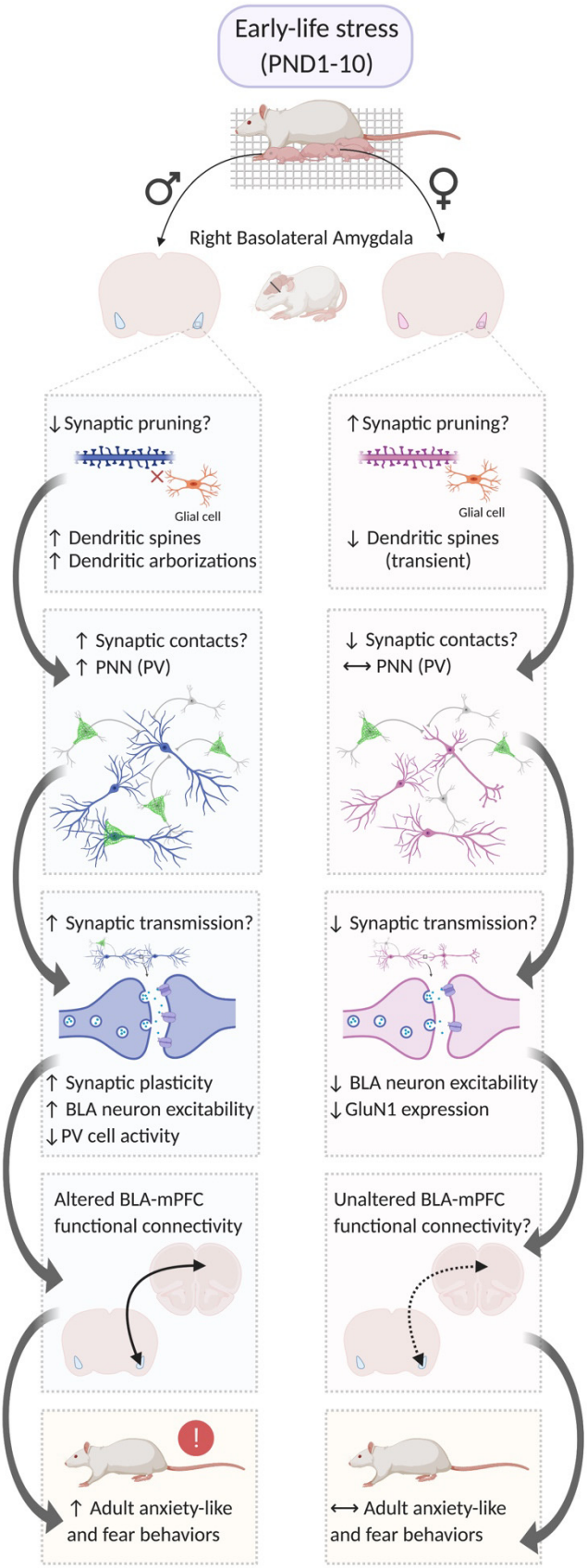
Early-life stress
(PND1-10)
♂
♀
Right Basolateral Amygdala
↓ Synaptic pruning?
Glial cell
↑ Dendritic spines
↑ Dendritic arborizations
↑ Synaptic pruning?
Glial cell
↓ Dendritic spines
(transient)
↑ Synaptic contacts?
↑ PNN (PV)
↓ Synaptic contacts?
⟷ PNN (PV)
↑ Synaptic transmission?
↓ Synaptic transmission?
↑ Synaptic plasticity
↑ BLA neuron excitability
↓ PV cell activity
↓ BLA neuron excitability
↓ GluN1 expression
Altered BLA-mPFC
functional connectivity
Unaltered BLA-mPFC
functional connectivity?
!
↑ Adult anxiety-like
and fear behaviors
⟷ Adult anxiety-like
and fear behaviors

Figure V-2. Working model of how limited bedding (LB) exposure induces sexually dimorphic effects in the developing right basolateral amygdala (BLA). Exposure to LB between postnatal days 1-10 might induce sex-specific changes in synaptic pruning in the developing BLA to increase neuron spine numbers and dendritic arborizations in males and decrease spine numbers in females (left and right amygdala sides combined), leading to either enhanced or reduced formation of synaptic contacts, respectively. In LB males, increased synaptic inputs to BLA neurons, in addition to enhanced perineuronal net (PNN) development, notably around parvalbumin (PV) interneurons, might contribute to enhanced synaptic transmission, as evidenced by increased BLA synaptic plasticity and neuron excitability and suppressed PV cell activity. In females, BLA neuron physiology may be "protected" from LB conditions because NMDAR GluN1 subunit expression and neuron excitability are decreased. We propose that right BLA hyperactivity in LB males alters the maturation of the BLA-medial prefrontal cortex (mPFC) circuit, resulting in impaired resting-state functional connectivity in this pathway and heightened anxiety-like and fear behaviors in adulthood. Created with Biorender.com.

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