The interaction between neonatal estradiol and early life stress on juvenile basolateral amygdala morphology and adult behaviour in rats

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Abstract

Prolonged exposure to adversity and chronic stress during early childhood presents a significant risk for the later development of psychopathologies as well as emotional, cognitive, and affective dysfunction. As early life stress (ELS) represents a diverse group of adverse experiences, its repercussions are similarly varied and sexually dimorphic in both humans and animal models. The corticolimbic circuit, particularly the basolateral amygdala, has been shown to be sensitive to ELS in a sexually dimorphic manner. However, to date, little is understood about the underlying mechanisms regulating the interactions between sex and early stress in the BLA and beyond. In this work, we are using the limited bedding (LB) paradigm, a well described model of ELS in rats during the first 10 days of life. We tested whether altering the estrogenic milieu in early life through neonatal injections of estradiol benzoate (EB) in females and of the aromatase inhibitor letrozole (Let) in males was sufficient to recapitulate the ELS-induced vulnerability phenotype of the opposite sex on morphology of principal neurons in the basolateral amygdala (BLA) and adult behavior. Our results show that EB treatment was sufficient to eliminate LB-induced morphological changes in the BLA of weaning females, independent of early changes in ERα expression in this region. In contrast, inhibition of aromatase function via the administration of Let in the early post-natal period did not substantially alter either ERα expression at PND10 or morphological response to ELS at weaning. In adult offspring, neonatal EB reduced the distance traveled but not the time spent in the center of the open field. EB treatment also increased social contact time in females across bedding conditions. Our findings indicates that estrogen plays a role in the mediation of some but not all aspects of ELS-mediated alterations in BLA morphology and adult behavior.

Résumé

L'exposition à l'adversité et au stress chronique au début de la vie représente un risque considérable pour le développement de psychopathologies à l'adolescence et l'âge adulte. Ces pathologies sont fréquemment associées à des dysfonctionnements émotionnels, cognitifs et affectifs. L'adversité en début de vie inclut diverses expériences négatives qui ont des répercussions très variées et généralement différentiées selon le sexe chez les humains comme dans les modèles animaux. Il a été démontré que le circuit corticolimbique incluant l'amygdale basolaterale (BLA) est très sensible à l'adversité et que les conséquences de l'adversité sur son fonctionnement varient en fonction du sexe des individus. Comme ce circuit est essentiel aux réponses émotionnelles, y compris l'apprentissage de la peur, un dysfonctionnement induit par l'adversité peut mener à une augmentation de l'anxiété. Par contre, les mécanismes sous-jacents des réponses différentiées selon le sexe de l'individu restent encore à clarifier. Dans ce travail nous utilisons un modèle expérimental d'adversité néonatale chez le rat et qui comporte une exposition à des conditions d'adversité, le « limited bedding » (LB) pour la mère et sa portée durant les 10 premiers jours de vie. Nous avons mesuré les changements morphologiques au niveau de l'amygdale basolaterale (BLA) induits par l'adversité en fonction du sexe des ratons. En induisant des changements du milieu œstrogénique au début de la vie, par des injections néonatales de benzoate d'estradiol (EB) chez les femelles et de l'inhibiteur de l'aromatase létrozole (Let) chez les mâles, nous avons évalué si ces modifications étaient suffisantes pour récapituler le phénotype de vulnérabilité induit par l'adversité du sexe opposé. Nous avons également évalué les conséquences de ces traitements sur le comportement a l'âge adulte. Nos résultats montrent que le traitement néonatal des ratons femelles avec EB est suffisant pour éliminer les changements morphologiques dans l'amygdale des jeunes rats au moment du sevrage et qui sont induits par l'adversité (LB). Ces effets ne sont pas reliés à des modifications précoces des niveaux d'expression des récepteurs aux œstrogènes (ER) dans cette structure. En revanche, l'inhibition de la fonction de l'aromatase via l'administration de letrozole au début de la période postnatale n'a pas d'effet significatif sur l''expression d'ERα ou sur les réponses morphologiques à l'adversité chez les ratons males au sevrage. Chez les adultes, le traitement avec EB chez les femelles a permis de réduire la distance parcourue dans une aire ouverte mais n'a pas modifié le temps passé au centre du champ ouvert. Nous avons également observe que l'administration néonatale de EB augmente la durée du contact

social chez les femelles, sans égard à l'exposition a de l'adversité ou non. Nos résultats indiquent que la masculinisation du cerveau des femelles par l'administration néonatale de EB modifie certaines, mais pas toutes les conséquences de l'adversité chez les femelles. Des interactions significatives sont apparues pour les changements morphologiques dans l'amygdale ainsi que certains comportements adultes.

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Contributions of Authors

During this project, I collaborated with Dr. Claire-Dominique Walker to design the experiments presented in this thesis. These experiments were conducted with assistance from Hong Long, whose help was invaluable. I took care of the animals and assisted Hong Long and Dr. Walker with tissue collection. I conducted the data analysis and have written this thesis with help from Dr. Walker who reviewed it at key stages and provided her advice and expertise.

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List of abbreviations

AI	Artificial Intelligence
ANOVA	Analysis of variance
AROM	Aromatase
BLA	Basolateral amygdala
BSA	Bovine serum albumin
BW	Body weight
CeA	Central Amygdala
E2	Estrogen
EB	Estradiol Benzoate
EPM	Elevated plus maze
ER	Estrogen receptor
FCG	Four-Core Genotypes
GD	Gestational day
GPER	G-protein coupled receptor
IHC	Immunohistochemistry
IL	Infralimbic
LB	Limited Bedding
LBN	Limited bedding and nesting
Let	Letrozole
MeA	Medial Amygdala
mPFC	Medial prefrontal cortex
mPOA	Medial preoptic area
MS	Maternal separation
NB	Normal Bedding
PBS	Phosphate-Buffered Saline
PFC	Prefrontal cortex
PL	Prelimbic
PND	Postnatal day
RCF	Repeated cross-fostering
SEM	Standard error of the mean
Veh	Vehicle
vHipp	Ventral hippocampus
VMH	Ventromedial nucleus of the hypothalamus

1. Background and Introduction

Brain development in rats does not follow a rigid predetermined pathway, instead neural plasticity provides a means for responsive brain development, wherein the environmental context interacts with and builds upon the developmental framework provided by internal factors, like genetics (Kolb & Gibb, 2011; Stiles & Jernigan, 2010). In early life, a finite period of neural plasticity provides the ability for one's environmental context to influence the creation of new neural connections and hone existing connections to better serve their needs. Later in development, as the period of plasticity ends and the brain's receptivity to the environment sharply decreases, any prior environmental influences on brain structure are made permanent as circuits stabilize and take their mature form. This process is largely adaptive, as it allows the brain to adapt in structure and function to best serve the individual and their specific environmental context. However, when the developmental environment is suboptimal or adversity is present, the initially adaptive process has the ability to induce later dysfunction, as observed widely in rodents exposed to early life stress (ELS) (Buss et al., 2012; Del Giudice et al., 2011; McEwen, 2006; Pluess & Belsky, 2011). In the short term, the neural restructuring initiated in the face of ELS is constructive, altering the corticolimbic circuit and hypothalamus-pituitary-adrenal axis central to fear and stress responses to best support survival in a high stress environment. The dysfunction arises when the adaptions are made permanent as development progresses and circuits mature, while stressors they were adapted to dissipate with time. As a result, the once adaptive process of altering the circuitry to improve function and survival during stress, becomes maladaptive across the lifespan, as the fear and stress circuitry is primed for a chronic stressor that no longer exists (L. K. Davis et al., 2020). This maladaptation often manifests as an outsized reaction to a fearful or stressful stimulus, as witnessed in increased incidences of anxiety-disorders in humans, and anxiety-like behaviours in animal models. While either sex can be exposed to such stress during development, the degree of vulnerability to the long-term dysfunction it induces has been shown to be sex dependent. Rodent studies have demonstrated that sex differences in ELS outcomes depend on the type, timing, and duration of the chronic stressor (Kolb & Gibb, 2011; Stiles & Jernigan, 2010). While it is evident that sex interacts with other factors to influence one's specific vulnerability to the long-term detrimental effects of ELS, the specific correlates of sex responsible for this difference remain unclear.

One of the primary consequences of ELS is emotional dysregulation arising from disruptions in the emotional circuitry, specifically those pertaining to the corticolimbic circuit. Within this circuit, one region that demonstrated a distinct sex-differences in susceptibility to ELS in rodents is the basolateral amygdala (BLA), a region responsible for fear learning, fear inhibition, and fear memory. Male rodents exposed to ELS show altered neuron morphology in the BLA by weaning, with corresponding increases in anxiety-like and fear behaviours as adults (Guadagno et al., 2018). The BLA connects with other areas of the fear circuitry both within the amygdala and beyond, forming reciprocal connections to the central amygdala, prefrontal cortex (PFC), and ventral hippocampus (vHipp) (McDonald, 1998; Senn et al., 2014). Alterations in the BLA can result in dysregulation of stress and fear reactivity, promoting the development of anxiety-related disorders. Therefore, sex-differences in BLA sensitivity to ELS may contribute to sex-differences in the development of neuropsychiatric disorders involving a dysregulated fear circuitry.

Despite the observation of sex differences in susceptibility to ELS, there is little understanding of the underlying interaction between sex and ELS that results in a heightened sensitivity or resilience across the sexes. While sex differences can be activated following the large sex-hormone production that begins in puberty, sex differences in ELS response have been shown to exist in the prepubescent brain, suggesting they arise from sexual differentiation in early development. The presence of changes in the prepubescent brain is supported by prior work in our lab utilizing the limited bedding model of ELS, wherein we observed a male-specific susceptibility both in the structure and function of the BLA at PND21-28. A variety of factors that work both individually and in concert are known to promote the sex-typical development and patterning of the brain during development. These include genetic and epigenetic factors, microglia migration and activation, and sex differences in early gonadal function. ELS protocols are typically applied during the first week or two of neonatal life, and thus, coincide with the established sex difference in gonad activity in late gestation and early life. While the ovaries of females remain quiescent, not producing any gonadal steroids until puberty, in males, the testis become active in late gestation, resulting in a biphasic release of testosterone. This biphasic release spans late gestation and early life, with the first surge around gestational day (GD)17-18, followed by a second shortly after birth (PND0-5). The period of elevated serum testosterone that follows the second spike represents a critical period in which testosterone, via aromatization to estradiol, promotes a masculine organization of various brain circuits (Figure 1). Due to this temporal

overlap of estrogenic masculinization and ELS exposure, we hypothesize that the masculinizing effects of estrogens perinatally might contribute to the heightened morphological and behavioural vulnerability to ELS observed in juvenile males.

To better understand the potential interactions between ELS and gonadal steroid milieu in determining sexually dimorphic adaptations of the fear circuitry, we will examine the interactions of early neonatal estrogen and ELS on BLA neuron morphology and fear behaviour in males and females.

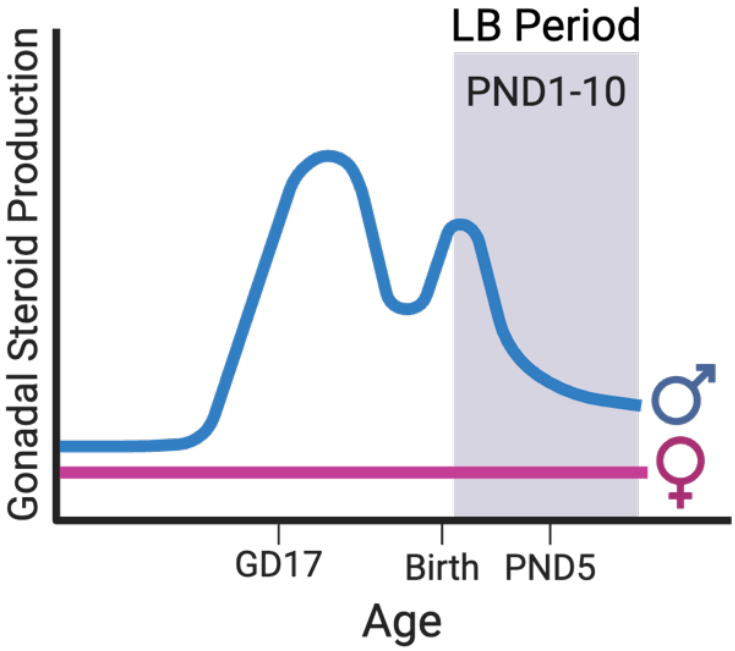

Figure 1. Gonadal steroid production in the perinatal period. Females (purple) do not produce gonadal steroids prior to puberty. Male (blue) rats exhibit biphasic androgen production, with peak gonadal steroid concentration between gestational days (GD) 17-18, and postnatal days (PND) 0-5. The critical period for sexual differentiation associated with gonadal steroid production coincides with the application of the limited bedding paradigm from PND 1-10. LB: Limited bedding. Figure created with BioRender.com.

1.1 Early Life Stress In Humans and Animal Models

Epidemiological and observational work in human populations has reported a strong link between early adverse life experiences and lasting consequences on cognitive and emotional health (Bremner & Vermetten, 2001; Gould et al., 2012; Pechtel & Pizzagalli, 2011).

In humans, a broad range of experiences during development may constitute early life stress, including but not limited to neglect, emotional, sexual, and physical abuse, in addition to environmental factors like poverty or loss of a parent. Chronic exposure to these stressors during early life increases the child's risk for the later development of psychiatric disorders, including anxiety, depression, post- traumatic stress disorders, and substance-use disorders (Carr et al., 2013; Green et al., 2010; Lupien et al., 2009; Repetti et al., 2002; Schore, 2000; Weber et al., 2008). Early life stress therefore represents a large risk factor for the development of mental health disorders, with an estimated 45% of childhood-onset disorders and 30% of later onset disorders having some association with ELS (Green et al., 2010; VanTieghem & Tottenham, 2018). Data from children raised in high stress environments, such as periods of war or famine, allowed for the initial detection and later dissection of the adverse effects of ELS (Clarkin, 2019; Joshi & O'donnell, 2003; Roseboom et al., 2006). However, the study of human data, whether observational or epidemiological, is not suited to the delineation of converging risk-factors nor mechanistic investigation of ELS consequences.

Animal models of ELS provide a means to experimentally measure and monitor the impact of ELS, with fewer ethical constraints relative to human studies. In the 1940s it was observed that altering the dynamic between offspring and its caregiver in early life had long term behavioural consequences on the offspring. This observation informed the basis of current ELS models, that destabilize the maternal-offspring relationship to induce stress in the young offspring, across various animal species, however we will focus on rodent models here. In rodents, the most commonly used models of ELS are maternal separation (MS) and limited bedding and nesting (LBN) or a variation of it (WALKER, 2017). In the MS model, the maternal-offspring relationship is altered by limiting the offspring's access to its mother. Similar to MS, the repeated cross-fostering (RCF) rodent model, seeks to induce stress by altering the maternal-offspring relationship. RCF, like MS, also relies on removal of the maternal figure, however instead of leaving the pups alone and returning the biological mother after a set time period as done in MS, in RCF, the biological mother is removed and replaced with a different lactating female usually

within the first 24-48 hours after birth and repeatedly thereafter. This constant change in caregiver disrupts the formation of attachment bonds, similar to the MS model, however unlike MS, pups in the RCF model always have access to a caregiver (Landers & Sullivan, 2012). In contrast to MS and RCF which require separation, the limited bedding and nesting (LBN) experimental paradigm models a slightly different experience, wherein the caregiver remains present and available, but due to environmental challenges, is unable to exhibit high quality or reliable care. In LBN, by limiting the amount of bedding and nesting materials accessible to the dam, experimenters create an impoverished environment for pup rearing (Molet et al., 2014; Walker et al., 2017). Unable to build a satisfactory nest in these conditions, the dam becomes stressed, hindering her ability to reliably care for her young. As young pups rely on consistent maternal care for homeostatic regulation prior to their ability to self-regulate, the fragmentation and erratic nature of the dam's behaviour interferes with her ability to regulate pups' physiology (Hofer, 1984, 1996b, 1996a; Shair et al., 2003). As LBN uses inadequate nesting material to induce stress, it more closely resembles human instances of resource scarcity and poverty, whereas MS and RCF model the experiences of children lacking a primary caregiver, such as children in the foster system. As the human experience of ELS encompasses a broad range of stressors, all three models are useful for their ability to recreate different characteristics of ELS, our lab however, focuses on the use of LBN.

In rodents LBN has been associated with a broad range of structural and functional alterations across emotional, cognitive, and stress-related systems (Walker et al., 2017).One of the most prominent consequences of LBN observed in mice and rats is its impact on anxiety, with LBN having been associated with increased anxiety-like behaviours across behavioural assays and in both juvenile and adult rodents (Malter Cohen et al., 2013; Wang et al., 2012). In 3-month-old mice, exposed to LBN from PND2-9, decreased time spent exploring the center arena of the open field and decreased time spent in the illuminated compartment of the light-dark box were observed compared to controls. Another metric of anxiety, novelty-induced hypophagia paradigm, which has been shown to be sensitive to anxiolytic and anxiogenic treatments was also impacted by LBN exposure. Malter et al. observed that a history of LBN increased anxiety-like feeding behaviours in both male and female mice at PND 21, 29, and 63. In rats, a history of LBN was associated with increased anxiety behaviours in the elevated plus maze (EPM) with both sex and age-specific effects. Males but not females exhibited an LBN-associated increase in anxiety-like behaviours,

with LB males, making fewer entries into and spending less time in open arms, compared to controls and LB females (Molle et al. 2013). A similar study of exclusively males also observed an LBN-associated increase in anxiety in the EPM in young adulthood. At 10 weeks of age, males with a history of LBN (PND2-9), exhibited a 20% decrease in entries to the open arms of the maze, compared to controls (Maniam et al., 2016). This LBN-associated increase in anxiety-like behaviour in the EPM, presents in early adulthood, but does not persist into middle-age. At 10-12 months of age, there was no significant differences between male rats reared in normal or limited bedding conditions on time spent exploring open arms in the EPM (Maniam et al., 2016).

While there is robust and consistent evidence of LBN's ability to induce increased anxiety-like behaviours, there is limited work assessing LBN's impact on the structures that underlie these behavioural effects. One of these studies comes from our lab, where we previously demonstrated that LBN was associated with dendritic hypertrophy and increased spine density in the BLA at PND10 and 18-22, exclusively in males. Further, there was a trend toward increased BLA volume in preweaning males exposed to LBN. These structural changes were mirrored by increased evoked synaptic function (fEPSP), measured from BLA neurons *in vitro*, which, like the structural changes, occurred only in males. Unfortunately, this study from our lab represents some of the only work assessing LBN-induced early structural changes in the fear circuitry, as most structural studies of the LBN focus on the hippocampus. Data from the hippocampus does provide support for ELS's ability to induce changes in brain structure both during development and into adulthood. Immediately following LBN in mice (PND9), hippocampal maturation appears to be accelerated, evidenced by an earlier decrease in markers of hippocampal neurogenesis. In late adolescence, LBN-induced volumetric deficits become apparent, but are limited to the dorsal hippocampus (Bath et al., 2016).Concurrently, adolescent rats with a history of LBN displayed disrupted hippocampal microstructure, with significantly decreased dendritic length and complexity, consistent with aberrant patterns of local connectivity observed during this period (Molet et al., 2016). LBN changes are not limited to developmental periods and can induce progressive impairment, as demonstrated by the initiation of adult-onset dendritic atrophy of CA1 pyramidal neurons and the concomitant expansion of mossy fibers in the CA3 in rats (Brunson et al., 2005). Observations in the hippocampus demonstrate LBN's ability to induce qualitative and temporal alterations in brain structure, promoting the functional and behavioural alterations observed following ELS.

1.2 The development of the Fear circuitry

Detection and response to threat, play an important role in the continued survival of an animal and involves the adequate functioning of the corticolimbic circuit in both processing of emotional stimuli and activation of fear responses as well as later fear inhibition and extinction to restore homeostasis (Figure 2). This circuit includes primary nodes such as the amygdala, primarily the basolateral amygdala (BLA), the medial prefrontal cortex (mPFC) and ventral hippocampus (vHipp). The abundance of ELS-induced modifications tied to a dysregulated corticolimbic circuit is posited to arise at least partially, from the temporal overlap of development of the corticolimbic circuit and the timing of ELS. In rodents, the critical period for the establishment of this circuit co-occurs with the time in which ELS models, including LBN, are applied. The BLA receives sensory inputs from the cortex and thalamus through its dorsal area, the lateral amygdala (McDonald, 1998; Turner & Herkenham, 1991). The basal amygdala receives fewer direct sensory inputs but is the source of important reciprocal connections to other regions of the fear circuitry, the prefrontal cortex (PFC) and ventral hippocampus (vHipp) (McDonald, 1998; Senn et al., 2014). The BLA also projects to the central amygdala, which is the main output region of the amygdala. In particular, the CeA projects to a "relay" structure in the BNST, which then influences the activity of the HPA axis, an important player in determining responses to stressors(McDonald, 1982a). Reciprocal BLA connections with the prelimbic (PL) and infralimbic (IL) regions of the mPFC are most important to regulate fear acquisition and extinction, respectively (Krabbe et al., 2018). The basolateral amygdala is composed of 80% glutamatergic principal neurons and 20% GABAergic interneurons (McDonald, 1982b; McDonald & Augustine, 1993). Excitatory BLA principal neurons, as the main sites of synaptic plasticity in fear learning, are considered critical to fear conditioning although BLA interneurons also have been shown to play an important role (Szinyei et al., 2007).

While the developmental timing of corticolimbic circuit development overlaps with exposure to ELS, imparting risk for altered circuit development, distinct aspects and connections within the circuit have further minute differences in their developmental timeline. This is notable as processes of fear conditioning and extinction occur along distinct pathways and connections, presenting the possibility of differing sensitivities to ELS. While fear conditioning is dependent primarily on BLA to PL mPFC pathways and vHipp to PL pathways, fear extinction is mediated by BLA-IL mPFC connections, vHipp inputs, alongside regional crosstalk between the IL- and

PL-PFC and vHipp (Burgos-Robles et al., 2017). Ascending BLA to PL mPFC pathways responsible for fear conditioning mature earlier, reaching adult-like laminar distribution by PND11 (Bouwmeester et al., 2002). Whereas descending projections from the mPFC to BLA responsible for fear extinction are not mature until PND13 (Bouwmeester et al., 2002). The period of development and maturation of ascending BLA-mPFC pathways is therefore more susceptible to ELS, as their development coincides with the period of LBN induced ELS (PND1-10). Further, the maturation of these connections coincides with the onset of fear learning in pups around PND10 (Moriceau et al., 2009; Moriceau & Sullivan, 2006; Sullivan et al., 2000; Sullivan & Holman, 2010). The sensitivity of these connections to ELS may also be influenced by sex, as their developmental period coincides with the peak period for the effects of gonadal steroids on sexual differentiation of the brain (McCarthy et al., 2017). Taken together the specific alterations observed following ELS rely on varying temporal factors related to the development of the corticolimbic circuits.

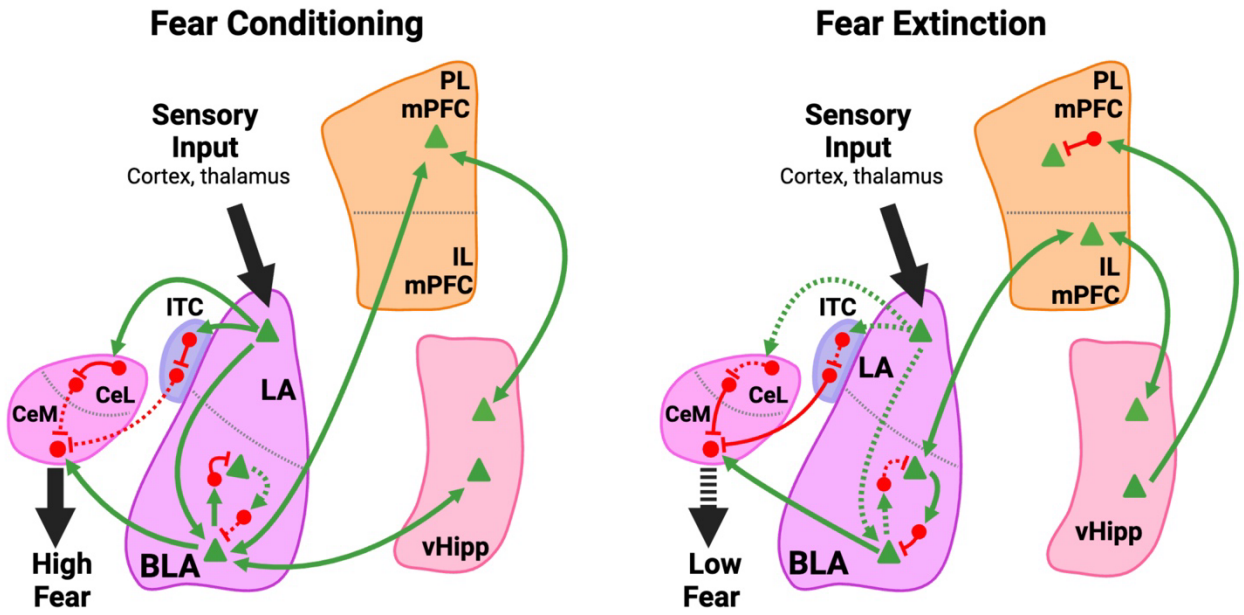

Figure 2. Amygdala and corticolimbic circuitry implicated in A) fear conditioning and B) fear extinction. Glutamatergic neurons are represented by green triangle, GABAergic neurons are represented by red circles. Dotted and solid lines represent weakened and strengthened connections, respectively. BLA, basolateral amygdala; CeL, lateral division of central amygdala; CeM, medial division of central amygdala; IL mPFC, infralimbic medial prefrontal cortex; ITC, intercalated cell masses; LA, lateral amygdala; PL mPFC, prelimbic medial prefrontal cortex; vHipp, ventral hippocampus. Figure adapted from Guadagno et al. (2021) and created with BioRender.com.

1.3 Mechanisms of sex differences: the role of early gonadal steroids

The classical hypothesis for sexual differentiation of the developing brain and the resulting sex-differentiated behavior focuses primarily on the direct role of gonadal hormones perinatally although many extragonadal factors also impact the sexually dimorphic development of the brain. These factors include direct effects of the sex chromosome complement on differential gene expression, differences in both the nature and extent of epigenetic regulation, and microglial influences resulting in alterations in synapse formation and pruning (McCarthy, 2020).

The effects of gonadal steroids during critical developmental periods, are known as organizational effects, as they induce lasting changes in the organization of neural circuits, promoting sex-typical responses to stimuli. The determination of which sex-typical organization is produced arises in part from differences in the gonadal steroid presence and signalling during critical periods in early life. In males the gonads are active, with the testes producing two surges in testosterone, while in females, the ovaries remain quiescent with no gonadal hormone secretion during the neonatal period promoting a female organization. Although they produce androgens, the testes exert their hormonal masculinizing effects on the brain primarily through the activation of estrogen receptors, requiring the conversion, or aromatization, of testosterone into estradiol (E2) by aromatase within the brain. Inhibition of aromatase, and therefore the inability to convert testosterone into E2, could result in the absence of masculinization of certain tissues, despite functioning testes and sufficient androgen production. As aromatase is an essential first step in the induction of masculinization, regions rich in aromatase and estrogen receptors are more likely targets of gonadal steroid induced masculinization.

1.3.1 Estrogen Receptors

In order to impart its primarily masculinizing influences, estrogen must bind to receptors in the tissue of interest. There are two predominant types of estrogen receptors, classical nuclear estrogen receptors (ER), either alpha or beta, and G-protein coupled receptors (GPER). These ER and GPER receptors vary in their cellular localization, signalling pathways, and time required for their effects. GPERs are localized primarily to the plasma membrane but can also be found in the endoplasmic reticulum. As the name implies, GPER signalling occurs through multiple G proteins and regulates ion channels, resulting in rapid nongenomic effects such as the generation of second messengers, including calcium ions and nitric oxide, as well as the activation of tyrosine kinase

and protein-lipid kinase pathways (Prossnitz & Barton, 2023). GPER activation in neurons has been associated with cellular signalling that promotes neuronal survival, while other cellular consequences include cellular adhesion, migration, and proliferation (Roque & Baltazar, 2019).

Additionally, through its various signalling cascades, GPER activation exhibits indirect regulation of gene expression, upregulating the expression of genes including *c-fos, cyclin D2* and *Bcl-2* (Albanito et al., 2015; Madeo & Maggiolini, 2010). In contrast, classical nuclear estrogen receptors (ER) are members of the nuclear receptor superfamily and function as transcription factors (Kumar & Thompson, 1999). Divided into two subtypes, ERα and ERβ, who share similar sequence homology and mechanism of action, they differ primarily in their physiological functions as well as their patterns of expression and distribution in tissue (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). In their unbound state, unlike many other nuclear receptors, ERs are predominantly (~95%) found in the nucleus, with the remaining in the cytoplasm and requiring translocation upon activation (Hager et al., 2000). Following estrogen binding, the receptors dimerize and bind to estrogen response elements on promoter regions of DNA, initiating transcriptional activation (Klinge, 2001). Alternatively, activated receptors can indirectly influence transcription through protein-protein interactions with other transcription factors in the nucleus (Klinge, 2001). Considering they function through the modulation of transcription, rather than through rapid cellular cascades, the biological effects of ERs are much slower than those of GPERs (Kumar & Thompson, 1999). While all three of these estrogen receptor types are present in the adult BLA of both sexes, ERα is the primary subtype found in the rat BLA, while ERβ is the primary subtype found in the central amygdala (Österlund et al., 1998). Because of its prominence in the BLA, ERα, represents a likely target for estrogen-mediated effects of ELS.

1.3.2. Estradiol (E2)-mediated sex dimorphism in brain regions linked to reproductive behavior

Estradiol exerts organizational effects across the brain but has been most studied in regions responsible for sex-typical copulatory behaviours, such as the medial preoptic area (mPOA) and ventromedial nucleus of the hypothalamus (VMH). In the developing mPOA and VMH perinatal estradiol is a key mediator in the development of masculinized and defeminized copulatory behaviours, respectively. In the mPOA E2 exposure during the critical organizational period decreases the rate of apoptosis, resulting in males having higher neuronal density and greater mPOA volume despite having similar rates of neuronal proliferation as females (Arai et al., 1996). Treatment of neonatal females with exogenous estradiol is sufficient to equalize these sex-specific

volumetric differences in the mPOA (Arai et al., 1996). In addition to volumetric differences, E2 also plays a role in modulating synaptogenesis in the mPOA. The VMH is similarly impacted by E2, however the presence of E2 acts to defeminize behaviour rather that masculinize in this region important in female copulatory behaviours. Reducing the presence of E2 in early life can induce sex-atypical behaviours, such as the presence of lordosis in adulthood in neonatally castrated males (Brown-Grant, 1975; Feder & Whalen, 1965; Gladue & Clemens, 1978; Gladue & Clemens, 1982). While these areas have been extensively studied due to their reproductive significance, E2's organizational effects may affect other brain regions and behavioural outcomes including fear behaviour.

1.3.3. E2 and sexual dimorphism in the fear circuitry

Unlike the highly sexually dimorphic medial amygdala (MeA), the BLA shows little effect of sex until adulthood in the rat. Up to PND90, there are no observed sex differences in volume or neuronal number in the BLA (Rubinow et al., 2009). Further, there is no significant effect of sex on the morphology of pyramidal neurons in the healthy BLA in the first postnatal month (Ehrlich et al., 2012). Only in adulthood do sex differences emerge, with adult males having higher spine density on pyramidal BLA neurons, compared to females. This effect appears to be mediated by testosterone, and not estradiol, in males, as the administration of an aromatase inhibitor, letrozole, does not impact spine density in adult males (Bender et al., 2017). This suggests that sex differences in spine density in the adult BLA arises from activational rather than organizational gonadal steroid effects. However, there does appear to be some organizational effects of gonadal steroids on adult dopamine content in the BLA since neonatal castration abolishes this sex difference.

Despite a lack of current evidence suggesting sex differences in BLA volume and neuronal morphology prior to adulthood in normal conditions, sex does appear to factor into BLA development in the presence of stress. In the limited bedding model, we previously showed that ELS differentially impacts BLA morphology and synaptic responses exclusively in neonatal males (Guadagno et al., 2018), suggesting that the developing male BLA might possess some pre-existing vulnerability to ELS. Although various aspects of sex contribute to the formation of distinct masculine and feminine brains, the timing of the gonadal steroid surge makes it a likely candidate for this sexually dimorphic response to ELS. The temporal overlap of chronic LB-induced stress with the peak of E2-mediated neuronal masculinization suggests that gonadal steroids may act as

the central mediator in the sensitivity of males to ELS, wherein the convergence of hormones and stress result in changes to the fear circuitry not seen in healthy animals. In this work, we will specifically examine the interaction between E2 and ELS on BLA structure.

1.4 Alternate Factors affecting Sexual Dimorphism of the Brain

Although gonadal steroids are critical for sexual differentiation of the developing brain, sexual dimorphism in brain structure and function arises from additional factors including direct genetic effects of the sex chromosome complement (Arnold & Chen, 2009), epigenetic regulation, and microglial influences that affect synapse formation and pruning (Lenz & McCarthy, 2015).

Sex chromosomes play a role in the sexually dimorphic development of the brain, both directly through the expression of specific gene and indirectly through the role of the Y-linked SRY gene, important for gonadal differentiation and development of the male phenotype. Importantly, the sex chromosome complement can induce sex differences independent of gonadal differentiation as demonstrated using the Four-Core Genotypes (FCG) and XY* models in which the direct chromosomal influences can be separated from those arising indirectly from gonadal action (Arnold & Chen, 2009). One such use of the FCG model found that aromatase immunoreactivity in the amygdala of XY mice was greater than observed in XX mice on gestational day 15 (GD15), providing evidence of isolated chromosomal regulation of aromatase expression in prenatal life (Cisternas et al., 2015). In addition to the ability to isolate specific chromosomal or gonadal effect, by experimentally manipulating the gonadal steroid milieu in XX and XY mice, the FCG model can also be used to assess how differences in chromosomal regulation helps to shape sexual dimorphic responses to the gonadal steroids. This same group reported that XX, but noy XY, neurons derived from the amygdalae of E16 FCG mice, exhibited increased aromatase expression following the application of gonadal steroids in culture, indicating the chromosomal complement works in concert with gonadal steroids to shape sex differences in aromatase expression in early life (Cisternas et al., 2015). Other studies have reported that ERβ expression is influenced by the SRY chromosomal complement, with XY cells having higher expression, suggesting that chromosomal complement can alter both how much estradiol is present and the availability of its receptors.

Together with gonadal steroid production, sex differences in developmental programing and behavior may arise through epigenetic alterations in early life. Environmentally controlled

epigenetic regulation via DNA methylation, histone acetylation or the production of microRNAs can lead to the induction or suppression of gene expression. Epigenetic regulation has previously been tied to sex-specific rearing behaviours, such as preferential grooming of male offspring by the mother (Edelmann & Auger, 2011; Gegenhuber et al., 2022; Nugent et al., 2015). Indeed, preferential anogenital licking of male offspring has been associated with increased methylation of the ERα promoter and reduced ERα expression in the developing amygdala of males (Edelmann & Auger, 2011)

Microglia through their roles on synapse formation and pruning act as critical sculptors of developing neural networks. Sex differences in microglia therefore present an opportunity for sexually dimorphic neural development, including the production of sex-specific vulnerabilities to ELS. Further, as the bulk of microglial colonization of the brain occurs prenatally, sex differences in synaptic patterning may exist prior to the onset of ELS, allowing differential response and therefore vulnerabilities to these stimuli (Schwarz et al., 2012). Sex differences in microglia colonization have been observed across the brain, in both areas important for patterning sex-typical reproductive behaviours, such as the preoptic area (POA), and non-reproductive regions, such as the hippocampus, parietal cortex, and amygdala (Lenz et al., 2013). Many of the same areas that observe sex difference in colonization also exhibit sex differences in morphology. By PND4, sexual dimorphic patterns of microglia morphology can be observed in the parietal cortex, hippocampus, and amygdala. As microglia morphology is tied to function and activity state, these sex differences in microglia morphology contribute to those seen in the development of the surrounding environment (Vidal-Itriago et al., 2022). Work in the POA has shown that altering the microglial state is sufficient to alter the sex typical development and synaptic patterning. During the critical period of sexual differentiation of the POA, both the total number of microglia and the number in the active morphological state is significantly greater in males. Considering microglia are implicated in regulation of spines density and synaptic pruning, inhibition of microglia in the POA during this critical period, blocks the masculinization of dendritic spine density resulting in the absence of male-typical reproductive behaviour in adulthood (Lenz et al., 2013). While the influence of sex on microglia number and morphology in the prenatal BLA has yet to be explored, significant sex differences have been observed in the broader amygdala, however the dynamic of these sex differences vary across development (Schwarz et al., 2012). The interaction between sex and microglia morphology is especially dynamic in the developing amygdala, as females exhibit

greater ameboid microglia at birth, with the sex effect reversing at PND4 and again at PND30 (Schwarz et al., 2012). In adults, no sex differences in amygdala microglia were found. Considering the distinct characteristics of individual amygdala subregions and the dynamic interactions between age and microglia, with the data currently available, it is difficult to make any inferences on the impact of sex on microglial colonization and morphology in the developing BLA.

In addition to the direct influence microglia have on neuronal circuits, they may also induce sex differences through differences in the production of inflammatory mediators such as cytokines and chemokines. Indeed, sex differences in the concentration of these factors have been observed in the developing hippocampus, amygdala, and cortex (Schwarz et al., 2012). Notably, the release of these factors from microglia in the POA have been associated with morphological alterations in this region, notably increased spine number and stability, similar to the changes witnessed in the BLA of males following ELS (Lenz et al., 2012).

1.5 Summary

Being exposed to early life adversity has long been associated with psychopathology and adverse emotional outcomes in humans and has been modelled in animal work using rodents and primates. Following ELS, rats display anxiety-like behaviour, with changes noted in the corticolimbic circuit structures that underlie emotional and stress responses, most notably the BLA. However, similar to human psychopathology, vulnerability to ELS-induced dysfunction has been shown to be sexually dimorphic. The creation of a sexually dimorphic brain and related behaviors is contributed by factors including the sex chromosomes present, microglial influence, differential epigenetic regulation, and the perinatal gonadal steroid milieu. As gonadal steroids peak in early life along the same time as ELS exposure and have been demonstrated to be essential in the sex-typical development of highly sexually dimorphic regions, they represented a likely starting point for the study of the sex difference in ELS response, focusing in this particular study, on the masculinizing influence of estrogens during the early postnatal period.

2. Specific Aims

Previous data from our lab have demonstrated a male-specific alteration in the morphology of BLA neurons following ELS in neonatal rat pups, however the cause for this sex difference has yet to be elucidated. One potential factor responsible for the increased susceptibility to ELS in males might be related to the conversion of high testosterone secretion into estradiol in the perinatal period, which has masculinizing effects in the brain. Alternatively, the lack of ELS-induced morphological changes in the BLA of females might be caused by extremely low estrogen levels perinatally. To modify the sex steroid milieu in the early postnatal period, we will block estradiol production in males with administration of an aromatase inhibitor, Letrozole and we will provide high doses of exogenous estradiol to females via injections of estradiol benzoate (EB). Aim 1 seeks to examine the interaction between estradiol signalling in the BLA and sex-differences in ELS susceptibility to induce morphological changes in the BLA. Aim 2 investigates whether the early gonadal steroid milieu interacts with ELS to induce long-term behavioral alterations in the adult offspring.

Aim 1: Determine whether the early gonadal steroid milieu is important to determine sex-differences in the effects of ELS on morphological characteristics of amygdala neurons and whether these effects are mediated by changes in the expression of estrogen receptors in this structure.

Aim 1a—*Determine whether there are sex-differences in ER-alpha expression in the BLA in early life and whether ER-alpha expression is impacted by early life stress and/or postnatal estradiol treatment.*

In this first sub-aim, we will use immunohistochemistry to examine the expression and density of ERα in neonatal rats from litters raised under either normal (NB) or limited (LB) bedding conditions following neonatal injection of either control or treatment targeted at sex-specific estradiol signalling. We will examine the interaction between rearing conditions and gonadal steroid milieu on ERα expression in the BLA.

Aim 1b—*Determine whether altering the early gonadal steroid milieu in males and females is sufficient to restore sex-specific morphological changes in the BLA following early life stress.*

In this second sub-aim, we will determine whether estradiol exposure during the first week of life is sufficient to promote a masculinized morphological response to ELS in females, including increased spine density and number in BLA neurons. Alternatively, inhibiting the conversion of testosterone into estradiol, using an aromatase inhibitor might induce a more feminized morphological phenotype in male BLA neurons. To investigate changes in BLA morphology, we will use reconstructed neurons in Golgi-stained brain sections, measuring changes in neuron length, spine density, and neuronal branching.

Aim 2: *Determine the effects of perinatal estradiol on adult behavior in rats exposed to early life stress.*

The second aim of this project will build on the morphological work of aim 1, by investigating whether the interaction between ELS and sex steroid milieu induces, at least in part, the lasting behavioural changes previously observed in males exposed to ELS. Using the same ELS and hormonal treatments as in aim 1, we will examine adult behavioral responses (PND68 onward) in several tests previously shown to be linked to stress and emotional circuits. These include the open field test, novel object recognition, and social interaction tests, as well as operant fear conditioning and fear extinction.

3. Methods

3.1. Animals

Pregnant female Sprague Dawley rats (Charles River, Kingston) were received on gestation day (GD) 14 and housed on a 12hr light: 12hr light cycle with *ad libitum* access to water and rat chow. Parturition day is considered postnatal day (PND) 0. On PND1, litters were culled to 10-12 pups, maintaining an equal male to female ratio when possible. Pups and dams were weighed at PND 1, 3, 7, 10, and 21. Pups kept for adult behaviour were weaned and moved to housing with same sex littermates at PND21. All procedures were approved by the University Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council of Animal Care.

3.2 Early life stress: Limited Bedding paradigm

On PND1, litters were randomly assigned to either limited (LB) or normal (NB) bedding condition, which was maintained until PND10. Litters assigned to the LB condition were placed on a metal grid above 1 inch of sawdust and are provided with one-half of a sheet of paper towel for nesting material. Litters assigned to NB, were placed directly on the 1 inch of sawdust bedding and are also provided one-half sheet of paper towel. Cages were changed on PND1, 4, and 10 when all litters were returned to NB conditions. To measure changes in maternal behaviour induced by the bedding condition, we recorded maternal behaviour over a 24-hour period between PND5-6. We then analysed two one-hour periods from each light phase for time spent nursing, pup- and self-grooming, and frequency of behavioural fragmentation. A total of 24 litters were used in these studies.

3.3 Hormonal Manipulations

In order to modulate the sex steroid milieu during the sensitive period of early postnatal life, we injected estradiol benzoate (EB) in female pups to masculinize their brain and an aromatase inhibitor, Letrozole (Let), in males to prevent conversion of testosterone into estradiol and thus, reducing the impact of the postnatal testosterone surge on brain masculinization. We waited a minimum of 12 hours following birth prior to initial handling and administration of the first injection, to minimize interference with early dam-pup bonding. The second injection was administered 48 hours later. As most births occurred in the afternoon or evening, injections

occurred on PND1 and PND3. In females, 0.05 mL of EB (0.1 mg EB/rat; stock solution: 2 mg EB/mL sesame oil) was administered subcutaneously to induce brain masculinization. As EB is dissolved in oil , control female pups received 0.05 mL of sesame oil, a neutral oil, subcutaneously, to control for injection stress. Male pups received 0.05 mL of the aromatase inhibitor letrozole (1 mg/kg BW) intraperitoneally, to block the ER effects of the postnatal surge in testosterone present in males. Male controls received I.P. injections of 0.05 mL Vehicle (5% Tween 80, ethanol 5% ion Saline) to control for the injection stress. In some litters, pups of each sex and bedding group were not injected (naïve) to control for the stress of the injection. However, we found no significant differences between naïve and control pups for our endpoints and therefore, we removed the naïve group in subsequent litters.

3.4 Tissue collection and allocation

A total of 90 pups from 19 different litters were sacrificed at two distinct time points, either PND10 or PND21 for immunohistochemistry or morphological analysis, respectively. For immunohistochemistry, PND10 pups (n=3-5 of each treatment group) were anesthetized with a ketamine-xylazine cocktail (0.1 mL, subcutaneous; 5 mg ketamine, 0.5 mg xylazine in PBS) and transcardially perfused with 0.9% saline-heparin (5 USP units/mL heparin; 5min) followed by 4% Paraformaldehyde (PFA, 20 mins). Following perfusion, brains were collected and kept in 4% PFA at 4^{o} C overnight. The following day, brains were placed in 30% sucrose in distilled water solution and maintained at 4° C until dehydrated (1-2 days) and then frozen and stored at -80° C prior to slicing for immunohistochemistry. Tissue for morphological analysis were collected on PND21 (n=6-10/group) and prepared for Golgi staining. In this case, pups were perfused with ice-cold 0.9% saline-heparin solution (5 min). Brains were collected and kept in 20% Golgi-Cox solution (35 mM $K_2Cr_2O_7$; 38 mM $HgCl_2$, and 43 mM K_2CrO_4 in dH_2O) and maintained in the dark at room temperature for 6-8 hours. After the initial 6-8 hours in the solution, the original Golgi-Cox solution is drained and replaced with fresh solution and tissues are left in the dark at room temperature overnight. The following day, the Golgi-Cox solution is replaced for the second time and tissues are kept in the dark at room temperature for 14 days. Tissues were then transferred into 30% sucrose (tissue protectant) and kept at 4° C for 2 weeks prior to sectioning and developing.

3.5 Detection of ERα by Fluorescent Immunohistochemistry

Antibody Selection

For the detection of ERα in the brain, we utilized a rabbit polyclonal antibody against Estrogen Receptor alpha (PA1-309) purchased from Invitrogen. Fluorescent visualization of ERα was achieved by goat anti-rabbit Alexa 488. The primary antibody was selected as it has been widely used in brain tissue, including in rats (Sakuma et al., 2008). We validated the specificity of the primary antibody by performing the ICC with the omission of the primary antibody. The lack of fluorescent signal from the sections treated with all subsequent steps to the primary antibody addition demonstrated that there was no non-specific signal that could be detected by the secondary antibody. (Figure 13B).

Immunohistochemistry

PFA-perfused brains of PND10 pups were sliced on a cryostat in 20 μm sections collected in micro-tubes and frozen. Free floating sections containing the basolateral amygdala (BLA) and other control regions such as the MeA and VMH were washed with Phosphate-Buffered Saline (1x PBS, 0.137M NaCl, 0.0027M KCl, and 0.0119M phosphates), and incubated 30 min with a blocking solution of 3% Bovine serum albumin (BSA) in PBS containing 0.1% of Triton X. Sections were then incubated with Anti-ERα antiserum (PA1-309, Invitrogen) diluted 1:500 for 45 minutes at room temperature then overnight at 4°C. After washing in PBS, sections were incubated with secondary antibody, goat anti-rabbit Alexa 488 (1:2000 in BSA solution) for 2 hours in the dark at room temperature. After washing with PBS, sections were mounted on slides and coverslipped with fluoromount with DAPI and left to dry before analysis under the microscope. To determine the relative abundance of ERα in the BLA, the ERα levels in two control regions known for their abundant ERα expression, the medial amygdala (MeA) and ventromedial hypothalamus (VMH), were also measured. A subgroup of NB-reared animals, either male or females (n=3 per injection group) totalling 12 animals, were selected to undergo separate immunostaining of control regions in addition to that of the BLA. Brain sections containing the MeA and VMH, were immunostained for ERα following the same protocol used for BLA-containing sections.

Microscopy and cell quantification

Images of the IHC stained sections were captured as Z-stacks at 20x magnification on an Olympus BX63 automated fluorescence microscope. Multi-area time lapse imaging was used to acquire tiled images of the entire BLA region bilaterally. Detection and exposure time remained consistent across animals.

For quantification of single IHC for ERα, we used ImageJ to convert z-stacks images to z-projections and ERα and DAPI channels were aligned and merged. Cell counts were completed manually in QuPath software. For each region, the BLA, MeA, and VMH, the region was first outlined, with micrometers (μm) as the unit of length. Cells were then manually counted within the outlined area, then converted to cell density using the surface area of the outlined region. Due to the time-consuming nature of manual counting, in place of analyzing every consecutive section for each region of interest, we instead analyzed a curated subset of 3-5 images per region. To accurately measure the entire region of interest, images selected for analysis spanned the entire rostro-caudal dimension of the nucleus with images being selected from equivalent bregma positions across subjects. The individual images selected at each bregma-range were selected according to image and stain clarity, minimizing the presence of abnormal stain characteristics (e.g. bright spots) and poor tissue quality (e.g. tears or breaks in region of interest).

3.6. Morphological analysis of BLA neurons using Golgi Staining

Saline perfused brains for Golgi-staining were sliced into 200μm coronal sections using a vibratome and mounted on 2% gelatin-coated slides. The Golgi-stain was then developed, in a dark fume hood using 100% ammonium hydroxide (40 mins) followed by fixative (Kodak Fix for film) diluted 1:1 in dH_2O (40 mins). Following development, sections were dehydrated in serial alcohol rinses (50%, 70%, 95% and 3x 100%) and cleaned with xylene prior to being coverslipped with Fisher Permount. Slides were left to dry at least 2 weeks at room temperature before morphological analysis.

Golgi-stained neurons were manually traced at 100x using the Neurolucida software (MicroBrightField, Williston, VT) and an Olympus BX51TF microscope with Hamamatsu camera by an experimenter blind to bedding and injection condition. Digital images were captured with a 20X objective. A minimum of three neurons were analyzed per animal (6-10 animals/group). Both branched and Sholl analyses were completed on reconstructed neurons using Neuroexplorer

(MicroBrightField, Williston, VT), to determine total dendritic length, dendritic spine number, and number of branch points, for whole neurons (branched analysis) and as a function of the radial distance from the soma (Sholl analysis). To calculate spine density (spines/μm), the total spine number was divided by the total dendritic length for each neuron.

3.7. Adult Behavioural tests

Behavioural testing was performed on a total of 34 adult rats (PND62-75) from 10 either LB or NB litters. For each bedding condition, 6-7 oil and 7-8 EB female rats were tested and compared to 3 vehicle males. After weaning at PND21, rats were transferred to a reverse 12 h light/12h dark cycle housing room (lights off at 9 AM). Animals were same-sex, group housed and left to grow until adulthood for behavioral testing. Adult rats (PND62-75) were tested sequentially in a battery of behavioural tasks performed during the dark phase of the circadian cycle. Rats were first tested in the open-field for 2 consecutive days, followed by a single testing day for social interaction tests, and cued-fear conditioning on the final testing day. All tests were performed in the dark under red light between 9:00h and 14:00h. Rats were moved to the experimental room and left to acclimate for a minimum of 30 minutes prior to each testing session. All experimental equipment (open field, fear chamber) were cleaned between subjects with Peroxyguard. As hormonal fluctuations throughout the estrous cycle can alter behaviour in cycling females (Marcondes et al., 2001), vaginal smears were taken following the social behaviour test on for determination of the stage of the estrous cycle.

Open field

The open field test is used as a test of anxiety as well as habituation to the field for the subsequent novel object recognition and social behaviour tasks. The open field test is used as a measure of locomotor activity as well as anxiety-like behaviour (Prut & Belzung, 2003; Seibenhener & Wooten, 2015). Animals were placed in an open field (1 m^2) for a period of 5 minutes and their activity was tracked with a video camera placed above the center of the open field.

Social Behaviour

Social interaction represents a widely accepted behavioural assay for anxiety-related behaviours, as administration of anxiogenic drugs is sufficient to reduce social behaviour

(Overstreet et al., 2002). Each adult rat to be tested was marked on the head with a permanent marker for tracking purposes and placed in one corner of the open field arena with a same-sex peripubertal animal (PND33-37) placed in the opposite corner. The animals were then left to interact for 5-minutes and social interactions were video-recorded. Social interaction was manually analyzed and measuring the number of contact bouts and the total time of social contact. For analysis, any approach and initiation of contact by the adult towards to juvenile was considered a contact bout, while total time spent in contact was calculated using a stopwatch and included any close contact between the animals regardless of whether it was initiated by the juvenile or adult. To be considered contact for our use, we required an active interaction, such as sniffing, within close proximity.

Cued fear conditioning, fear extinction

Adult animals were exposed to a protocol of fear conditioning, followed by fear extinction 24hrs later. day. For fear conditioning, animals were placed in operant boxes containing a metal rod floor that can deliver mild electrical shock to the footpads. Fear conditioning used an unconditioned stimulus (US: 0.5 sec electric shock) of varying intensity according to the sex (intensity: 0.5 mA for males, 0.7 mA for females; duration: 0.5 s) that was preceded by and co-terminated with, a tone as the conditioned stimulus (CS: 80 dB tone, duration: 30 sec). On day 1, animals were placed in the chamber for 5 minutes and presented with two habituation tones alone followed by six tone-shock pairings with varying inter-trial intervals (40-240 s). After testing, rats were returned to their home cage. Fear conditioning was measured by the presence of freezing in response to the CS. Freezing is defined as the lack of any non-breathing related movement (Stevenson et al., 2009) and was determined using FreezeScan (Clever Sys Inc., Reston, VA) software.

3.8 Statistical Analysis

Data were analyzed using GraphPad Prism versions 10. Maternal behaviour data were analyzed using two-way mixed design ANOVA with bedding as between-subjects factor and light phase as within-subjects factor. Pup weight gain data was analyzed by two-way ANOVA, with bedding and injection as between-subjects factors. Post-hoc comparisons were computed using Bonferroni's multiple comparisons test. The branched morphological analysis data were analyzed by two-way ANOVA, with bedding and injection as between-subjects factors. Significant interactions were

decomposed with subsequent simple effects tests and post-hoc comparisons were computed using Bonferroni's multiple comparisons test. The Sholl morphological analysis data were analyzed by three-way mixed design ANOVA, with bedding and injection as between-subjects factors, and radial distance from the soma as within-factor. Significant interactions were then decomposed by subsequent simple effects tests. ERα density data from the BLA were analyzed using two-way ANOVA, with bedding and injection as between-subjects factors. Regional ERα density data were analyzed using mixed effects analysis, with region as within-subject factor and injection as between-subjects factor. Significant interactions were decomposed with subsequent simple effects tests and post-hoc comparisons were computed using Bonferroni's multiple comparisons test. Adult behavioural data was analyzed two-way ANOVA, with bedding and injection as between-subject factor, post-hoc comparisons were computed using Bonferroni's multiple comparisons test. Male adult behavioural data was excluded due to limited sample size. Significance was set to $p < 0.05$.

4. Results

In this project we aimed to investigate the role of estradiol signalling in the development of sexually dimorphic vulnerability to ELS. To investigate this, we used treatments that alter signal transduction at ERs, increasing estradiol signalling in females through the administration of EB, and decreasing estradiol signalling in males through treatment with an aromatase-inhibitor, letrozole, in effect masculinizing females and demasculinizing males. Due to the nature of these treatments, the impact of sex and treatment are inextricably linked within our treatment groups (EB-females and Let-males). To account for this, when analyzing the effect of treatment by sex, treatment groups will be contrasted with controls of both sexes (oil-treated females and veh-treated males). Comparisons within the same sex provides a quantification of the treatment effect on the sex-typical response to ELS, while comparison with the opposite sex control provides quantification of the treatment's ability to recreate the sex-typical response to ELS of the opposite sex, and therefore the degree to which sex-differences in estradiol signalling during the early postnatal period mediate the sexual dimorphic response to ELS.

4.1 Effect of bedding on maternal behaviour and pup weight

Analysis of the maternal behaviour recorded between PND5-6 in mothers from all our cohorts is displayed in Figure 3. The behaviours measured were nursing (active and passive), pup grooming, self-grooming, and eating, we also assessed the fragmentation of maternal behaviour. Two-way ANOVA with bedding as between factor and light phase as a within factor showed no significant interaction between bedding and light phase, nor was there a significant effect of bedding across the parameters measured. However, fragmentation (Fig 3A), nursing (Fig 3B), and self-grooming (Fig 3D) showed significant effect of light phase, (Fragmentation: $F(1, 22) = 22.68$" $P < 0.0001$, Nursing: $F(1, 22) = 24.16$, $P < 0.0001$), Self-grooming: $F(1, 22) = 26.91$, $P < 0.0001$). Fragmentation and self-grooming increased in the dark phase, while nursing behaviours were highest in the light phase. Due to the behavioural differences across light and dark phases, we then ran t-tests for the same parameters to assess bedding effect during the light phase. There was no significant effect of bedding during the light phase, however nursing and self-grooming showed a trend toward significant bedding effects (Nursing $t(22)=1.716$, $P = 0.1002$, Self-grooming $t(22)=1.745$, , $P = 0.0949$), with LB mothers showing increased nursing and self-grooming relative to their NB counterparts.

Next, we determined pup weight gain up to PND10 (Fig 4A) or PND21 (Fig 4B). We performed a two-way between factors ANOVA to analyze the effect of treatment and bedding on pup weight gain, at PND10 and PND21, respectively. At PND10, we observed a significant main effect of bedding on pup weight gain from PND1 (Fig 4A; $F(1, 68) = 81.37$, $P < 0.0001$), with lower weight gain in LB-reared pups. We also observed a significant main effect of treatment ($F(3, 68) = 2.756$, $P = 0.0490$), however subsequent pairwise comparisons using Bonferroni's multiple comparisons test failed to find any significant differences between treatment pairs. There was no significant bedding by treatment interaction. At PND21, there was no significant main effect of bedding or treatment, or significant bedding by treatment interaction.

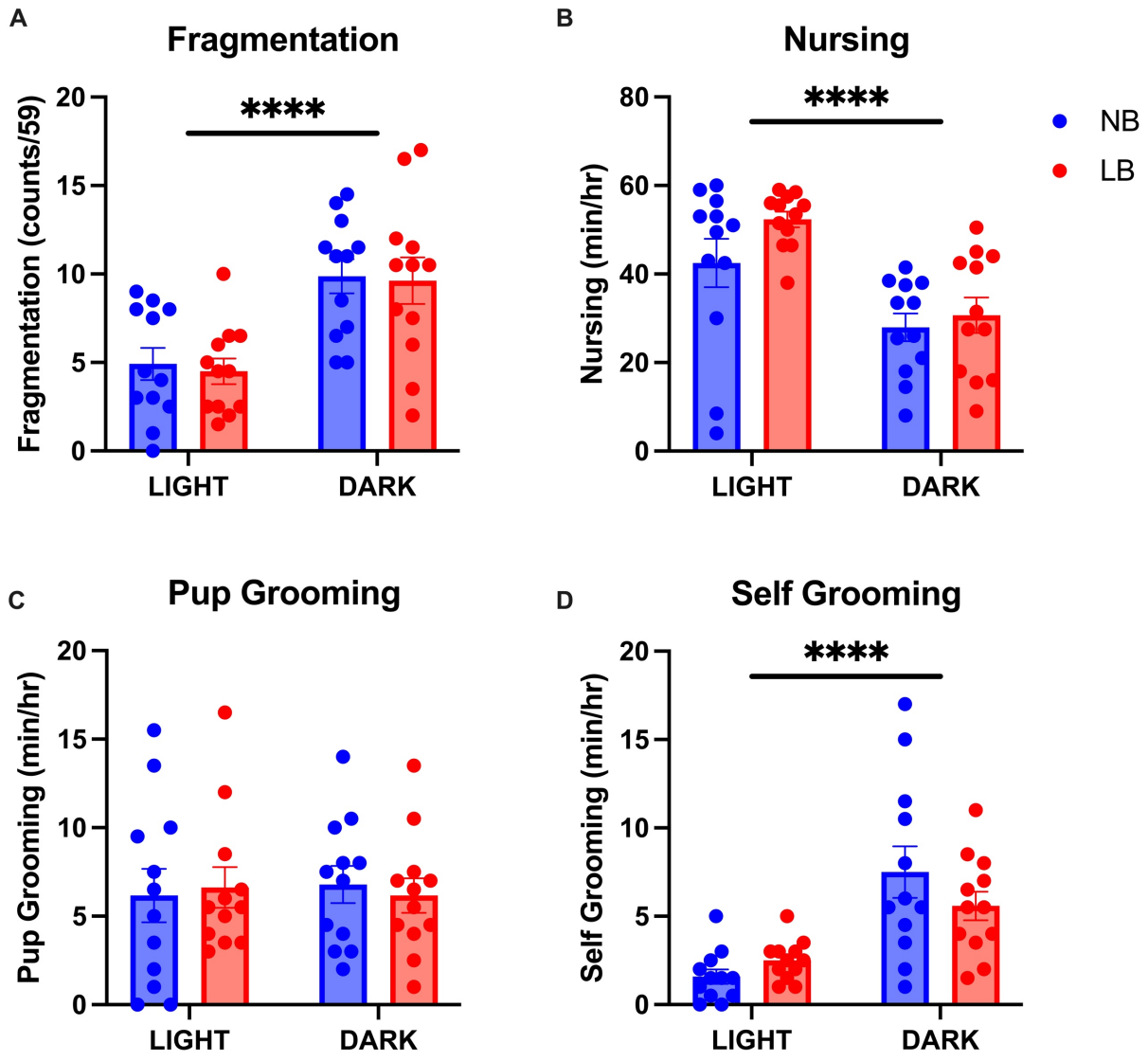

Figure 3. Characteristics of maternal behavior in NB and LB mothers in the light and dark phase of the circadian cycle and across all experimental cohorts (n=12). Maternal behaviour was observed on PND5-6 and scored over 2 one-hour periods in each light condition. A: Fragmentation of behavior, B: Total nursing time, C: pup grooming time, D: self-grooming time. All variables except pup grooming showed significant impact of light phase, but no bedding effect or light by bedding interaction.
Two-way ANOVA. Values represent mean ± SEM of 12 mothers per bedding condition.

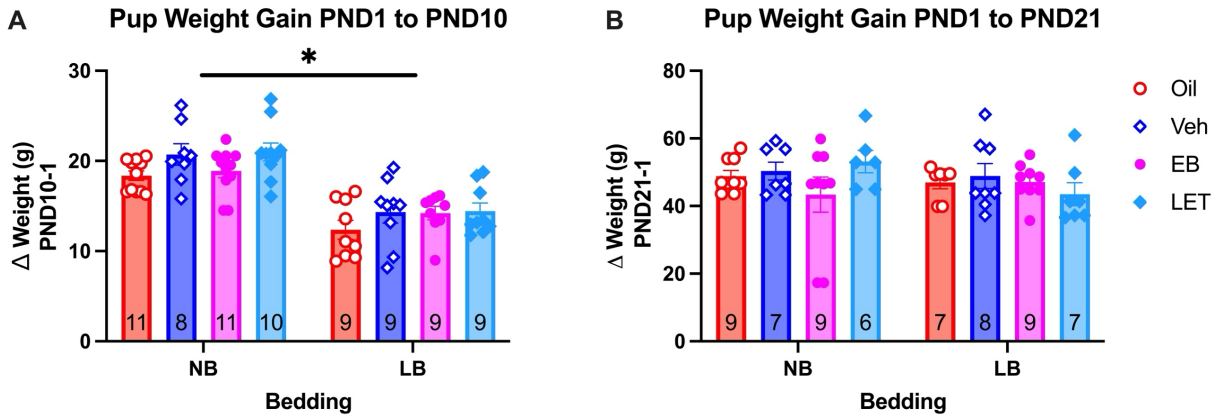

Figure 4. Pup body weight gain as a function of bedding (NB and LB) and neonatal treatment measured on PND10 (A) and PND21 (B). Changes in body weight were calculated as the delta BW between each age and PND1. At PND1 and PND3, males were treated with either Veh or Let and females were treated with either oil or EB. At PND10, but not PND21, there was a significant effect of bedding ($p < 0.0001$), pups reared in LB had significantly lower gains in body weight compared NB pups, regardless of treatment. Similarly, at PND10 but not PND21, there was a significant treatment effect. ($p = 0.0490$) however, subsequent Bonferroni's multiple comparisons test, yielded no significant pairwise differences in treatment effect. There was no significant treatment by bedding interaction at either PND10 or 21.
Two-way ANOVA. Values represent mean ± SEM. of 8-11 pups/group at PND10 and 6-9 pups/group at PND21. The number of samples is indicated within of the bars.

4.2 Effect of sex steroids and bedding on basolateral amygdala (BLA) morphology

We performed both branched and Sholl analysis of Golgi-stained BLA neurons of PND21 pups from either NB or LB mothers and receiving the various neonatal treatments as indicated in the methods section. Representative photographs of Golgi-stained neurons in female pups are presented in Figure 5.

Branched Analysis of BLA Neurons:

We examined four morphological parameters of BLA neurons, dendritic length, branching, total spines, and spine density, comparing both female groups to the control male group in Figure 6 and both male groups to the control female group in Figure 7. For all parameters, we first performed a two-way ANOVA with injection (treatment) and bedding as between-subject factors.

When comparing EB-treated females to controls of both sexes, two-way ANOVA for dendritic length showed no main effects of bedding or treatment, but did yield a significant interaction between bedding and treatment (Fig 6A; $F(2, 40) = 3.677$, $p = 0.0342$). In oil-treated females, Bonferroni post-hoc test showed that LB rearing significantly increased dendritic length relative to NB ($p = 0.0244$). Similarly for branching, two-way ANOVA showed no main effects of bedding or treatment, but a significant interaction between bedding and treatment (Fig 6B; branching: $F(2, 40) = 5.268$, $p = 0.0093$). In oil-treated females, Bonferroni post-hoc test showed that LB rearing significantly increased dendritic branching relative to NB ($p = 0.0117$). In the NB females, EB treatment significantly increased branching compared to Oil ($p = 0.0301$). In the LB condition, oil-treated females exhibited significantly increased branching compared to Veh-treated males ($p = 0.0413$). Total spines (Fig 6C) and spine density (Fig 6D) did not exhibit a significant main effects or interactions.

When comparing letrozole (LET)-treated males to controls of both sexes, two-way ANOVA for dendritic length, showed no significant interaction, but did yield significant main effects for both bedding, with greater length in LB, and treatment (Fig 7A; bedding: $F(1, 39) = 6.333$, $p = 0.0161$, Treatment: $F(2, 39) = 3.381$, $p = 0.0442$). Bonferroni post-hoc test on the main effect of treatment showed a strong trend towards greater dendritic length in oil-treated females compared to Let-treated males ($p = 0.0687$). Two-way ANOVA for dendritic branching showed no main effects of bedding or treatment, but did yield a significant interaction between bedding and treatment (Fig 7B; $F(2, 39) = 5.510$, $p = 0.0078$). In oil-treated females, Bonferroni post-hoc test

showed that LB rearing significantly increased dendritic branching relative to NB ($p = 0.0097$). In the LB condition, oil-treated females exhibited significantly greater branching compared to both Let- and Veh-treated males (Let: $p = 0.0209$; Veh $p = 0.0345$). Two-way ANOVA for spine density showed no significant main effect of treatment nor a significant interaction but did yield a main effect of bedding, wherein spine density was greater in NB (Fig 7D; $F(1, 39) = 7.597$, $p = 0.0088$). Total spines (Fig 7C) did not exhibit a significant main effects or interactions.

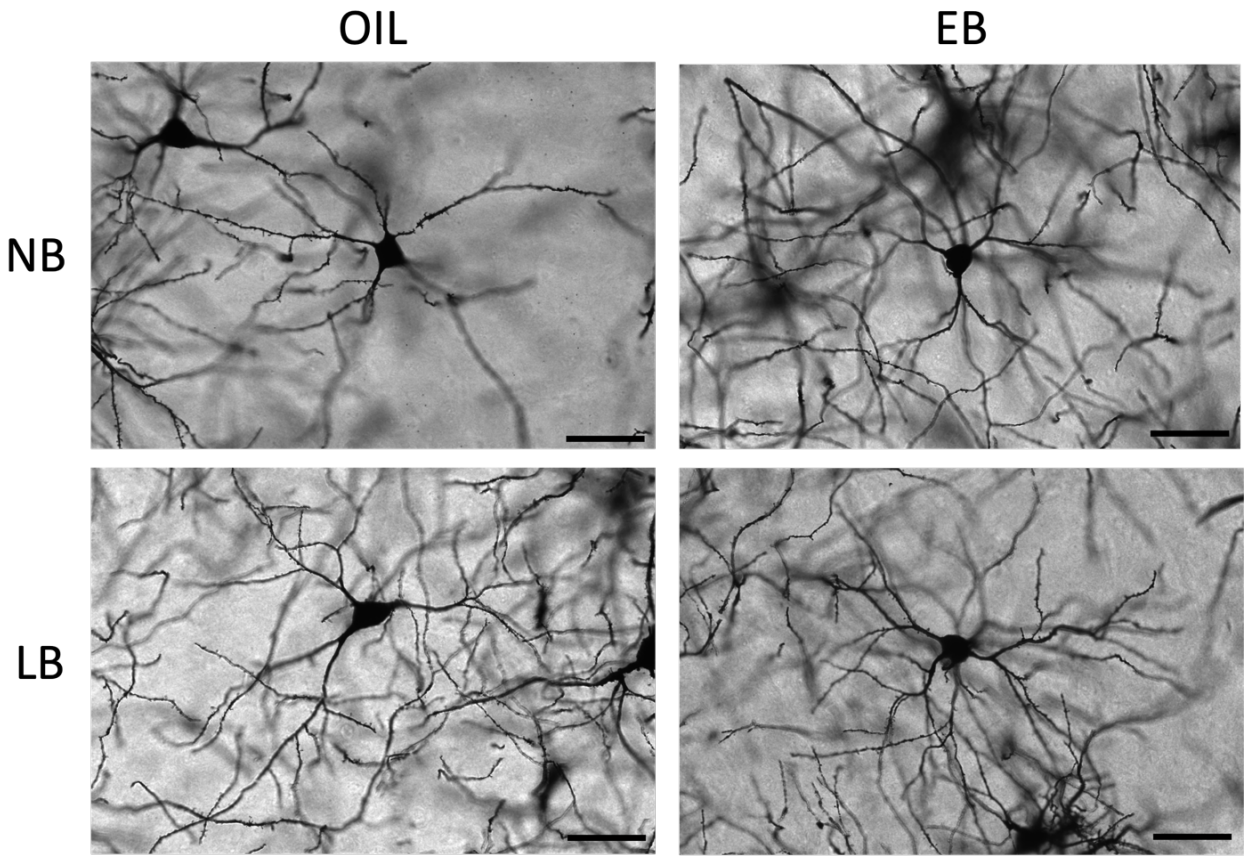

Figure 5. Magnified digital images (40x objective) of Golgi-stained NB and LB neurons of the BLA in Oil- or EB-treated female PND10 pups. Scale bars = 50 μm

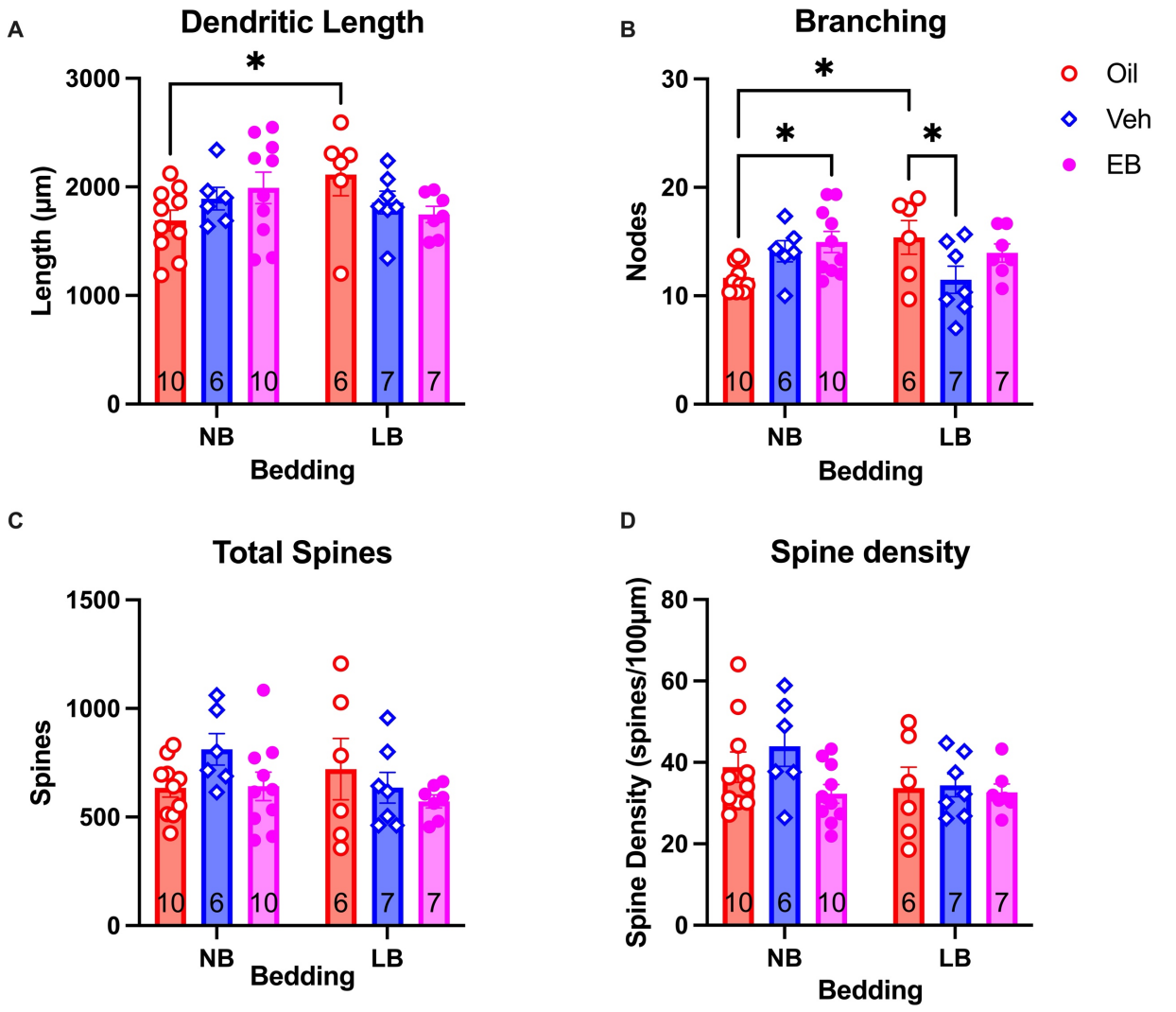

Figure 6. Morphological analysis of BLA neurons in PND21 females (control and EB-treated) and xmale controls (Veh) reared in either normal bedding (NB) or limited bedding (LB) conditions. Variables measured are dendritic length (A), dendritic branching (B), total spines (C) and spine density was calculated as the ratio of spines over length (D). Neither, bedding nor treatment has significant individual effects; however, a significant bedding-treatment interaction was observed in dendritic length ($p = 0.0345$) and branching ($p = 0.0093$). For dendritic branching, Bonferroni's multiple comparisons tests observed three significant interactions between bedding and treatment groups. In NB, but not LB reared animals, dendritic branching was significantly greater in females treated with EB than control females ($p = 0.0301$). In LB, but not NB reared animals, dendritic branching was significantly greater in female controls compared to male controls ($p = 0.0413$). Control females were the only group to show a significant difference across bedding conditions, with significantly greater branching in control females reared in LB compared to NB ($p = 0.0117$). Two-way ANOVA. Values represent mean ± SEM of 3 neurons analyzed/animal and 6-10 animals/group (indicated in the bars). *$p < 0.05$

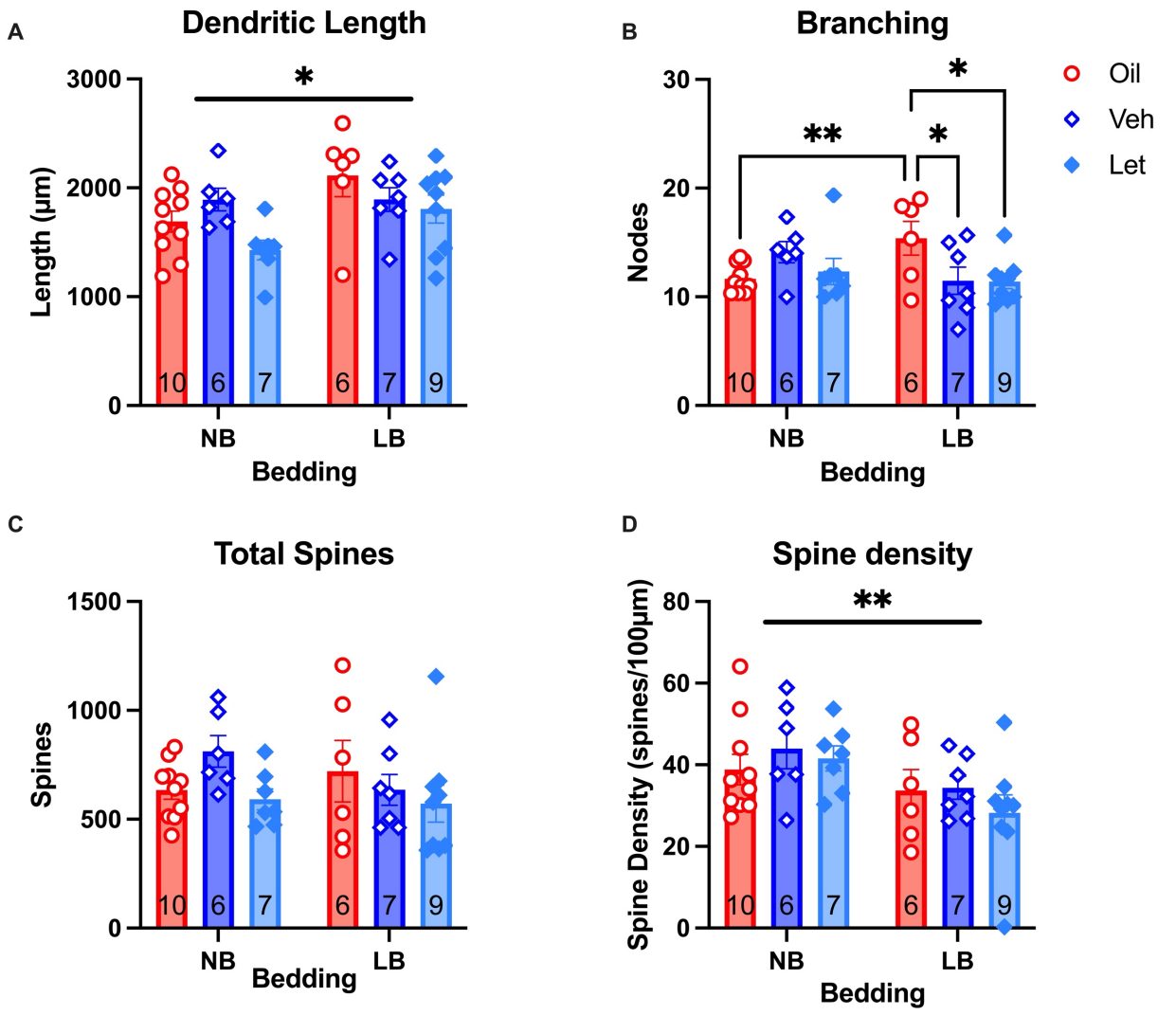

Figure 7. Morphological analysis of BLA neurons in PND21 males (Veh and Let-treated) and female controls (Oil) reared in either normal bedding (NB) or limited bedding (LB) conditions. Variables measured are dendritic length (A), dendritic branching (B), total spines (C) and spine density was calculated as the ratio of spines over length (D). Bedding had a significant effect on dendritic length ($p = 0.0161$), with LB rearing associated with increased dendritic length. There was also a significant effect treatment ($p = 0.0442$) on dendritic length. A significant bedding by treatment interaction was observed on dendritic branching in males ($p = 0.0078$). Further, control females reared in LB exhibited significantly greater branching than those reared in NB ($p = 0.0097$). Total spines showed no significant individual nor interaction effects. However, there was a significant effect of bedding ($p = 0.0088$) on spine density, with NB-rearing being associated with higher spine density.

Two-way ANOVA. Values represent mean ± SEM of 3 neurons analyzed/animal and 6-10 animals/group (indicated in the bars). *$p < 0.05$, **$p < 0.01$

In addition to the above analyses, we also ran two-way ANOVAs between control and treatment within each sex, with bedding and treatment as between-subject factors (Figure 8). In females, two-way ANOVA for dendritic length showed no main effects of bedding or treatment, but a significant interaction between bedding and treatment (Fig 8A; $F(1, 29) = 6.156$, $p = 0.0191$). In oil-treated females, simple main effects test showed that LB rearing significantly increased dendritic length relative to NB ($p = 0.0381$). In NB, we observed a trend towards greater length in EB-treated females ($p = 0.0850$), while the opposite trend was observed in LB ($p = 0.0907$). Similarly, two-way ANOVA for dendritic branching showed no main effects of bedding or treatment, but a significant interaction between bedding and treatment (Fig 8B; $F(1, 29) = 6.278$, $p = 0.0181$). In oil-treated females, simple main effects test showed that LB rearing significantly increased dendritic branching relative to NB ($p = 0.0109$). In NB, EB treatment significantly increases branching ($p = 0.0093$). Total spines (Fig 8C) and spine density (Fig 8D) did not exhibit a significant main effects or interactions.

In males, two-way ANOVA for dendritic length showed no main effects of bedding, but did yield a significant main effect of treatment (Fig 8E; $F(1, 25) = 5.028$, $p = 0.0341$) and a near significant interaction (Fig 8E; $F(1, 25) = 3.215$, $p = 0.0851$). A simple main effects test found LB rearing increased dendritic length in Let-treated males ($p = 0.0221$). In NB, vehicle treatment yielded greater dendritic length ($p = 0.0116$). Two-way ANOVA for dendritic branching showed no significant main effect of treatment nor any significant interaction, however we did observe a trend towards a main of effect of bedding (Fig 8F; $F(1, 25) = 3.034$, $p = 0.0938$), with greater branching in NB conditions. Two-way ANOVA for total spines showed no significant main effect of bedding nor any significant interaction, however we did observe a trend towards a main of effect of treatment (Fig 8G; $F(1, 25) = 3.034$, $p = 0.0938$), with greater total spines in NB conditions. Two-way ANOVA for spine density showed no significant main effect of treatment, nor a significant interaction, but did yield a significant main effect of bedding (Fig 8H; $F(1, 25) = 8.265$, $p = 0.0081$), with greater spine density in NB reared males.

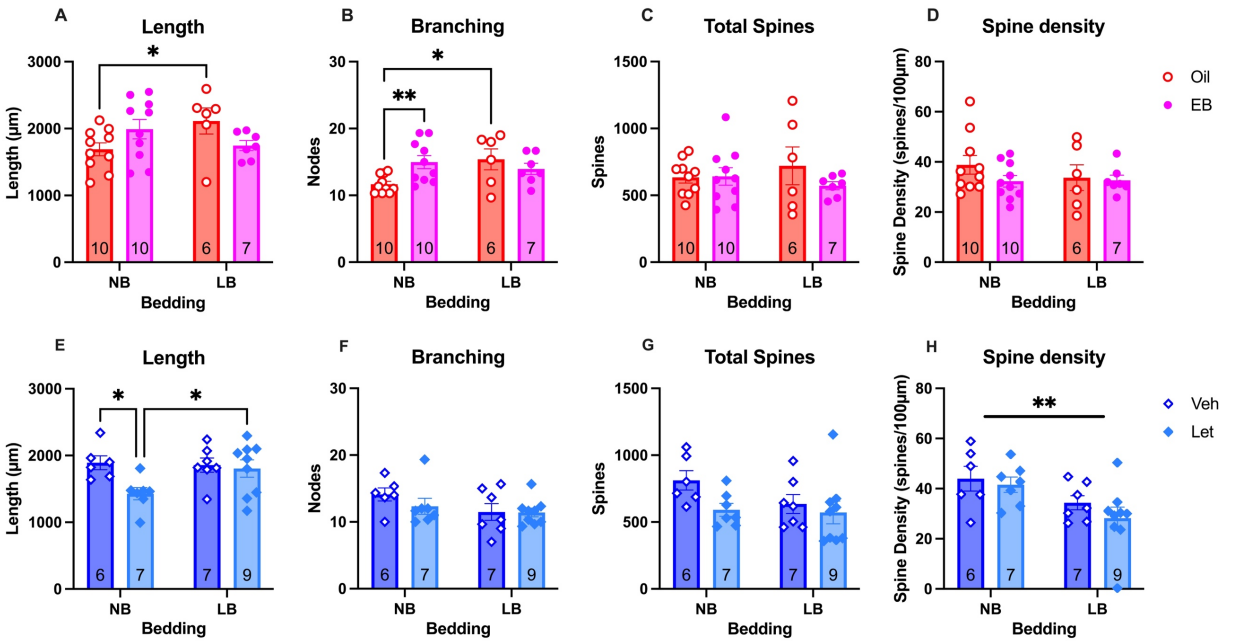

Figure 8. Morphological analysis of BLA neurons in PND21 male and female pups from either normal bedding (NB) or limited bedding (LB) mothers. Comparison in each sex between control and treatment, female pups received either oil or Estradiol benzoate (EB) (panels A-D) while male pups received either vehicle (Veh) or Letrozole (Let) (panels E-H). Variables measured are dendritic length (A, E), dendritic branching (B, F), total spines (C, G) and spine density was calculated as the ratio of spines over length (D, H). There was a significant bedding-treatment interaction observed on dendritic length in females, with post-hoc tests indicating that oil-treated pups reared in LB showed increased dendritic length compared to NB-reared pups (p= 0.0124). There was also a significant bedding-treatment interaction on branching in females (p = 0.0181). Post hoc tests indicated that in NB but not LB bedding, EB-treated females had significantly greater dendritic branching compared to those treated with oil (p = 0.0093). Further, LB-rearing was associated with significantly higher branching than NB-rearing but only in oil-treated females (0.0109). In males, there was a significant treatment effect observed on dendritic length (p=0.0341), and a significant bedding effect on spine density (p = 0.0081). There was no significant bedding by treatment interaction on any morphological parameter measured in males.
Two-way ANOVA. Values represent mean ± SEM of 3 neurons analyzed/animal and 6-10 animals (indicated in the bars).
*p < 0.05, **p < 0.01

Sholl Analysis of BLA Neurons

We performed a Sholl analysis to examine changes in morphological variables as a function of distance from the soma. We used a cutoff of 140 μm from the soma for these analyses, corresponding to the shortest dendritic length in our samples. We first performed a three-way ANOVAs in each sex, with bedding and treatment as between-subject factors and radial distance from soma (radius) as a within-subject factor.

In females, analysis of dendritic length (Fig 9A) and branching (Fig 9C) yielded a significant three-way (bedding x treatment x radial) interaction and spine density (Fig 10C) yielding a near significant interaction (length: $F(13, 377) = 1.768$, $p = 0.0464$; branching: $F(13, 377) = 1.998$, $p = 0.0200$, spine density: $F(13, 377) = 1.627$, $p = 0.0753$). Simple effects tests conducted across radials at each bedding-treatment combination in females, found that radials had a significant effect on all bedding-treatment pairings on both dendritic length and branching ($ps < 0.0001$). Simple effects tests conducted between treatment at each combination of bedding and radial distance, revealed that at certain radials (50, 60, 70, 100 μm) treatment with EB significantly increased dendritic branching in normal bedding reared females ($p = 0.0336$), EB only significantly increased branching relative to oil at 40μm ($p = 0.0084$), neither length or spine density were significantly impacted at any radial distance. Simple effects tests conducted between bedding conditions at each treatment and radial distance combination and revealed that at radials 50, 70 and 130 μm, rearing in LB increased branching relative to NB-rearing, but only in those females treated with oil ($ps < 0.05$), however there was no significant effect on dendritic length or spine density. All morphological characteristics yielded a significant effect of radial distance from the soma (Length: $F(2.879, 83.48) = 123.1$, $p < 0.0001$; branching: $F(6.811, 197.5) = 44.28$, $p < 0.0001$; spines: $F(3.013, 87.39) = 110.9$, $p < 0.0001$; spine density: $F(5.132, 148.8) = 145.9$, $p < 0.0001$)

In males, there was no significant three-way interaction on any of the morphological parameters measured, however, analysis of both dendritic length (Fig 11A) and total spines (Fig 12A) yielded a significant bedding x radial interaction, with a near significant interaction shown in spine density (Fig 12C) (length: $F(13, 325) = 1.859$, $p = 0.0340$; spines: $F(13, 325) = 2.744$, $p = 0.0010$; spine density: $F(13, 325) = 1.735$, $p = 0.0527$). Simple effects tests conducted across radials at each bedding condition, for length, spines, and spine density, yielded significant radial at NB effects ($ps < 0.0001$) and radial at LB effects ($ps < 0.0001$) for length, spines, and spine

density. All morphological characteristics yielded a significant effect of radial distance from the soma (length: $F(3.353, 83.81) = 105.6$, $p < 0.0001$; branching: $F(5.497, 137.4) = 33.48$, $p < 0.0001$, spines: $F(3.223, 80.58) = 94.39$, $p<0.0001$; spine density: $F(6.009, 150.2) = 130.5$, $p < 0.000$)

Branched Analysis with 140 μm Cut-Off

As the Sholl analysis used a cut-off of 140 μm from the soma, we paired this analysis with a corresponding branched analysis using the same 140 μm cut-off. We analyzed this data using two-factor (bedding x treatment) between subject ANOVA for each morphological parameter.

In females, two-way ANOVA for both dendritic length (Fig 9B) and branching (Fig 9D) showed no main effects of bedding or treatment, but did yield near significant interactions between bedding and treatment (length; $F(1, 29) = 3.219$, $p=0.0832$, branching: $F(1, 29) = 3.969$, $p=0.0558$). Post-hoc analysis showed that EB treatment increased both length and branching, but only in NB conditions. A near significant increase in branching was observed in LB, relative to NB conditions, in oil-treated pups. Total spines (Fig 10B) and spine density (Fig 10D) did not exhibit a significant main effects or interactions.

In males, two-way ANOVA for both dendritic length (Fig 11B) showed no main effects of bedding or treatment, but did yield a near significant interaction between bedding and treatment ($F(1, 25) = 3.145$, $p=0.0883$). Post-hoc analysis showed that Let treatment decreased length, but only in NB conditions. Analysis of both total spines (Fig 12B) and spine density (Fig 12D) yielded significant main effects of bedding (spines: $F(1, 24) = 12.08$, $p = 0.0020$; spine density: $F(1, 25) = 9.750$, $p = 0.0045$), with increased spines and spine density in males reared in NB relative to LB reared males. Total spines also yielded a significant main effect of treatment ($F(1, 24) = 6.582$, $p = 0.0170$), with Let-treated males having decreased spines relative to Veh-treated males. Dendritic branching (Fig 11D) showed no significant main effects of bedding, treatment, nor a significant bedding treatment effect.

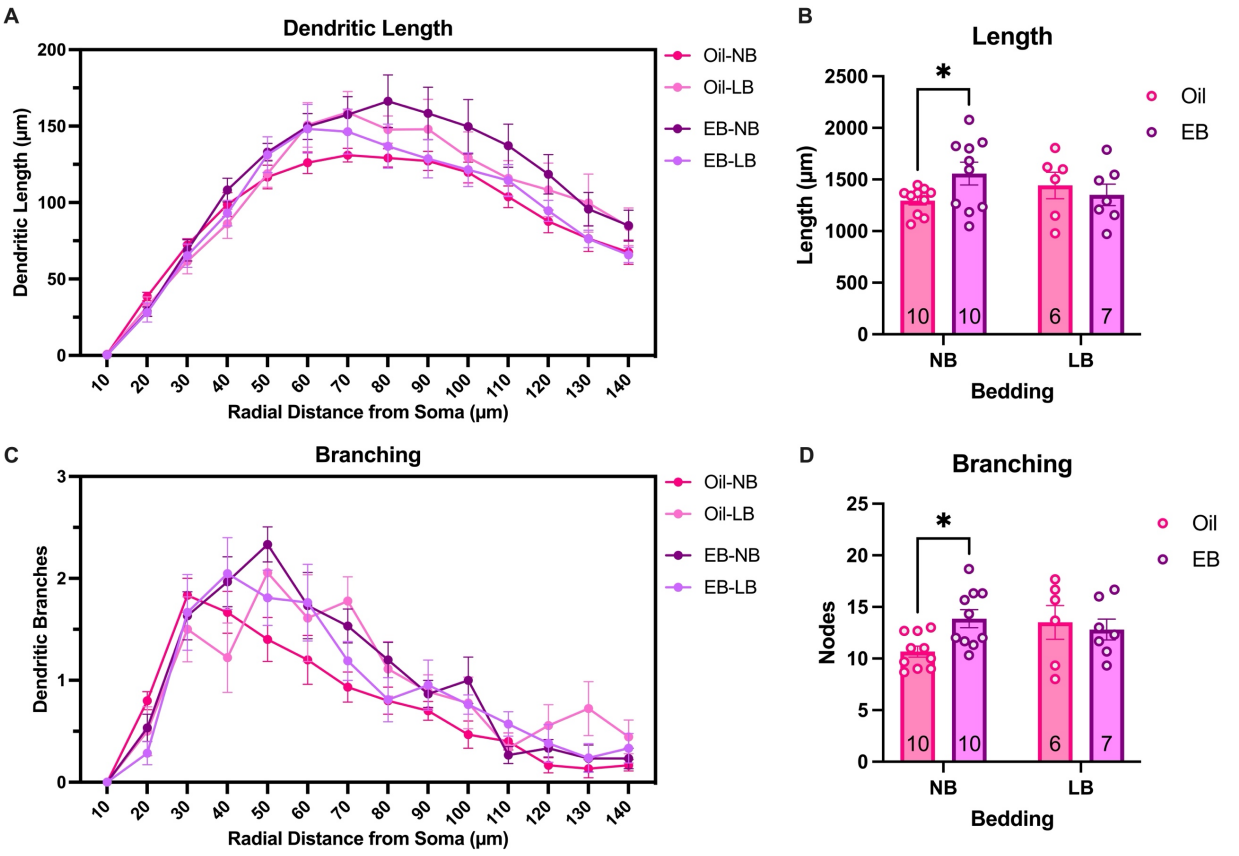

Figure 9. Sholl analysis of the effects of bedding condition and gonadal steroid treatment on the BLA neurons of control (oil) and EB-treated females at PND21 as a function of radial distance from the soma. Two morphological parameters, dendritic length (A, B), and dendritic branching (C, D), were assessed with both sholl analysis (A, C) and "cropped" branched analysis (B, D) assessing only the area within 140µm from the soma. There was no significant effect of bedding or treatment noted across sholl or branched analysis of dendritic length and branching. Sholl analysis of both dendritic length and branching did however find a significant effect of radial distance from soma ($p < 0.0001$). Sholl analysis also found a significant three-way interaction (radius x treatment x bedding) on dendritic length ($p = 0.0464$) and branching ($p = 0.0200$). There were no significant interactions noted in the branched analysis.
A, C: Three-way ANOVA. B, D: Two-way ANOVA. Values represent mean ± SEM of 3 neurons analyzed/animal and 6-10 animals/group.

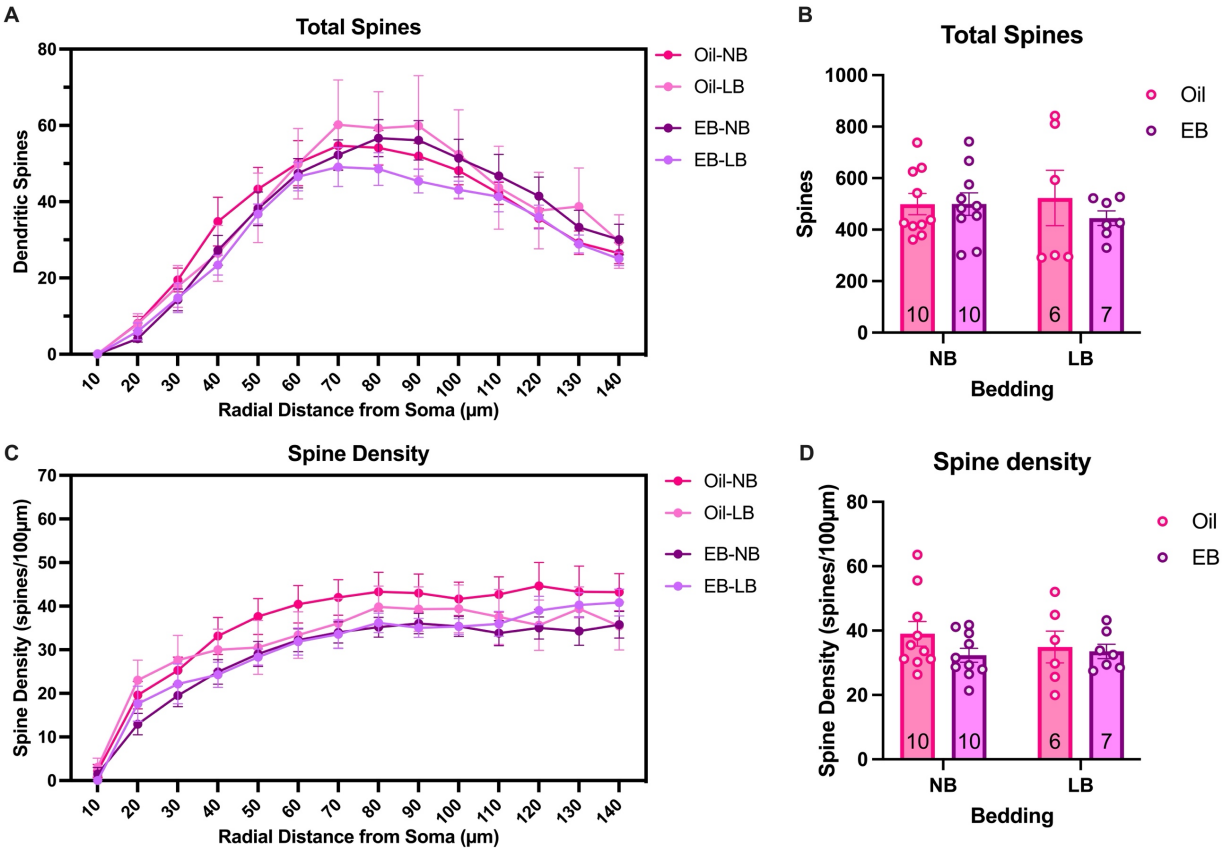

Figure 10. Sholl analysis of the effects of bedding condition and gonadal steroid treatment on the BLA neurons of control (oil) and EB-treated females at PND21 as a function of radial distance from the soma. Two morphological parameters, total spine number (A, B), and spine density (C,D) were assessed with both sholl analysis (A, C) and "cropped" branched analysis (B, D) assessing only the area within 140µm from the soma. There was no significant interactions, nor individual effects of bedding or treatment noted across sholl or branched analysis of spine number and spine density. Sholl analysis of total spine number and spine density did however find a significant effect of radial distance from soma ($p < 0.0001$).
A, C: Three-way ANOVA. B, D: Two-way ANOVA. Values represent mean ± SEM of 3 neurons analyzed/animal and 6-10 animals/group.

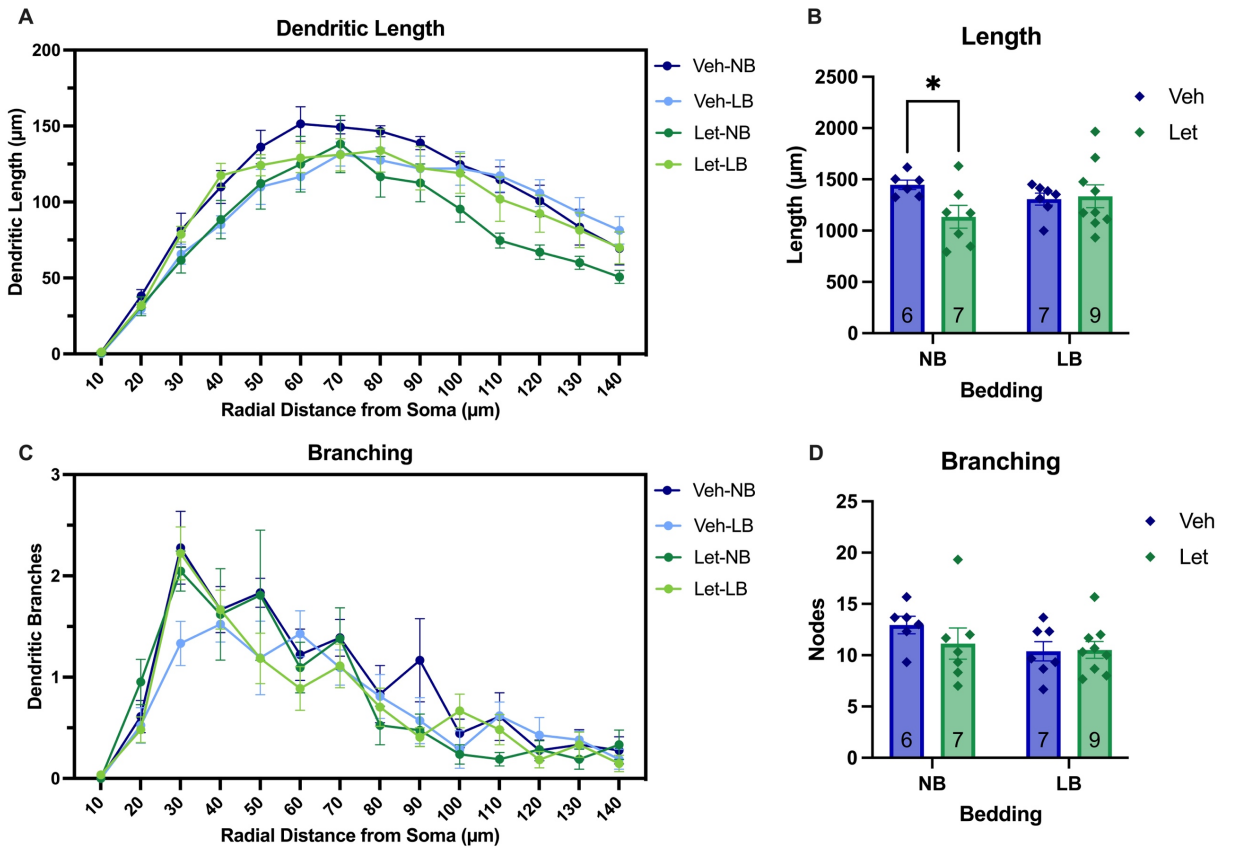

Figure 11. Sholl analysis of the effects of bedding condition and gonadal steroid treatment on the BLA neurons of control (Veh) and Let-treated males at PND21 as a function of radial distance from the soma. Two morphological parameters, dendritic length (A, B), and dendritic branching (C, D), were assessed with both Sholl analysis (A, C) and branched analysis (B, D) assessing only the area within 140µm from the soma. There was no significant effect of bedding or treatment noted across Sholl or branched analysis of dendritic length and branching. Sholl analysis of both dendritic length and branching did however find a significant effect of radial distance from soma ($p < 0.0001$). Sholl analysis also found a significant two-way interaction (radius x bedding) on dendritic length ($p = 0.0340$). There were no significant interactions noted in the branched analysis. A, C: Three-way ANOVA. B, D: Two-way ANOVA. Values represent mean ± SEM of 3 neurons analyzed/animal and 6-9 animals/group.

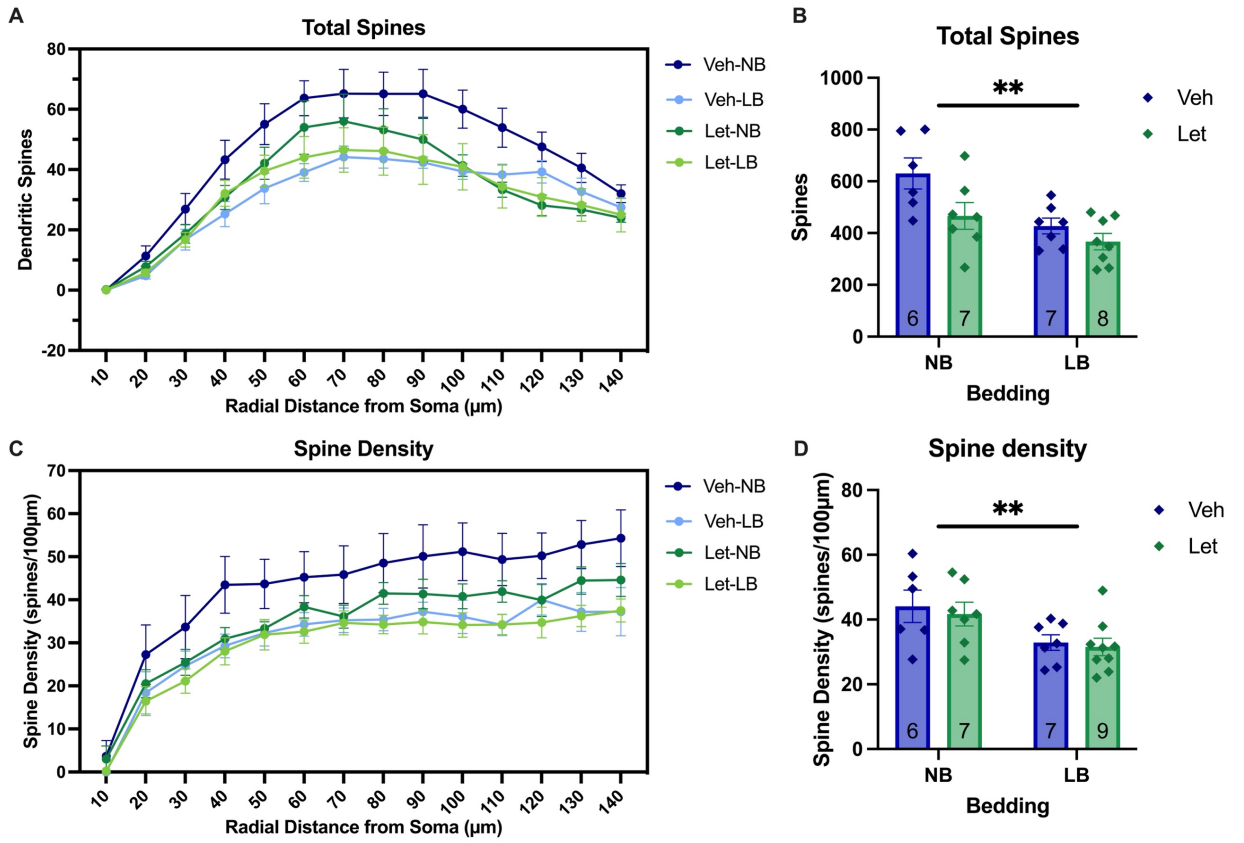

Figure 12. Sholl analysis of the effects of bedding condition and gonadal steroid treatment on the BLA neurons of control (Veh) and Let-treated males at PND21 as a function of radial distance from the soma. Two morphological parameters, total spine number (A, B), and spine density (C, D) were assessed with both sholl analysis (A, C) and branched analysis (B, D) assessing only the area within 140μm from the soma. Sholl analysis found no significant effect of treatment on either total spines or spine density, however there was a significant effect of bedding on spine density ($p = 0.0164$), and a significant effect of radial distance on both total spine number and spine density ($p<0.0001$). Sholl analysis also found a significant two-way interaction (radius x bedding) on total spine number ($p = 0.0010$). Branched analysis similarly found a significant effect of bedding on both spine number ($p = 0.0020$) and spine density ($p = 0.0045$), in addition to a significant treatment effect on spine number only ($p = 0.0020$). There were no significant interactions observed on spine number or density in branched analysis.
A, C: Three-way ANOVA. B, D: Two-way ANOVA. Values represent mean ± SEM of 3 neurons analyzed/animal and 6-9 animals/group.

4.3 Effect of sex steroids and bedding on ERα density in BLA at PND10

To determine if some of the observed treatment related morphological effects in the BLA could result from changes in the expression of estradiol receptors in PND10 neonates, we performed immunohistochemical detection of ERα in three regions, our region of interest, the BLA, as well as the medial amygdala (MeA) and ventromedial hypothalamus (VMH) (Fig 13). Both MeA and VMH are implicated in the effects of estradiol on reproductive behavior and thus, would serve as positive "controls" for the BLA region. We focused on females and compared EB-treated females to controls of both sexes (oil-treated females, vehicle treated males), and only analyzed those reared in NB conditions. A two-way, mixed-effects model (REML), with treatment as a between-subjects factor, and region as a within-subjects factor, yielded a significant region x treatment (Figure 13b; $F(4, 11) = 14.56$, $p = 0.0002$). Simple main effects tests conducted between treatments at each region indicated a significant treatment effect in the MeA and VMH, but not the BLA (MeA: $F(2,16) = 4.62$, $p = 0.0261$, VMH: $F(2,16) = 15.61$, $p = 0.0002$). Subsequent pairwise comparison with Bonferroni's multiple comparisons test, indicated that in the MeA, females treated with EB had significantly lower ERα density relative to those treated with oil ($p < 0.05$), while ERα density in Veh-treated males failed to differ significantly from females of either treatment group ($p > 0.05$). Pairwise contrasts in the VMH, indicated that Veh-treated males had significantly greater ERα density compared to both oil- and EB-treated females ($p< 0.001$), while EB- and Oil-treated females were not different. Simple effects tests conducted between regions at each treatment type indicated a significant region effect on all three treatment groups (Oil: $F(2,12) =93.06$, $p <0.001$; VEH: $F(2,12) = 211.81$, $p <0.001$; EB: $F(2,12) = 75.72$, $p <0.001$). Subsequent pairwise comparison with Bonferroni's multiple comparisons test, indicated that ERα density is significantly decreased in the BLA relative to both the MeA and VMH, across all three treatments ($p < 0.001$).

We next examined whether early EB treatment and limited bedding impact ERα concentrations in the BLA, we performed immunohistochemical detection of ERα in the BLA of PND10 pups as indicated in Figure 14. A two-way ANOVA with treatment and bedding as between-subject factors failed to yield significant effects of treatment ($F(2, 22) = 0.4869$, $p = 0.6210$) or bedding ($F(1, 22) = 1.194$, $p = 0.2863$) or a significant bedding by treatment interaction, $F(2, 22) = 0.1572$, $p = 0.8555$).

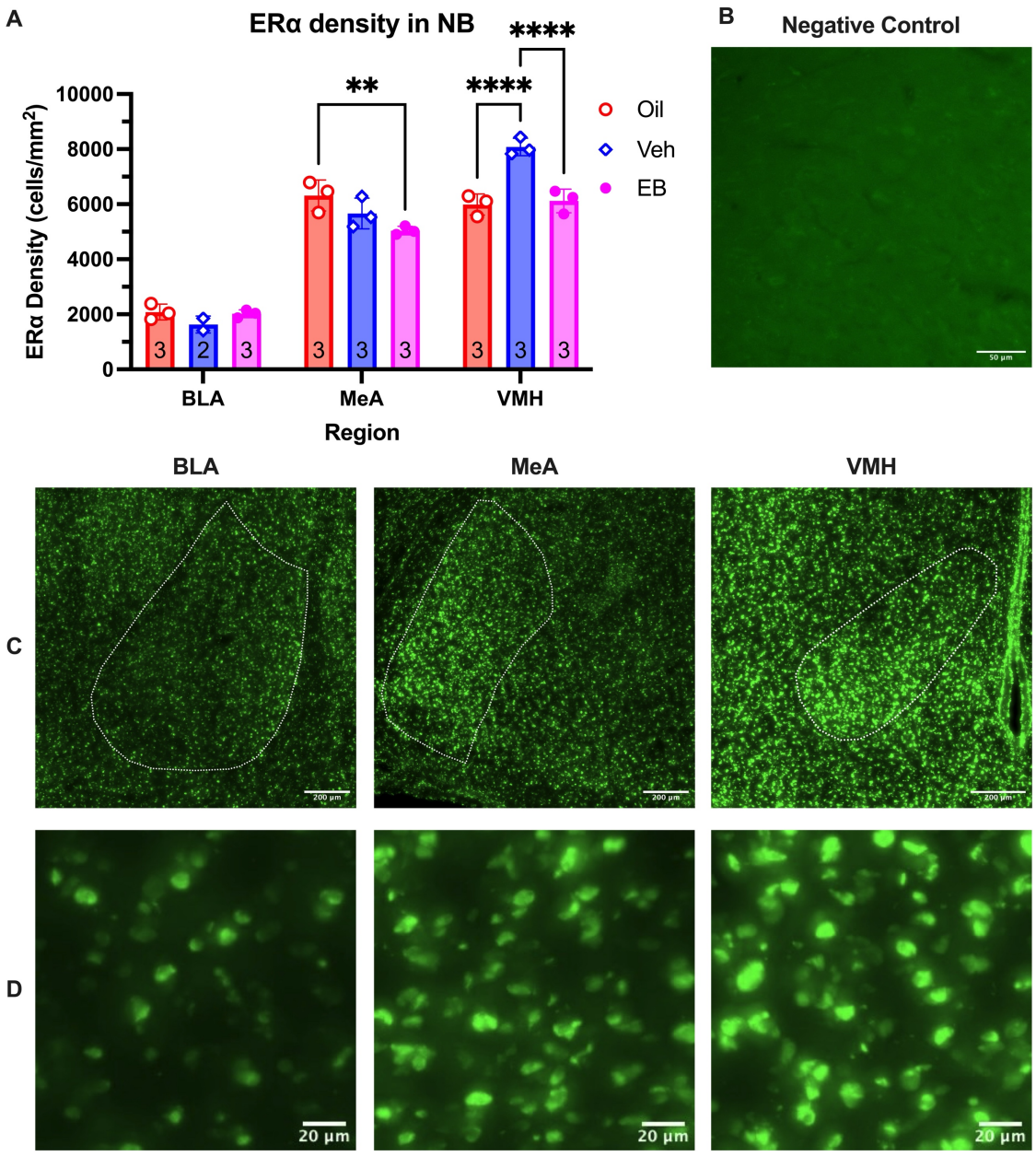

Figure 13. Effect of treatment and region on ERα density. A) Assessing ERα density in the BLA, VMH and MeA of PND10, across females treated with either EB or oil, and vehicle-treated males. There was a significant region by treatment interaction ($p < 0.001$), with a significant effect of region at each treatment group ($p < 0.001$) and a significant effect of treatment in both the MeA ($p = 0.0261$) and VMH ($p < 0.001$), but not the BLA.

B: Image of control ICC for ERα in the BLA performed in the absence of primary antibody (20X) Scale bar = 50µm. Note the absence of significant fluorescent signal demonstrating primary antibody specificity. C-D: Magnified digital images (20x objective)), of ERα immunoreactivity in the BLA, MeA, and VMH of oil-treated females at PND10 (C, D; Scale bars = 200µm and =20µm, respectively.)

Two-way mixed factors ANOVA. Values represent mean ± SEM of a minimum of 3 sections per region per subject, with 3 animals/treatment (indicated in the bars).

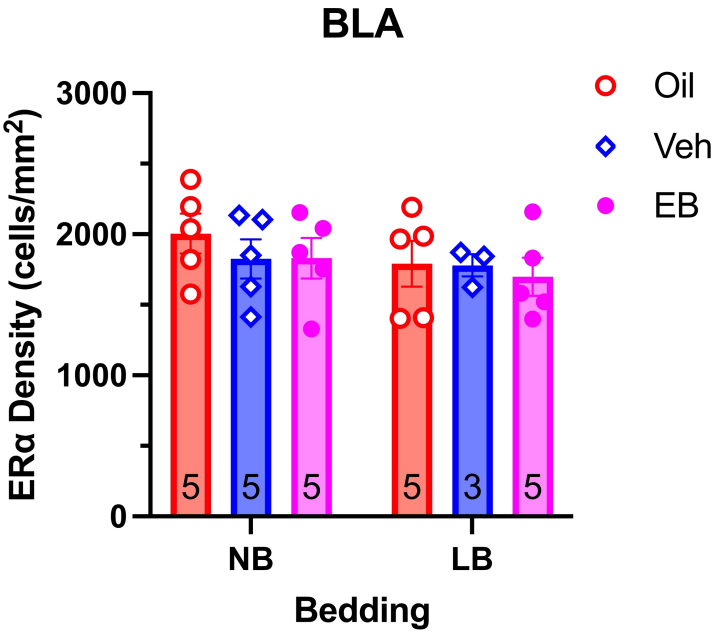

Figure 14. Effect of bedding condition and treatment on ERα density in the BLA of PND10 females (control and EB-treated) and male control (Veh) pups. There was no significant main effect of bedding or treatment, nor significant bedding by treatment interaction.
Two-way ANOVA. Values represent mean ± SEM of a minimum of 3 sections per subject, with 3-5 animals/group (indicated in the bars).

4.4 Effect of early sex steroid manipulations and bedding condition on adult fear- and anxiety-related behaviours

We performed 3 separate tests related to BLA function on adults between PND 62 and 75. We focused on the effect of exogenous estrogen, comparing EB- and Oil-treated females.

Open-field testing

We analyzed both the time in center and total distance traveled (locomotion) while in the open field, using a two-factor (Bedding x Treatment) between subjects ANOVA. Analysis of time spent in the arena center (Fig 15A) revealed no significant main effects of bedding or treatment and no significant bedding by treatment interaction. Analysis of locomotion (Fig 15B), however, did yield a significant main effect of treatment ($F(1, 24) = 10.07$, $p = 0.0041$), with locomotion lower in EB-treated compared to Oil-treated females. However, the interaction term and main effect of bedding were not significant.

Social Contact

We assessed social interactions with a juvenile rat of the same sex and determined, both total contact time and bouts of initiation of contact (bouts) as shown in Figure 16. For total contact time, a two-way ANOVA, with bedding and treatment as between factors revealed a significant main effect of treatment (Fig 16A; $F(1, 24) = 8.754$, $p = 0.0068$), with EB treated females spending more time in social contact with same-sex juveniles, relative to those treated with oil. The bedding by treatment interaction was not significant. Analysis of the number of bouts of social contact, revealed no significant main effects of bedding or treatment, nor a significant bedding by treatment interaction (Fig 16B).

Fear conditioning

We assessed fear conditioning in females by measuring the percent time freezing during six tone-shock pairings (Figure 17A-B). The data was analyzed using a three-way mixed design ANOVA, with tone as a within-subjects factor and bedding and treatment as between-subjects factors. While the three-way interaction and all two-way interactions were not significant using the Greenhouse-Geisser correction for sphericity (Fig 17A), the ANOVA did yield a significant main effect of tone ($F(3.531, 84.75) = 70.79$, $P<0.0001$). Subsequent pairwise comparisons using Bonferroni's multiple comparisons test indicated that all tone pairs except, 3-6, 4-6, 5-6, and 4-5,

were significantly different ($p < 0.05$), with freezing increasing with each subsequent tone and plateauing at tone 4. In addition to the analysis of freezing responses during all 30sec tones, we calculated the mean percentage of freezing across the six tones (Fig 17B). We analyzed mean freezing using a two-factor (bedding x treatment) between subject ANOVA, there was no significant main effect of bedding or treatment, nor a significant bedding by treatment interaction. In addition to freezing during the tone, we also assessed freezing behaviour during the intertrial intervals (ITI; Figure 17C-D). We analyzed percent freezing during each ITI, using a three-way mixed design ANOVA, with ITI as a within-subjects factor and bedding and treatment as between-subjects factors. As seen in freezing during the tone-shock pairing, the three-way interaction and all two-way interactions were non-significant (Fig 17C), however, the ANOVA did yield a significant main effect of ITI ($F (3.620, 86.88) = 46.89$, $p <0.0001$). Subsequent pairwise comparisons using Bonferroni's multiple comparisons test indicated that all interval pairs except, 2-6 and 3-5, were significantly different ($p < 0.05$), with percent freezing increasing with each subsequent ITI until peaking at interval 4, before decreasing across intervals 5 and 6. We also analyzed the mean percent time freezing between across all 6 intervals using a two-factor (bedding x treatment) between subject ANOVA (Fig 17D). We found no significant main effect of bedding or treatment, nor a significant bedding x treatment interaction factor.

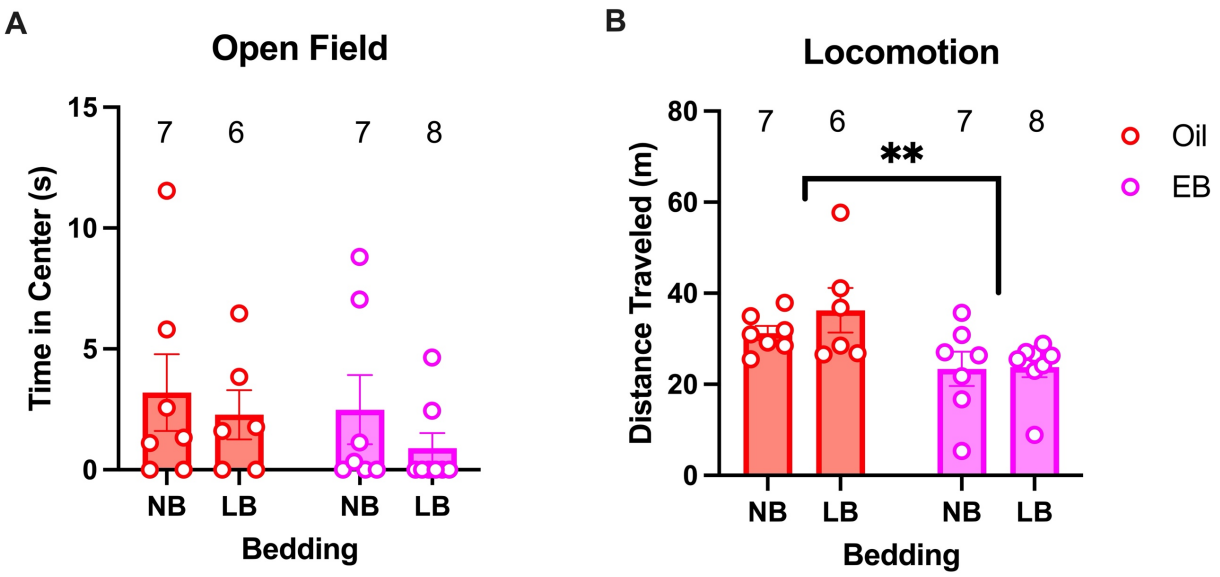

Figure 15. Open field task as a proxy for anxiety-like behaviours in adult females reared in either NB or LB conditions and treated neonatally with either oil or EB. The amount of time spent in the center arena of the open field (A) and the total distance traveled (locomotion; B) was measured in over a 5-minute period. The time spent in the center arena (A) was not significantly different across bedding or treatment groups, nor was there any bedding-treatment interaction. However, there was a significant effect of treatment ($p = 0.0041$) on locomotion, with oil-treated animals travelling greater distances compared to EB-treated animals. There was no significant bedding effect nor bedding-treatment interaction.
Two-way ANOVA. Values represent mean ± SEM of 6-8 animals/group (indicated above the bars).

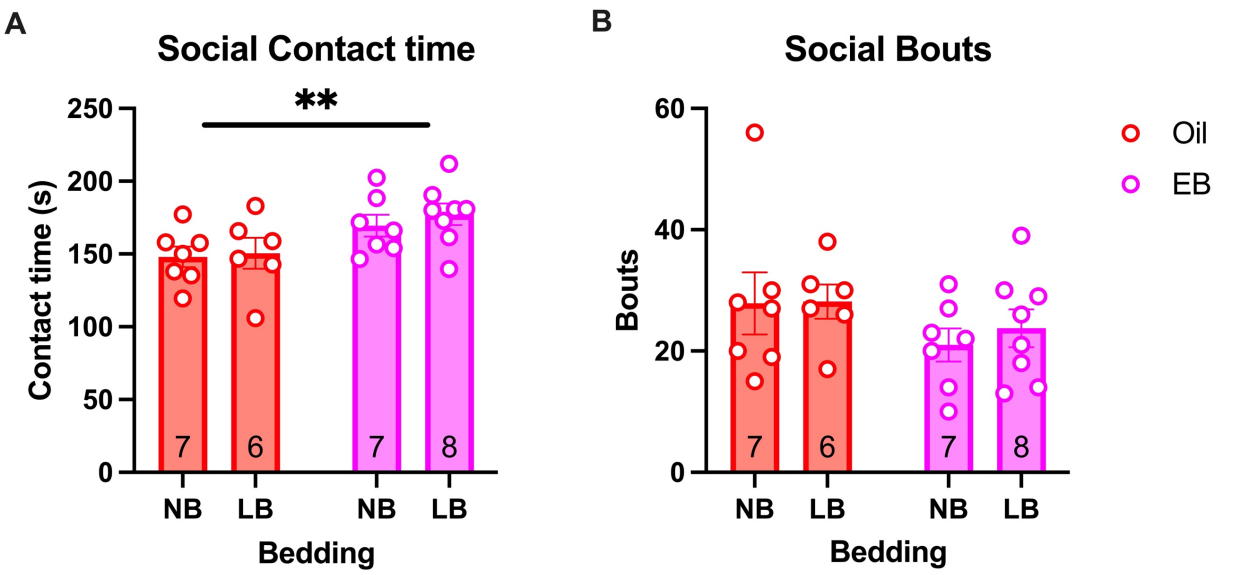

Figure 16. Analysis of social behaviour in adult females reared in NB and LB and treated neonatally with either oil or EB following habituation to context. Social interaction test, measuring time in contact with (A) and number of approach bouts initiated by the adult to a same sex juvenile (B). Social contact time showed a significant effect of treatment ($p = 0.0068$), with EB-treated adults spending more time interacting with the juvenile compared to oil-treated adults. There was no bedding effect nor bedding by treatment interaction on social interaction time. Neither treatment nor bedding significantly altered the number of social approaches made by the adults, and there was no bedding by treatment interaction on approaches.
Two-way ANOVA. Values represent mean ± SEM of 6-8 animals/group (indicated in the bars).

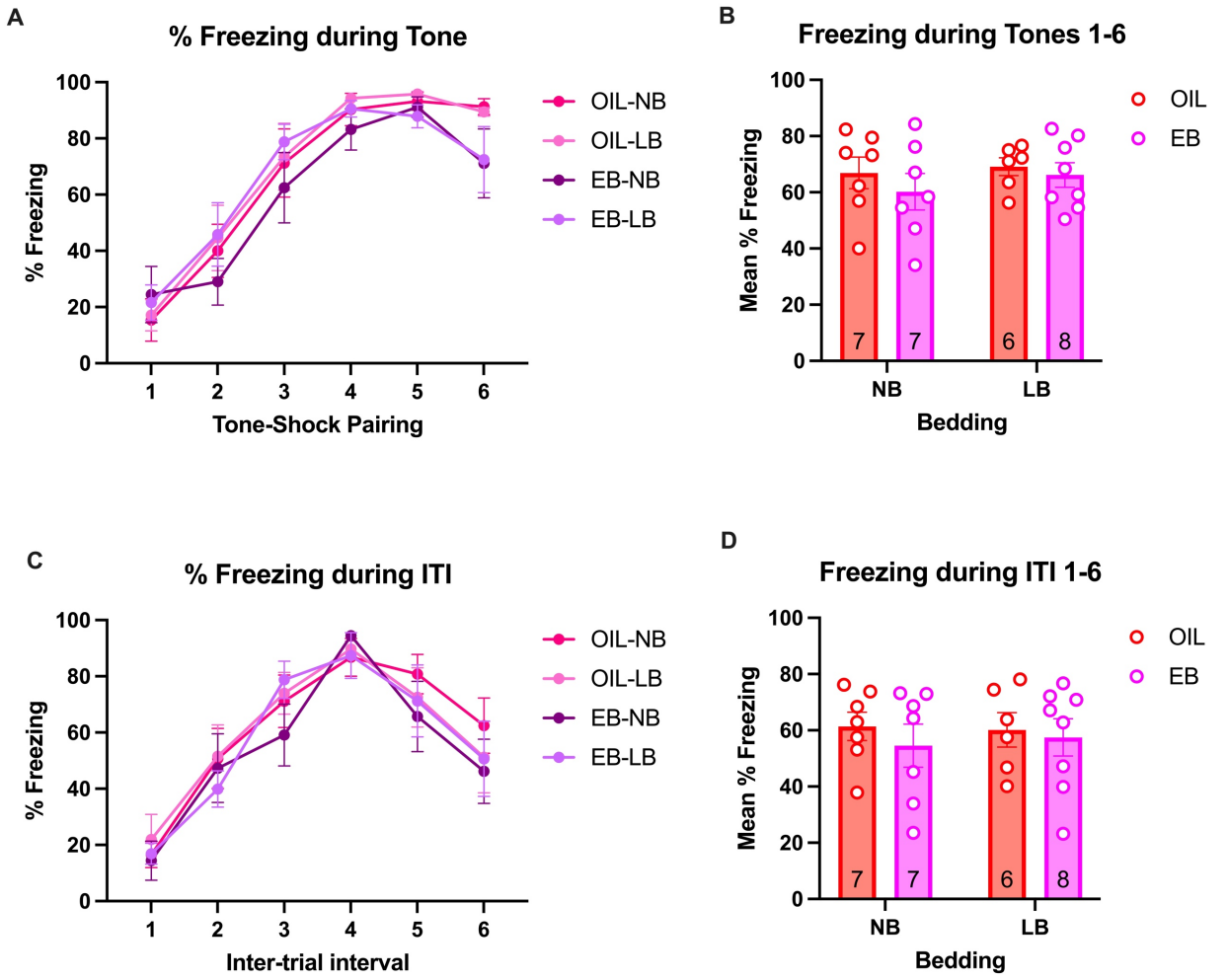

Figure 17. Fear conditioning in adult females reared in either NB or LB conditions and treated neonatally with either oil or EB. Fear behaviour measured as percent time freezing during tone-shock pairings (A, B) or intertrial intervals (ITI; C, D). When assessing freezing during the 6 tone-shock pairs (A), there was no significant effect of treatment or bedding or any interactions, however freezing was significantly affected by tone number ($p < 0.0001$). Analysis of the mean percent time freezing across all 6 tone-shock pairings (B), failed to show any significant bedding or treatment effect nor a significant bedding by treatment interaction. Freezing during ITI (C) showed no significant effect of bedding or treatment, nor any significant interactions, however freezing was significantly affected by ITI number ($p < 0.0001$). Analysis of the mean percent time freezing across all 6 intervals (D), found no significant bedding or treatment effects, nor any interactions.

A, C: Three-way ANOVA. B, D: Two-way ANOVA. Values represent mean ± SEM of 6-8 animals/group.

5. Discussion

General Overview of results

In this work, we sought to examine the interaction between neonatal estradiol and early life stress (LB) on juvenile basolateral amygdala morphology and adult amygdala-related behaviours in rats. Our findings were that LB induced significant dendritic hypertrophy exclusively in oil-treated (control) females at PND21, with increased total dendritic length and branching. LB-induced morphological alterations were not present in EB-treated females, nor males regardless of treatment group. Further, aromatase-inhibition with Letrozole was insufficient to recreate the neuronal hypertrophy witnessed in oil-treated females following LB, suggesting that the morphological changes witnessed in oil-treated females are not dependent on early estrogens. In keeping with our morphological findings, we found no significant effect of bedding nor treatment on ERα expression in the BLA. Interestingly, investigations of associated regions found a significant effect of treatment and sex in the MeA and VMH, respectively. While we found no bedding effect across social and fear conditioning tests in adulthood, oil-treated females did show increased anxiety-like behaviours compared to those treated with EB. We did however find a bedding-treatment interaction in the open field tests, with a habituation-like pattern of behaviour in the Oil- but not EB-treated females across the two testing days.

ELS model: Maternal Care and Pup Growth

The LB paradigm used here to model ELS has been shown to disrupt maternal care behaviour and has been shown to increase the fragmentation of these behaviours in both rats and mice (Glynn & Baram, 2019). LB's influence on pups themselves is most consistently observed in the decrease in preweaning body weights in pups reared in LB, relative to NB controls (Walker et al., 2017). While changes in maternal behavior with the LB conditions were modest in our experiments, being limited to variations across circadian light cycle, there were sufficient to cause at minimum a metabolic stressor for the pups, measured by reduced pup weight gain by the end of the 10-day protocol. Interestingly, the difference in growth normalized between the return of all animals to normal bedding at PND10 and weaning at PND21. This suggests that LB pups are able to account for the suppressed growth that occurred while in LB, with an acceleration in weight gain upon their return to normal conditions, allowing them to catch up to their NB peers. Retardation of physical growth in early life is associated with aberrant neurodevelopment, even in those who

exhibited preweaning catch-up growth (Alexeev et al., 2015; Jou et al., 2013). The same metabolic stresses and deficits in energy availability that limit weight gain and physical growth, also stifle other aspects of development, including that of the brain. Due to the rapid and time-sensitive nature of brain development, limitations in the energy available for sustained growth and development during this period has the potential to induce far more lasting and detrimental effects relative to what is seen in physical growth. Despite the presence of later accelerations in growth, as much of neural development is limited to set intervals defined by critical periods, these areas may not be able to similarly rectify the developmental setbacks associated with suppressed energy availability in early life, preventing neural development from being able to catch up to their NB peers in the way body weight can. While we did not find any differences in maternal behaviour across LB and NB dams, this difference in body weight provides a likely method in which LB can influence development of stress systems and induce lasting changes on neural function and behavior. Decreased pup growth, measured by weight gain across the ELS period, has been consistently observed in LB, both in rats and in mice (Guadagno et al., 2018; Kanatsou et al., 2017; Naninck et al., 2015; Pardo et al., 2023; Rice et al., 2008; Shupe & Clinton, 2021). Additionally, when maternal behaviour is measured at multiple time points daily across the length of the LB paradigm, the observed degree of fragmentation is inversely proportional to pup weight gain (Rice et al., 2008). While associated to fragmentation in LB, reductions in pup weight have been similarly observed in both rat and mice following maternal separation (Maghami et al., 2018; Moriceau et al., 2009; Orso et al., 2020). It appears that reduced weight gain in pups is similarly associated with either interruptions in maternal care in the case of separation, or fragmentation of maternal care with the LB paradigm.

Morphology

Interaction between ELS and Estradiol

At weaning age, both LB rearing and neonatal estradiol exposure modify neuronal morphology in the BLA, with LB promoting neuronal hypertrophy in oil-treated (control) females and to a lesser extent in Let-treated males. Oil-treated females reared in LB experienced a significant increase in both dendritic length and branching relative to their NB-reared peers. Interestingly, this effect was absent in EB-treated females and in Veh-treated males, suggesting that brain masculinization of females via EB treatment eliminated the effect of LB on BLA neuron morphology. In males,

preventing estradiol conversion by Letrozole treatment yielded a significant increase in dendritic length in LB conditions, without increase in dendritic branching. In the present studies, Veh-treated males (control) did not experience significant LB-induced alterations in BLA neuron morphology, but Oil-treated (control) females did. Together this suggests that early life exposure to estradiol (Veh males and EB-treated females) may be protective, preventing or counteracting the morphological changes induced by LB. In the absence of estradiol, as seen in control females, LB induces neuronal hypertrophy, resulting in neurons that are both more branched and have a greater total dendritic length. Similarly, by inhibiting the formation of estradiol in males, Letrozole treatment can increase the sensitivity of BLA neurons to LB-induced modification, however the observed hypertrophy was limited to dendritic length. However, due to the dynamics of the treatments and the gonadal milieu in which they are applied, unlike EB's ability to recreate male morphology, letrozole treatment did not fully recreate the female-typical sensitivity in males. Due to the absence of counteracting gonadal steroid dynamics, as our neonatal EB injections occurred within the critical period for masculinization, EB treatment was sufficient in recreating male-typical morphology in females. In contrast, as the testes are active in late gestation, males have already undergone one of the two peaks in androgen production by birth, therefore our neonatal Letrozole injections were only able to partially inhibit the formation of estradiol and any subsequent masculinization, explaining Letrozole's limited capacity to recreate female morphology.

In contrast to prior work in our lab, wherein the neuronal hypertrophy observed following ELS was accompanied by a significant increase in spine number(Guadagno et al., 2018), spine number did not increase alongside dendritic length and branching following LB in our study. Neither males nor females, regardless of treatment, experienced any significant LB-induced changes in spine number. However, males exhibited greater spine number in NB, an effect that was blocked by Letrozole treatment, while females regardless of treatment exhibited similar spine number to Let-treated males. The ability of Let to block this effect, while EB failed to recreate it, suggests that estrogen, while essential, does not act alone to modulate this sex difference. Rather, spine number is likely modulated by a combination of estradiol and extragonadal factors, such as microglia, which work in tandem and are therefore both required to produce the male-typical patterning of dendritic spines. In contrast to spine number, there was a bedding effect observed in spine density; however it was limited to males, despite the moderate observations in other

morphological characteristics. This arose due from two separate phenomena, in control males LB was associated with a moderate increase in spines but did not alter length, while in Let males, LB significantly increased dendritic length without impacting spine number, the result was both groups experiencing a net decrease in spine density following exposure to LB conditions.

Morphology in Non-Stressed Conditions

While both EB and Let treatment reliably recreated the change observed between NB and LB-reared offspring typical of the opposite sex, the ability of our treatments to recreate the opposite sex's specific morphological patterning varied across the bedding groups. When reared in NB conditions, exogenous estradiol treatment in females resulted in dendritic length near-identical to, but slightly exceeding, length in Veh (control) males. Similarly, letrozole treatment of males decreased dendritic length to a degree similar but slightly below that typical of oil (control) females. Taken together either treatment yielded a somewhat hyperbolic, extending slightly above or below, recreation of the dendritic length typical for the estrogenic condition it mirrors. The effect observed on branching in those reared in NB is similar, with both treatments mirroring the relative branching observed in the opposite sex control, however both EB and Let treatment yielded branching slightly exceeding their respective opposite sex controls. Taken together the effects of treatment observed in NB, most prominently in those treated with EB, suggests that the sexual dimorphism observed in morphological patterning in non-stressed conditions may be primarily driven by estrogenic signalling in the perinatal period. While our findings suggest that the morphological influences of estrogens in early life primarily shape neuron structure, increasing dendritic length and branching, it has been reported in adults that estrogen's role shifts to promote the formation of synapses and connections, primarily through the modulation of spine density Estrogen's influence is most apparent in the hippocampus, wherein both exogenous administration of estrogens and peaks in naturally cycling estrogens have been shown to increase dendritic spines in CA1 pyramidal neurons, without impacting length and branching (Woolley et al., 1990). Studies measuring the effects of exogenous estrogen in gonadectomized adults have observed similar estrogen-induced increases in spine density, however similar studies are lacking in the BLA. The limited data in the adult BLA exclusively examine sex differences, often failing to observe any across morphological parameters, including spines(Blume et al., 2017; Koss et al., 2014). However, studies separating the basal and lateral nuclei, do observe sex differences and estrous cycle effects but they are limited to the basal nucleus, with peak spine numbers in diestrus,

suggesting that combining the nuclei might obscure potential sex differences in adulthood (Blume et al., 2017).

Morphology in ELS Conditions

In contrast to the clear influence of estrogen on morphology in non-stressed conditions, when exposed to early life stress estrogenic modulation is far less influential (Guadagno et al., 2018). Interestingly, we found little influence of sex or treatment on dendritic length in LB-reared animals. In LB, control females exhibited slightly greater dendritic length relative to control males, although the effect was not significant. Due to the absence of any sex difference, the treatment effect proved to be insignificant. In contrast to a moderate, effect of treatment in females, Let treatment failed to alter dendritic length in LB conditions. Unlike length, dendritic branching in LB-reared offspring did appear to be sexually dimorphic, with control females exhibiting a near significantly higher branch points in LB compared to LB males. Despite the sex difference, treatment effects were not significant for this variable. Notably, in stark contrast to what we observed in NB, in LB conditions, EB only had a minor masculinizing effect on branch points and Let treatment similarly had no influence on dendritic branching in LB males. These results suggest that, contrary to the control NB conditions, estradiol does not appear to represent a significant mediator of morphological patterning in those reared in LB and that a yet unknown aspect of ELS hamper's estradiol's ability to directly influence morphological patterning in the BLA. While there is ample evidence of gonadal steroids impacting stress responses and sensitivity to stress, evidence of the reverse, i.e. the ability for stress to modulate the gonadal steroid milieu, has also been observed. Stress and glucocorticoids both inhibit the hypothalamic and pituitary signals that regulate gonadal steroid production and downregulate gonadal steroid production at the level of the gonads (Chrousos et al., 1998; Vermeulen, 1993). These effects are increased when the stress is chronic or repetitive. However, the influence of stress on the gonadal steroid milieu was not limited to hormonal production but also impacted receptors. Stress associated with reduced maternal care in the neonatal period (the low licking and grooming mothers) alters reproductive behaviour later in life, associated with a reduction in ERα in brain areas associated with the control of these behaviours including the VMH (Cameron, 2011; Cameron et al., 2011). While we did assess ERα density in this study, it was limited to the BLA and we cannot rule out that other areas or receptor subtypes may be implicated in mediating the interactions between estrogen and early stress.

Impact of Estradiol and ELS on ERα density

In keeping with our morphological findings, we anticipated that the presence of greater concentrations of estradiol may result in the downregulation of ERα receptors, resulting in lower relative ERα density in control males and EB females, at least in the absence of early life stress. Within the BLA neither treatment nor stress significantly altered the ERα density. While the literature investigating the influence of exogenous estradiol on ERα availability in the BLA is limited, our observations are consistent with other reports of the absence of any sex difference in ERα protein (Pérez et al., 2003) or mRNA (Cao & Patisaul, 2013)during the second week of life in postnatal rats. We broadened our analysis in NB reared pups to include two control regions, the medial amygdala (MeA) and ventromedial hypothalamus (VMH), two regions implicated in sexually dimorphic social and reproductive behaviours. Relative to the BLA, the MeA and VMH, exhibited significantly greater concentrations of ERα at PND10. Further differentiating them from the BLA, ERα density in the MeA and VMH were found to be sensitive to treatment and sex, respectively. Similar to our observations in the BLA, and consistent with reports of ERα protein (Pérez et al., 2003) and mRNA (Cao & Patisaul, 2013) levels during the second week of life, there was no significant difference between ERα density across the MeA of control males and females. We did however observe a significant downregulation of ERα in the MeA when females were treated with EB. ERα density in the MeA therefore is not significantly impacted by sex, but is impacted by estradiol availability, with estradiol decreasing ERα density. In the VMH however, we noted the opposite, as we observed significantly greater ERα density in males relative to both control and EB-treated females. While we did observe a significant sex difference, EB treatment failed to either block this sex effect or result in a downregulation of ERα. These results agree with recent observations of a sex difference in ERa density in in specific VMH subpopulations.

Adult Behaviour

There are several instances in the literature that document morphological changes after stress in many brain regions and the association with stress-related behavioral modifications (Bessa et al., 2013; Eiland et al., 2012; Kim et al., 2014). In our previous work, we observed LB-induced dendritic hypertrophy of pyramidal BLA neurons that was associated with heightened anxiety-like behaviour in male rats (Vyas et al., 2002). In the present work, as this represented an

initial investigation of the long-term interaction between estrogens and LB, we focused primarily on females. We chose to assess females, as treatment in females more completely altered the estrogenic state, as males treated with Let still experienced prenatal estradiol production. Further, we observed LB-induced changes more prominently in females in early life.

Despite exhibiting different morphological sensitivities to LB, neither EB- nor Oil-treated females exhibited any ELS-induced change in anxiety-related behaviours. Notably, control females who exhibit increased length and branching when reared in LB did not exhibit increased anxiety-behaviours compared to those reared in NB, contrasting what is observed in males. While this may suggest the presence of a sex-difference in the relationship between BLA morphology and anxiety behaviour, as these measurements were taken at different time points it does not represent definitive evidence of a sex difference. Interestingly, a similar study in our lab using the same timepoints in males, did observe an association between neuronal hypertrophy at weaning and later anxious behaviour (Guadagno et al., 2018). While assessment of adult morphology would be required for confirmation and closer association, it is possible that these discrepant results arise from sex differences in the trajectory of BLA morphological development across adolescence until adulthood and that some of the morphological changes we document at weaning age are eliminated as adults. It is known that BLA maturation diverges along sex-specific trajectories following puberty (Guily et al., 2022), which may result in males maintaining this hypertrophy, while females exhibit reparative action, returning to a non-hypertrophic state therefore reducing the behavioural consequences of stress. While it is possible that the discrepancy between what we observe in oil-females and the expected relationship between neuronal hypertrophy and anxiety is due to sex differences, it may also be the result of the degree and types of morphological changes observed. In addition to being observed exclusively in males, the studies that found this link all observed increases in either the number or density of dendritic spines, in addition to increased length and branching, whereas our oil-treated females did not exhibit any changes in spines. Further, heightened spine density on BLA neurons is associated with and can be used as a predictor for greater anxiety behaviour in rats (Adamec et al., 2012), and stress-induced spinogenesis has been associated with anxiety behaviour both with and without associated increased in dendritic length and branching (Mitra et al., 2005). Considering the association between dendritic spines and anxiety-like behaviour, the absence of LB-induced spinogenesis in the BLA of oil-treated females

may explain the absence of a corresponding behavioural alteration in the presence of LB-induced dendritic remodelling. Regardless of the cause, it appears that the morphological changes observed in oil-females at PND21 were not sufficient to induce later anxiety-like behaviour, and further analysis, including assessment of adult morphology is required to untangle the relationship between altered morphology and behaviour in females.

Limitations and future studies

Maternal Behaviour and the LB paradigm

In the current study, we did not observe any significant differences in maternal care behaviours between bedding conditions, nor did we observe, increased fragmentation in the LB condition, a characteristic that is often, but not always reported in studies using the LB paradigm either in rats or mice (Baram et al., 2012). The observation of significant metabolic stress in the form of suppressed growth in LB pups suggest, however that LB conditions did produce a significant stress to the pups during the first 10 days of life. How do we reconcile reductions in body weight in pups with lack of significant changes in maternal behavior? Considering the fact that methods used to measure maternal behaviour vary across research groups, it is possible that rather than representing an issue with our paradigm, the lack of observable change in dam's behaviour may be attributable to the method and time points used for the quantification of maternal behaviour (Guadagno et al., 2018; McLaughlin et al., 2016). While dams and their litters were exposed to LB from PND1-10, maternal behaviour was only recorded for a 24-hour period from PND5-6, with quantification further limited to four one-hour blocks, with one observation per minute. Thus, the maternal behaviour quantified and analyzed represented only a snapshot of the dam's care during the 10-day protocol. The days and time points assessed for maternal behaviour differ across research groups, with some choosing to record for shorter periods more frequently, either every second day or daily for the length of LB period (E. P. Davis et al., 2017; Orso et al., 2020). The choice to assess behaviour along the entire period may better encapsulate the changing response of the mother to her environment, that is not apparent in the quantification of a single day. As maternal behaviour was scored manually, we concentrated on a 24hr period with repeated scoring.. Considering that the circadian clock also influences behaviour, we found that the measurement of multiple time points across the day better represents maternal behaviour, compared to assessing only one time point across multiple days. Experimentally, reaching a better

time and age resolution should be accomplished once we will be able to automatically and reliably quantify maternal behavior using AI-associated tools such as modifications of DeepLabCut for instance.

Neonatal treatment

Organizational effects of gonadal steroids in males, in particular via testosterone secretion occur both during the late prenatal (GD18) and neonatal periods. In our study, we injected the aromatase inhibitor in male pups within the first 12-24h of birth and on PND3 to reduce the effect of the postnatal testosterone surge, without affecting the prenatal surge. This prenatal estradiol exposure may underscore the inability of Letrozole-treated males to fully recreate the morphological effects of LB seen in females, such as the absence of increased arborization following LB.

Because of this, our treatment only sufficiently recreated the postnatal steroid milieu, as our female treatment does not include prenatal estradiol exposure, nor does our male treatment block the aromatization that occurs prior to PND1. This makes our female treatment slightly more robust compared to our letrozole treatment, as females at least do not experience any other hormonal influences prior to their treatment, while males may have had prior in utero exposure to organizational effects of testosterone (via estradiol). It is clear that female EB treatment led to masculinization of behavior in adolescence as we recently reported that adolescent social play behavior of EB females exhibited all the characteristics of male play behavior and that LB conditions enhanced play behavior to a larger extent in EB females compared to Oil-treated females (Kraatz et al., 2024). Future experiments should attempt to apply treatments and techniques that effectively block the entire perinatal gonadal effect, such as the prenatal application of Letrozole or the use of gene silencing techniques targeting ERα. Unfortunately, the application of these treatments in utero is difficult and has the potential to induced significant undesirable effects. To treat males prenatally, letrozole would have to be administered to the pregnant dam, effectively treating both dams and pups regardless of sex. In addition, to the undesirable hormonal effects in the dam, which could impact the remaining gestation and birth, the treatment could act as a stressor for pups, further blurring the distinct effects of prenatal and early life stress.

Changes in animal colony and prior stress: comparison with previous work in our lab

Prior work in our lab observed a sexually dimorphic response to LB, wherein males but not females experienced LB-induced neuronal hypertrophy (Guadagno et al., 2018). While analysis of our control-treated males and females similarly yielded a sex difference in morphology following LB, the sexual dimorphism we observed was reversed. While there are a variety of factors that might contribute to the divergent findings, we experienced a large, unexpected change in our animal colony between these studies. While this project and our prior study utilized animals of the same strain (Sprague-Dawley) obtained from the same vendor (C River), the animals originated from different colonies housed at different facilities. While the use of different colonies can result in variation in mating time and offspring growth that impacts later results, slight differences are also to be expected in our LB model. As the stressor employed is reliant on the mother's response to her impoverished environment with the lack of nesting material, some degree of variability is expected across animal colonies and housing facilities that might alter maternal behavior during LB conditions. As the animals used originated from two different facility locations, in addition to colony differences, the cohorts also experienced different transportation time and procedures before reaching our animal facility. As our experiments require the transport of pregnant dams, increased transportation time might represent an additional prenatal stressor for the pups utilized in our studies. In addition to differences in transit time, the gestational day in which the rats are in transit differs between the new and old colonies. In our prior study the dams were received later in gestation (GD17-18) relative to the dams used in the present study (GD13-14). Changes in the gestational time points at which the mother is in transport or acclimating to her new environment, both of which may act as stressors, might directly influence brain development of the pups and/or alter the resulting maternal behavior. Similar to stress during early post-natal life, increased prenatal stress, such as that related to transport, has the ability to alter neurodevelopment and impact stress systems, possibly contributing to the differences observed between these studies (Kraszpulski et al., 2006). To control for the potential effects of transportation stress on the outcomes of LB exposure in the offspring, we would have to conduct a similar study with animals bred in our facility.

In addition to differences arising from external factors, differences in the study protocol itself may have influenced our results. The current study requires the use of early life injections, which both may be stressful for the young pups but also requires greater handling. As our previous

experiment did not require any handling beyond initial weighing and culling of the litter after birth, there is significantly more interaction and handling by the experimenters in this group. It is unclear, how this difference would impact pups, with both increased handling and the transient stressor of the injections. Whatever influence it may have, at least it would be equalized across all experimental groups, regardless of bedding conditions or injections received.

Conclusion

In conclusion, our results demonstrate the presence of a complex and reciprocal influential relationship between environmental stress and estrogenic signalling on neuronal morphology in the BLA and some aspects of adult behavior. The ability for stress to impact estrogenic actions is observed in the ability of ELS to abolish the estrogenic modulation of sex typical morphology observed in non-stressed conditions. Conversely, we found that estrogenic signalling decreases sensitivity to stress, with EB treatment successfully preventing ELS-induced neuronal hypertrophy observed in control females. Despite the observed interplay between ELS and estrogen on BLA morphology, we do not yet know how this interaction is modulated. The absence of changes in ERα in the BLA of neonates after EB treatment or early stress suggest that these receptors might not be implicated directly. However, modulation by other BLA estrogen receptors is still possible or inputs to BLA neurons from other ERα-rich regions that subtypes and are responsive to EB and/or stress effect might also indirectly modulate these effects. Future experiments should examine other receptor subtypes in the BLA and ERα-rich regions projecting to the BLA. Alternatively, changes in aromatase expression in the BLA induced by LB might also impact the response of male neonates to early stress. Future experiments will also measure changes in aromatase expression in the BLA. Finally, contrary to our expectation, our morphological changes at weaning were not associated with anxiety later in life. To assess why this is, assessment of lasting effects of early life gonadal steroid modulation and the possibility of compensatory actions in puberty and beyond are required.

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McGill

October 23, 2023

Animal Certificate

This is to certify that **Dr Claire Walker, Douglas Mental Health University Institute**, currently holds an approved **Animal Use Protocol # DOUG-8179** with McGill University and its Affiliated Hospital's Research Institutes for the following project:

Animal Use Protocol Title: Sex-dependent effects of early life stress on the development of the basolateral amygdala-prefrontal cortex circuit for fear conditioning

Start date: October 1, 2023

Expiration date: September 30, 2024

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.

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