Effect of early life stress on PV interneurons and perineuronal nets in the corticolimbic system of juvenile rats

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Abstract

Early life stress (ELS) predisposes individuals to neuropsychiatric disorders by modifying neurotransmission and plasticity in key brain areas involved in emotional processing and cognitive control. Particularly, impaired inhibition and aberrant neuronal firing of inhibitory interneurons might mediate some of the effects of ELS on the corticolimbic circuit and lead to increased behavioral fear in the long term. ELS might also cause oxidative damage to susceptible parvalbumin interneurons (PV INs) that are not encapsulated by perineuronal nets (PNNs), known to protect cells from oxidative stress. We tested whether ELS alters PNN density and its distribution around specific neuronal subtypes in the medial prefrontal cortex (mPFC) and basolateral amygdala (BLA) of juvenile rats and examined how these changes might associate with protection against oxidative stress. We used the limited bedding (LB) paradigm between postnatal day (PND)1-10 to induce ELS in male and female offspring. Brain tissue were processed on PND28 for two experimental endpoints: 1) identification of the neuronal subtype associated with PNNs using fluorescent RNA in-situ hybridization for VGAT and VGlut-1 coupled with immunohistochemical detection of PNNs; 2) detection of genes coding for enzymes implicated in the protection or sensitivity to oxidative stress including *Cat*, *Fis1*, *Dnm1l* and *Sod1* using quantitative PCR. Our results indicate that ELS does not modify the density of PNN or the ratio of VGLUT1 or VGAT cells harbouring PNNs in either the mPFC or BLA. However, LB males did exhibit an increased density of VGLUT1 cells expressing PNN in the infralimbic mPFC. In the BLA, ELS increased VGAT cell density in both sexes, suggesting the presence of immature GABAergic neurons and the possibility of neurogenesis in a typically non-neurogenic region. LB males exhibited increased *Cat* expression in the mPFC, suggesting increased antioxidant capacity. The juvenile BLA showed the opposite trend, indicating it remains vulnerable to oxidative damage induced by ELS. Taken together, these results emphasize that ELS has sex-and region-dependent effects on the corticolimbic system of juveniles, primarily by modifying the maturation of inhibitory interneurons in the BLA of male, but not female rats.

Résumé

L'exposition au stress en début de vie prédispose les individus à des troubles neuropsychiatriques en modifiant la neurotransmission et la plasticité neuronale dans les régions du cerveau qui sont principalement impliquées dans le traitement des émotions et le contrôle cognitif, faisant partie du système corticolimbique. En particulier, des changements précoces dans l'équilibre entre activation et inhibition neuronale ainsi qu'une activité dysfonctionnelle des neurones inhibiteurs pourraient être impliqués dans les effets à long terme du stress néonatal sur le fonctionnement du système corticolimbique. Ceci pourrait donc conduire à un phénotype anxieux a l'âge adulte. De plus, l'exposition au stress précoce peut également causer des dommages oxydatifs aux interneurones contenant la parvalbumine (PV INs) qui sont très sensibles lorsqu'ils ne sont pas encapsulés par les réseaux périneuronaux (PNNs), connus pour protéger les cellules du stress oxydatif. Dans notre étude, nous avons cherché à déterminer si l'exposition au stress précoce modifie la densité des PNNs et leur distribution autour de sous-types neuronaux spécifiques (glutamatergiques et GABAergiques) dans le cortex préfrontal médian (mPFC) et l'amygdale basolatérale (BLA) chez de jeunes rats juvéniles. Nous avons également cherche à savoir comment ces changements pourraient être associés à la protection des tissus neuronaux contre le stress oxydatif. Notre modèle de stress précoce était celui d'un appauvrissement environnemental pour la mère et sa portée, le « limited bedding » (LB) au cours des 10 premiers jours de vie. Nous avons récolté le cerveau des jeunes rats sous conditions de LB ou conditions contrôles (NB) a 28 jours de vie afin 1) d'identifier le phénotype des neurones (VGAT et VGlut-1 positifs) encapsules par les PNNs en utilisant la technique de RNAscope et immunohistochimie et de déterminer si leur densité est modifiée après un stress précoce de la même manière chez les mâles et les femelles et 2) d'effectuer la mesure d'expression des gènes codant pour les enzymes impliquées dans la protection ou la sensibilité au stress oxydatif, incluant *Cat, Fis1, Dnm1l et Sod1* par la technique de PCR quantitative. Nos résultats indiquent que l' exposition au stress précoce ne modifie pas la densité des PNNs dans le mPFC ou la BLA ni le ratio de cellules VGLUT1 ou VGAT abritant des PNN dans l'une ou l'autre structure. Cependant, chez les mâles LB, nous avons observé une densité cellulaire accrue de neurones VGLUT1 exprimant des PNN dans le mPFC infralimbique compare aux males NB. Dans la BLA, le stress

precoce augmente la densité des neurones exprimant VGAT chez les deux sexes, ce qui suggère la présence de neurones GABAergiques immatures et la possibilité d'une neurogenèse dans une région typiquement non neurogène. Malgré le manque de changement dans la densité des PNNs suite au stress précoce, nous avons observé que les mâles LB expriment une plus forte expression de Catalase dans le mPFC, impliquant une capacité antioxydante accrue dans cette région. La tendance inverse a été observée dans la BLA juvénile. Dans l'ensemble, ces résultats soulignent que l'exposition au stress précoce conduit a des effets dépendants du sexe et de la région sur le système corticolimbique des jeunes, principalement en modifiant la maturation des interneurones inhibiteurs dans la BLA des rats mâles, mais pas des rats femelles.

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First, I would like to thank Dr. Claire-Dominique Walker, my supervisor, for her continuous support, guidance, and encouragement throughout my Master's project. Her dedication to science and her students is truly inspiring. I would also like to thank my advisory committee, Dr. Naguib Mechawar and Dr. Tak Pan Wong, for contributing their expertise and their insightful suggestions to improve my project. This project would not have been possible without the technical support from Hong Long, thank you for your patience in teaching me so many techniques and helping with the troubleshooting in the lab. Thank you to the Mechawar lab for lending us their RNAscope oven and to Maria Antonietta Davoli and Claudia Belliveau for their technical advice. The services from the Molecular and Cellular Microscopy Platform at the Douglas were essential in completing the imaging experiments and thank you to Melina Jaramillo Garcia who helped set up the imaging and analysis. Completing this project during the pandemic was challenging, but I am so grateful for the welcoming community in our lab and at the Douglas. I want to thank my amazing lab mates, Jiamin Song, Simona Kobrinsky, Grace Kraatz, Iris Guo, Michael Ru, and Alyssa Guerra, for being so generous with their time and resources. Support from the Canadian Institute of Health Research made this project possible. Finally, a huge thank you to my parents, family, and friends who supported me throughout this time and helped me come this far!

Contribution of authors

During this project, I have had the opportunity to collaborate on the experimental design with Dr. Walker to conceive the experiments presented in this thesis. I have conducted these experiments with help from Hong Long, whose assistance was crucial in creating a specialized RNAscope protocol for our purposes. I took care of the animals and aided with the tissue collection alongside Hong and Dr. Walker. I performed the data analysis and have written this thesis with help from Dr. Walker, who kindly reviewed and improved it using her expertise.

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ELS	Early life stress
PL mPFC	Prelimbic medial prefrontal cortex
IL mPFC	Infralimbic medial prefrontal cortex
BLA	Basolateral amygdala
vHipp	Ventral hippocampus
PV IN	Parvalbumin interneuron
PNN	Perineuronal net
PN	Principal neuron
SOM	Somatostatin
PND	Postnatal day
E/I	Excitatory-inhibitory
CSPG	Chondroitin sulfate proteoglycan
MMP	Matrix metalloproteinase
ROS	Reactive oxygen species
LB	Limited bedding
NB	Normal bedding
WFA	Wisteria floribunda agglutinin
VGAT	Vesicular GABA transporter
VGLUT1	Vesicular glutamate transporter
ANOVA	Analysis of variance
SEM	Standard error of the mean
DCX	Doublecortin
BrdU	Bromodeoxyuridine
NSC	Neural stem cell

1. Background & Introduction

Individuals who are exposed to early life stress (ELS) are more susceptible to developing certain neuropsychiatric disorders, like anxiety and depression, later on in life[1]. Approximately 50% of adults with depression report at least one type of childhood trauma, like physical or emotional neglect or physical or sexual abuse, whereas only 20% of healthy controls report childhood adversity[1]. Children who were neglected during infancy and early life continue to exhibit increased levels of anxiety, even years after moving to a stable home[2]. These disorders are often associated with altered neurotransmission in key brain regions dedicated to emotional regulation, namely the corticolimbic system[3]. Indeed, the corticolimbic regions, including the prelimbic (PL) and infralimbic (IL) medial prefrontal cortex (mPFC), basolateral amygdala (BLA) and ventral hippocampus (vHipp), are highly influenced by the environment during early life and thus susceptible to early stressors, which can lead to aberrant brain development[4]. Notably, evidence from both human and rodent work indicates for instance, that the mPFC-BLA connectivity is altered after ELS and that the BLA becomes hyperactive in response to ELS[2,5–7]. As a result, ELS-exposed humans and rodents display more anxiety-like symptoms in response to fearful stimuli[2,5,6], suggesting that the neuronal changes occurring during ELS might contribute to symptoms of neuropsychiatric disorders. Alterations to inhibitory interneurons could be especially harmful because they regulate principal neurons at the soma and proximal dendrites, thus influencing the activity of the corticolimbic regions and connections. Since inhibitory interneurons, particularly parvalbumin expressing interneurons (PV INs), are primary regulators of corticolimbic activity and plasticity, we hypothesized that ELS might disrupt PV IN development in key regions such as the mPFC and BLA, leading to dysregulated perception and processing of threats in the long term. In this project, we will focus on how ELS influences PV INs in the male and female corticolimbic circuit in order to better understand the long-lasting consequences of altering normal development.

1.1. Corticolimbic circuitry

The corticolimbic system is essential for emotional regulation and fear processing in mammalian species including humans, nonhuman primates and rodents[4,8,9]. The circuit of fear shown in Figure 1

illustrates the neuronal connections between the mPFC, BLA, and vHipp, which are necessary for fear conditioning, extinction, and fear memory retrieval. Specifically, reciprocal connections between the PL mPFC and BLA drive fear conditioning, while IL mPFC-BLA connections promote fear extinction[10], indicating that connections between these structures are essential for fear acquisition. The vHipp projects to the mPFC and forms reciprocal connections to the BLA, which are necessary for providing contextual information[11] and fear memory consolidation and retrieval[12]. On a neuronal level, inhibitory interneurons in the BLA, mPFC, and vHipp strongly influence principal excitatory neurons (PNs) that connect these structures and extend to other brain regions to influence emotional regulation[13]. Glutamatergic projections from the vHipp end on principal neurons and interneurons in the mPFC and BLA, and PNs from the BLA project to other PNs in the vHipp to retrieve fear memories[12]. Projection neurons from the BLA target both PNs and inhibitory interneurons in the mPFC, and together with reciprocal connections from the mPFC to the BLA, these connections facilitate learned fear behaviour[10]. Additionally, the BLA projects to inhibitory interneurons, including somatostatin and PV INs, in the mPFC to drive feedforward inhibition[14]. Evidently, PN activity in the BLA is important in regulating circuit activity.

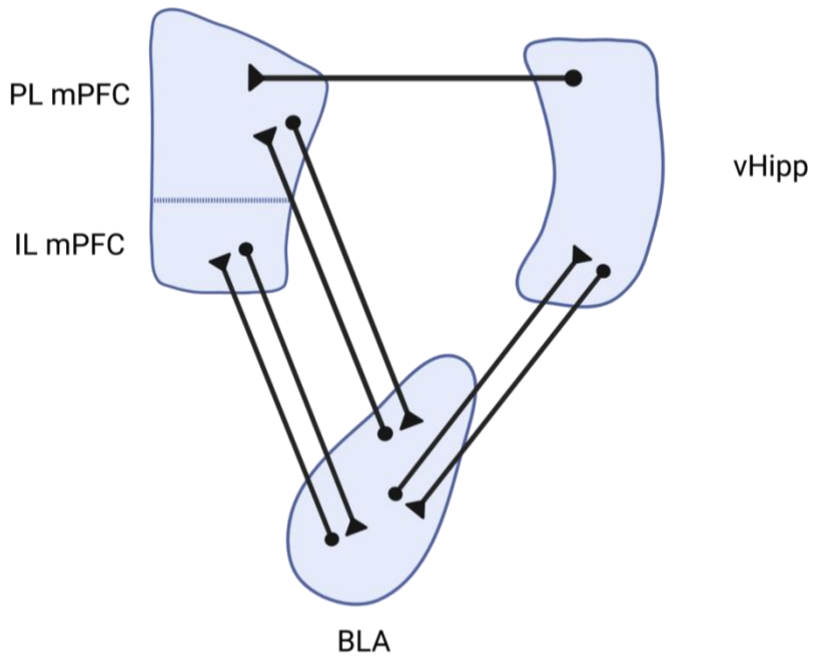

Figure 1. Projections between corticolimbic regions in the rodent brain. The basolateral amygdala (BLA) bidirectionally projects to the prelimbic (PL) and infralimbic (IL) medial prefrontal cortex (mPFC) and the ventral hippocampus (vHipp). The vHipp also projects to the PL mPFC, but receives no reciprocal inputs from the mPFC. Figure adapted from Guadagno, Kang, et al., 2018.

Principal neuron activity in the BLA is highly influenced by PV INs in the BLA because they constitute the majority of interneurons and regulate principal neurons at the soma and proximal dendrite sites, unlike somatostatin (SOM) neurons that inhibit principal neurons at more distal dendrites[11,13]. As shown in Figure 2, PV INs synapse on PNs close to the cell body, which strongly influences firing activity of PNs. Conversely, SOM interneurons synapse on the apical dendrites of PNs, which are further away from the cell body and have less influence on neuronal activity. Additionally, because of these structural differences and since PV INs have fast-spiking activity, they better modulate PN spiking activity compared to SOM neurons, which are more involved in regulating prolonged stimulation[13]. Corticolimbic circuitry can be disrupted by environmental influences like ELS, leading to aberrant connectivity. For example, juvenile and adult rodents exposed to ELS exhibit reduced vHipp volume and altered resting-state functional connectivity[7]. Previous results from our lab and that of others have documented a reduction in mPFC-BLA connectivity, complex alterations to vHipp-BLA circuitry, and hyperconnectivity of the mPFC with the vHipp[7,15]. Animals exposed to ELS are vulnerable to corticolimbic modifications because these connections are disrupted during critical periods in their development. Particularly, disruptions in mPFC-BLA circuitry can impact the formation of learned fear.

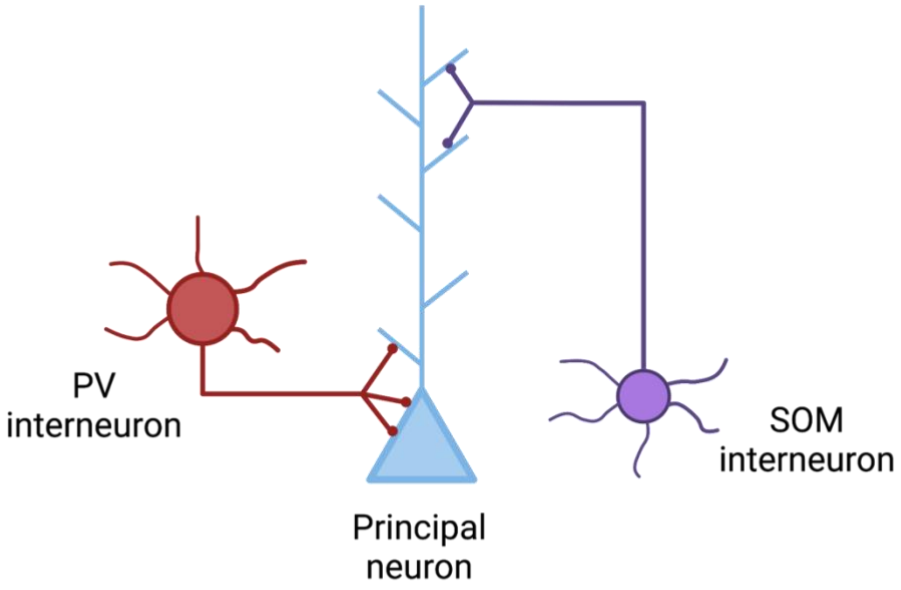

Figure 2. Dendrite sites where PV and SOM interneurons regulate PNs. PV INs regulate principal neurons at the soma and proximal dendrites, whereas SOM neurons regulate PNs at the distal dendrite sites. Figure created with BioRender.com.

1.2. Development of the mPFC-BLA circuit and critical periods

The mPFC-BLA circuitry is especially sensitive to environmental influences because connections between these regions mature postnatally. Ascending projections from the BLA to the mPFC form postnatally starting from postnatal day (PND) 7-9, while descending projections from the mPFC to the BLA develop from PND13-21 onwards [16], which results in delayed inhibition of fear responses compared to fear perception. Until mPFC-BLA connections are fully developed, offspring rely on caregiver presence to dampen BLA activity and regulate emotions in stressful situations[17]. Several models of ELS in rodents involving maternal separation or fragmented care indicate that pups reared in these conditions experience chronic stress and exhibit cognitive, social, and emotional deficits in adulthood[18]. Continuous quality care throughout early life is important because structures develop sequentially, so the activity of the BLA will influence the development of the mPFC, which matures later on[19]. The BLA and mPFC continue to develop into the late juvenile period and do not mature until adolescence, meaning that these regions remain sensitive to environmental influences due to a heightened state of plasticity.

Indeed, this heightened state of plasticity marks the critical period during which neurons are labile and therefore more susceptible to ELS. During critical developmental periods, external stimuli must align with cellular events in the neuron to promote experience-dependent learning of essential behaviours, like sensory processing, social behaviour, and emotional regulation[3,20]. To illustrate, Takao Hensch's seminal work in the visual cortex showed that mice experiencing visual deprivation during early life exhibited a delay in critical period opening and eyesight development because the inhibitory circuitry in the visual cortex was not properly formed[21]. The switch of GABAergic signals from excitatory to inhibitory that initially opens the critical period of plasticity is essential for selecting the most salient excitatory connections to mature[20]. Without environmental experience to stimulate PV INs, the number of synapses to principal neurons are decreased, the connections are weakened, and electrophysiological properties remain immature[19]. At the same time, abnormal environmental stimulation perhaps leads to the strengthening of aberrant neuronal networks, for example, the visual cortex of pups reared with one eye covered develops to favour representation of the uncovered eye. Evidently, neuronal circuitry does not form

properly if the critical period does not align with appropriate sensory experiences, suggesting that appropriate emotional responses may be impaired if ELS disrupts the critical period of mPFC and BLA development. In fact, rat pups deprived of sensitive caregiving during early life display impaired social behaviour towards their mother[22] and peers later on[23], increased anxiety-like behaviour[6], and hyperactivity in the right BLA[24].

Because inhibitory interneurons consolidate the activity and connectivity of PNs during the critical period, the observed increase in excitability of principal BLA neurons after ELS is possibly caused by a dysfunction in inhibitory interneuron activity. Indeed, PV INs are particularly influential for principal neuron activity, in both the BLA and mPFC, because they regulate principal neurons near the soma[10] and contribute to the excitatory-inhibitory (E/I) balance of the circuit[19] by producing oscillatory activity in the gamma frequency band. With the correct E/I balance, the critical period of plasticity opens and is maintained by a gradually increasing inhibitory tone[19]. However, the critical period is delayed if the inhibitory tone is lacking, as was observed in juvenile GAD65 knockout mice that were unable to acquire the functional connectivity associated with environmental stimuli at the appropriate time[25]. Similarly, chemogenetic inhibition of PV INs in adult rats reopens the critical period, so both studies reveal that inhibitory tone is necessary for brain structures to form in juveniles and remain stable in adulthood[26]. Alterations to PV IN due to ELS during the critical period could affect corticolimbic circuit maturation, rendering certain regions vulnerable to negative environmental experiences at times that are inappropriate for optimal circuit development. The closure of the critical period of plasticity coincides with PV IN maturation[3,27] and the appearance of perineuronal nets (PNNs)[28], mostly around PV INs.

1.3. Perineuronal net structure and function

At the end of the critical period, neuronal activity is stabilized by the consolidation of synaptic inputs, which occurs through the formation of PNNs around PV INs. PNNs are carbohydrate components of the extracellular matrix that form a lattice-like structure around neurons to stabilize synaptic inputs and reduce plasticity at the end of the critical period[29]. The detailed analysis of the PNN structure shows that a carbohydrate backbone composed of the polysaccharide hyaluronan is connected to chondroitin sulfate

proteoglycans (CSPGs), such as aggrecan and brevican, by link proteins, such as HAPLN1[28] (as shown in Figure 3). Specific components of PNNs serve various important functions, for example, HAPLN1, tenascin-R, and aggrecan are essential for protecting enveloped neurons from oxidative stress[30]. Mice with these components knocked out showed evidence of neuronal degeneration after the administration of the toxic agent $FeCl_3$. Furthermore, deleting brevican significantly reduced excitatory synaptic input to PV INs, resulting in altered E/I balance[31]. These essential PNN components are produced both by neurons and glial cells, in particular, neuronal cell surface enzymes produce the hyaluronan backbone, and astrocytes produce CSPGs[28]. However, because PNNs dynamically interact with the neurons they encapsulate, the exact composition of PNNs and developmental trajectory varies according to brain region[28]. For example, PNNs in the hippocampus showed higher signal intensity of aggrecan and brevican compared to PNNs in the amygdala[32]. Despite variability in PNN composition, PNN generally serve a similar function throughout the brain.

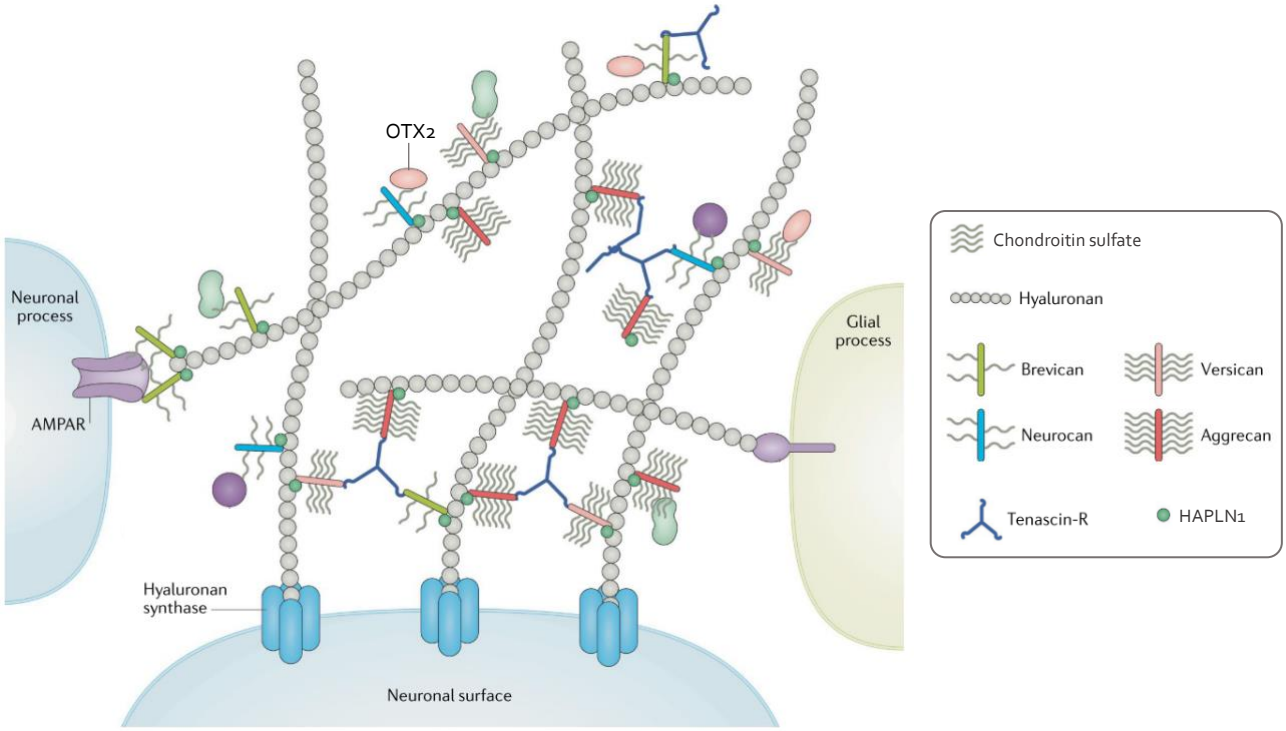

Figure 3. PNN structure and components. PNNs are carbohydrate structures that create a lattice-like formation around neurons to stabilize their synaptic input and protect against oxidative stress. PNNs are composed of a hyaluronan backbone attached to chondroitin sulfate proteoglycans (CSPGs) like brevican, neurocan, versican, and aggrecan, by link proteins like HAPLN1. The various PNN components are produced by neurons and glia. Figure adapted from Fawcett, Oohashi, & Pizzorusso, 2019.

Two major functions of PNNs are to strengthen synaptic inputs to the encapsulated neuron and to protect the neuron from oxidative damage. PNNs stabilize afferent inputs by regulating plasticity and controlling which molecules reach the encapsulated neuron. PNNs facilitate communication between brain regions by binding molecular messengers circulating within the ECM and delivering these molecules to the encapsulated neuron[33]. For example, PNNs are essential for delivering Otx2 circulating in the cerebrospinal fluid to PV INs in order to promote their maturation[33]. Another major function of PNNs is to protect the neuron from toxins in the extracellular matrix and oxidative stress. The anionic structure of PNNs repels oxidative cations like Fe^{3+} that could damage the encapsulated neuron[33]. As PNNs develop, they become denser, thus restricting the movement of growth factors, cell surface receptors, and ions around the encapsulated cell[34], which confers additional protection against toxicity, but also reduces synaptic plasticity by forming a compact structure that blocks AMPAR motility[33]. Thus, at the end of the critical period, the formation of mature PNNs consolidates PV IN activity and in turn, stabilizes the activity of efferents from these neurons to principal glutamatergic cells.

In most brain regions, PNNs primarily surround PV INs to stabilize the fast-spiking activity of PV INs and support the closure of the critical period. However, studies have shown that PNNs are also found around principal neurons in various brain regions like the cerebellum[35], hippocampus[36,37], amygdala[38], the parietal cortex[39] and sensory cortices[40], where their function is lesser known. The population of excitatory neurons encapsulated by PNN has not been studied extensively, but a study by Morikawa et al. investigating the amygdala found that more excitatory cells with PNN expressed c-Fos after fear conditioning compared to those excitatory cells without PNN in adult mice[38]. Since they found that PV cells with or without PNN showed no difference in c-Fos activation following fear conditioning, they proposed that PNN-expressing excitatory cells are more likely to be recruited during fear conditioning[38]. However, one major limitation of this study is that excitatory neurons were labelled with CAMKII, which has been shown to bind to a population of inhibitory interneurons as well[41]. Thus, a more specific method of excitatory cell labelling, like in-situ hybridization, should be used to investigate PNN colocalization with excitatory cells. Additionally, the effect of ELS on the population of excitatory cells with PNN has not yet been studied.

Excitatory cells without PNN might be functionally different from those with PNN because their activity and connectivity are more malleable.

Indeed, the formation of a mature PNN around a neuron stabilizes its synaptic inputs and activity, however these connections can become labile and plasticity can be restored by degrading PNN components, for instance, by administering chondroitinase ABC, an enzyme that will dissolve PNNs. A prime example of this has been documented by Gogolla and colleagues where chondroitinase ABC treatment to the BLA of adult mice was able to restore a period of fear memory erasure that is generally only observed in early developing rodents[42]. These results illustrate the integral role of PNN in consolidating fear memories following the critical period. Furthermore, the removal of particular PNN components by more specific agents also impairs synaptic and behavioural functioning[43]. Knocking out core PNN components, like chondroitin 6-sulfate or CSPGs, reduced hippocampal excitatory and inhibitory postsynaptic currents[44], and led to premature memory loss[45] in mice. Complete removal of PNNs has dramatic effects on memory retention and fear behaviour, but even dissolving key components considerably alters synaptic stabilization by PNNs. Evidently, the structural integrity of PNNs is necessary for proper functioning of the neurons they ensheathe. As an animal matures, PNNs improve in structural integrity and increase in density in most brain regions.

1.4. Sex specific alterations to PNN following ELS

In the mPFC and BLA, PNNs emerge around PND 14 and PND18, respectively, and increase in number and structural integrity until adulthood[28,46]. PNN colocalization with PV INs in the mPFC accelerates from the juvenile to adolescent period, and in the BLA continues to increase into adulthood in both males and females[47,48]. However, sex differences emerge if rats are exposed to ELS, perhaps due to the organizational effects of sex steroids during the perinatal period in rodents. Our previous data have shown that after ELS, PNN around PV INs increased in density only in the right BLA of males on PND28-29[24] and decreased in the mPFC of both males and females on PND20[49]. The corticolimbic system undergoes sexual differentiation during infancy[50], which may interact with the early environment to produce sexually dimorphic phenotypes expressed in ELS animals. Shortly before birth, a surge of testosterone in

male rodents masculinizes the brain via aromatization to estradiol and activating estrogen receptors, while the lack of estrogen receptor activation in female rodents feminizes the brain[50]. Since estrogen and androgen receptors are found on glutamatergic principal neurons and GABAergic interneurons in the amygdala, gonadal hormones can differentially alter neurotransmission in males and females[50]. Other factors, like microglial activation, can also produce sex differences in the brain and are implicated in sexually differentiated endpoints, like synaptic pruning[51]. Dendritic pruning occurs earlier in the mPFC of females compared to males, indicating that some aspects of development occur at different rates between the sexes[50]. Thus, the timing of ELS can interact with an organism's developmental stage to produce sex differences. Additionally, microglia secrete matrix metalloproteinases (MMPs) that degrade PNN components[28,52], so sex differences in microglial activation could also confer sex differences to the maintenance of PNN following ELS. Sex differences in the developing brain can alter the maturational trajectory of corticolimbic circuitry.

The observed PNN alterations in juveniles may indicate that ELS interacts with sex differences to produce changes in brain maturation. In the male BLA, ELS accelerates the maturation of PNNs[24], which may have a protective function and improve a young animal's resilience to stress. However, in the male and female mPFC, ELS is associated with a decrease in PNN[47], which may maintain a higher state of plasticity in these regions. Increased plasticity renders these regions more vulnerable to negative environmental experiences like ELS, which may be maladaptive. Male rats seem to suffer greater negative consequences of ELS in the BLA while females are unaffected in this region. This discrepancy in the demonstrated vulnerability of the sexes could be explained by each study's use of different ELS paradigms where females might be more sensitive to maternal separation and males more affected by limited bedding. Nevertheless, this research confirms that ELS differentially impacts males and females with respect to PNN maturation.

Notably, sex differences are also apparent regarding hemispheric alterations of PNN following ELS. Previous work from our lab has indicated that PNN are altered by ELS in a hemispheric-dependent way where the density of PNN surrounding PV INs increased in the BLA of PND28-29 only in male rats

and only on the right side[24]. The right BLA is known to be more involved in fear processing in humans[53], but lateralization of function is not as well documented in rodents. Nevertheless, some research suggests hemispheric differences in the rat fear responses since lesions to the right BLA produce greater deficits in fear learning than lesions to the left BLA[54]. Additionally, male PND18 rats exposed to ELS exhibited more resting-state fMRI connectivity alterations of the right versus left BLA to other brain regions, including the mPFC and hippocampus[7]. Since the BLA forms contralateral and ipsilateral connections to the mPFC, alterations in BLA activity conferred by changes in PNN density could affect the development of the mPFC. Like the BLA, functions in the mPFC are lateralized, where the right mPFC is more involved in stress reactivity[55]. Thus, ELS-induced alterations in the mPFC might also exhibit interactions between sex and hemisphere, but few studies have investigated lateralization effects regarding how ELS alters PNN density in the BLA and mPFC.

ELS also results in sex differences in the fluorescent intensity of PNNs, which is a proxy for their structural integrity and complexity. In the mPFC, adult males exposed to ELS showed an increase in intensity of PNNs surrounding PV cells[47], whereas adult females exposed to ELS exhibited a decrease in intensity of PNNs encapsulating PV INs[56]. In the BLA, only juvenile males displayed an increase in PNN intensity following ELS[24]. Essentially, PNNs that appear more intense have a higher density of components surrounding the neuron, which improves protection against oxidative stress[43]. Although alterations in PNN intensity occur independently from PNN cell density changes[57], PNN intensity typically increases with animal maturity[58], mirroring the increase in the number of PNN-encapsulated cells throughout development. In the mPFC, PNN intensity progressively increases from the juvenile period to adulthood in male and female rodents, while in the BLA, increases in intensity are seen between adolescence and adulthood[47]. PNN intensity in adulthood can be modulated by environmental stimuli since chronically stressed adult rodents presenting with a depressed phenotype exhibited an increase in the deposition of PNN components in the hippocampus, namely the CSPGs neurocan and brevican[59,60]. Thus, an increase in PNN components perhaps occurs to protect neurons from oxidative stress, but may also have detrimental consequences. For example, one study showed that reducing PNN structural integrity may have positive

outcomes, like reducing depressive symptoms. They found that partially degrading PNNs by activating an endogenous enzyme, matrix metalloproteinase 9 (MMP-9), increased excitatory activity in the cortex of mice, thus improving depressive indications[61]. Indeed, a delicate balance of PNN density and structural integrity is required to maintain proper circuit function and protect neurons against oxidative stress.

1.5. Oxidative stress

The structural integrity of PNNs surrounding PV INs is particularly important as PV INs are especially susceptible to oxidative stress because of their fast-spiking activity requiring a high metabolic demand and large number of mitochondria[62]. During energy production, mitochondria release reactive oxygen species (ROS) that can inflict oxidative damage on PV IN if produced in excess. ROS production increases during chronic stress due to the increased energy demands on mitochondria[63]. To satisfy the additional energy demands imposed by stress, mitochondria may undergo fission[64], a process that allows for a large increase in mitochondria within the cell[65] and as a consequence, a higher production of energy for the cell. Changes in mitochondria have been documented to occur after exposure to early adversity[66–69]. Following ELS, alterations in mitochondrial enzyme activity, mtDNA copy number and protein expression have been observed[66–68]. Indeed, a recent study by Ruigrok et al. found that ELS altered the expression of two genes, *Dnm1l* and *Fis1*, that regulate mitochondrial fission on P9 in the hippocampus and increased expression of *Fis1* in adult rats[70]. As depicted in Figure 3, *Dnm1l* encodes dynamin 1-like protein, which initiates fission and is recruited by the fission, mitochondrial 1 protein encoded by *Fis1*. Since fission is associated with increased ROS, this evidence of upregulated fission genes suggests a heightened oxidative state of the hippocampus following ELS. However, the oxidative state following ELS of other corticolimbic regions, like the mPFC and BLA, have yet to be investigated.

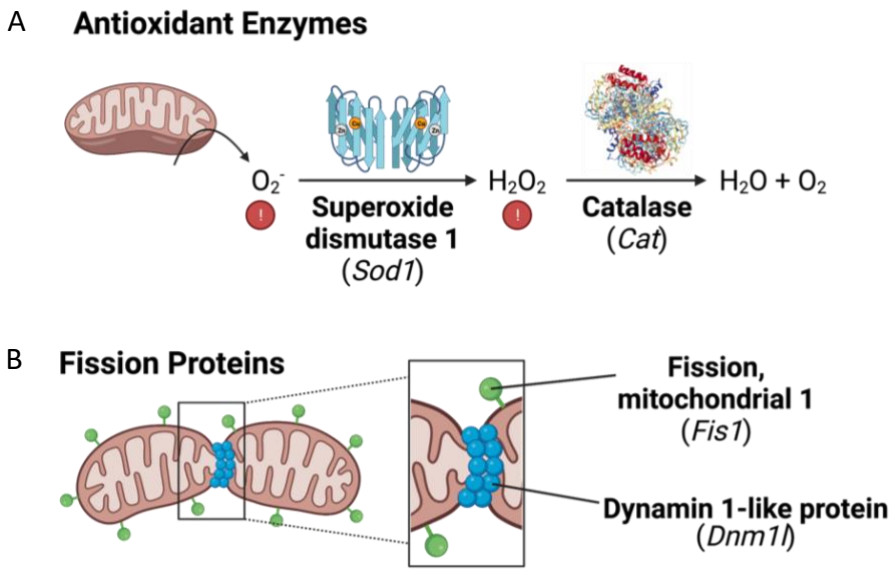

Figure 4. Antioxidant enzymes and fission proteins in the mitochondria. **A:** During energy production, mitochondria generate ROS, like the superoxide radical (O_2^-). The enzyme superoxide dismutase, which is encoded by the *Sod1* gene, breaks down the superoxide radical to hydrogen peroxide (H_2O_2), which is then broken down to innocuous molecules by the catalase enzyme (*Cat* gene). **B:** Dynamin 1-like protein, encoded by the *Dnm1l* gene, is recruited by fission, mitochondrial 1 (*Fis1* gene) to initiate fission. The process of fission generates ROS. Figure created with BioRender.com.

Due to the increase of ROS during stress, antioxidant enzymes like catalase and superoxide dismutase 1 (SOD1) must provide additional defense against ROS to maintain cellular functions[70]. However, stress may also impair the antioxidant capacity of a cell. Ruigrok et al. showed an increased expression of the *Cat* gene encoding catalase and decreased expression of *Sod1*, encoding superoxide dismutase 1, at PND9 after ELS exposure, variations that were not maintained in adulthood[70]. The upregulation in *Cat* might suggest an adaptive mechanism to cope with additional ROS induced by stress, but the decreased expression of *Sod1* suggests that increased levels of the superoxide anion could damage the cell (Figure 4). Excess ROS can impair mitochondrial function by damaging mitochondrial DNA, leading to altered production of respiratory chain enzymes, which affects energy production[63]. Many studies have confirmed that stress negatively impacts mitochondrial function in various brain regions, like the cortex and hippocampus, however the majority of these studies have been performed in male rodents[67]. One recent study investigating sex differences found that maternal separation during the first 3 weeks of life decreased mitochondrial energy metabolism in the PFC of female rats only[66]. Specifically, they found impaired activity of cytochrome c oxidase, an enzyme that consumes oxygen and generates energy as a member of the electron transport chain.

Other components that impact the function of PV IN, like DNA and synaptic proteins, are also sensitive to oxidative stress. For example, DNA oxidation marked by 8-oxo-dG is associated with down-regulated function of PV+ neurons[71] and it was found that maternal separation increases the amount of PV INs colocalizing with 8-oxo-dG in the mPFC of juvenile rats[72]. Furthermore, maternal separation altered the levels of proteins involved in glutamate signaling in adult rats[73], which may impact synaptic transmission. Indeed, the deletion of aldehyde dehydrogenase, a mitochondrial protein that protects against lipid peroxidation, decreased synaptic boutons and active zone area while the rest of the neuron was unaffected[74]. This evidence suggests that changes in mitochondrial protein expression can specifically alter synaptic transmission in disease.

Interestingly, ELS alterations to mitochondrial proteins seem to be more pronounced in juveniles compared to adult mice[68]. Directly following ELS at postnatal day 9, male mice exhibited altered expression

of several genes related to antioxydation in the hippocampus, but many of these changes disappeared by 10-12 months of age[70]. Unlike juveniles who may need to compensate for excess ROS using antioxidant mechanisms, adult animals are perhaps better protected against oxidative stress by their fully formed PNN. Chondroitin sulfate, a primary component of PNNs, has been shown to protect human neuroblastoma cells against oxidative stress by stimulating PKC/pAkt pathway and enhancing the synthesis of the antioxidant protein heme oxygenase-1[75]. Although plenty studies demonstrate the protective role of PNN against oxidative stress[30,76,77], more research is required to understand how ELS-induced alterations to PNN might associate with oxidative stress in the mPFC and BLA of males and females.

Ultimately, ELS impacts emotional development by altering neuronal and glial components of the corticolimbic system. In particular, PV INs are modified by ELS in important ways, notably through changes in PNN density, formation (intensity) and oxidative state. Even so, the scope of these changes has not been fully investigated, nor do we understand the precise role of PV INs to increase vulnerability to psychopathologies after exposure to ELS. Evidently, ELS causes oxidative damage to PV INs in the corticolimbic regions[71–74], however it remains unclear whether PV INs surrounded by PNNs are more protected from oxidative stress induced by ELS. Additionally, studies have yet to investigate if ELS modifies the distribution of PNNs around specific neuronal types, thereby enhancing their protection. Research has already shown that ELS changes PV IN and PNN colocalization in certain regions and varies between the sexes[24,47,49], but mechanisms underlying the effects of ELS on PV IN dysfunction remain unknown. In order to address some of these questions, we will examine the BLA and mPFC regions of male and female juvenile rats to determine if ELS alters the cell type harbouring PNNs and if oxidative pathways are altered as a function of PNN abundance. Because we previously found that ELS induced sex-dependent increases in PNN density in the BLA, but only on the right BLA, we will consider both sex and hemispheric differences in our studies.

2. Specific Aims

Previous work from our lab indicates that exposure to early adversity using the limited bedding paradigm increases PNN in the rat BLA in a sex- and hemispheric- dependent way[24]. However, we do not know whether ELS produces similar effects in other regions of the corticolimbic circuit and which is the phenotype of cells ensheathed by PNN. Previous studies using another model of early adversity (maternal separation) have documented changes in PNNs in the mPFC[47,49], but none of these studies have examined the distribution of PNNs around excitatory or total inhibitory cells. *Therefore, the first aim of this project is to determine if ELS modifies the distribution of PNN around inhibitory and excitatory cells in the BLA and mPFC, and if sex and hemispheric differences seen in the BLA are also observed in the mPFC.* To investigate this, we will use *in-situ* hybridization (RNAscope) to identify cells expressing VGAT and VGLUT1 to label inhibitory and excitatory neurons respectively, coupled with immunohistochemistry for *Wisteria floribunda* agglutinin (WFA) to label PNN. We have chosen to label all inhibitory cell types using VGAT because we are interested in comparing the ratio of PNN around excitatory and total inhibitory cell populations. Although PNN are known to primarily encapsulate PV INs, other types of GABAergic interneurons also harbour PNNs in juvenile rats[39,78]. We will measure cell density as well as the ratio of glutamatergic and GABAergic neurons surrounded by PNN in juvenile rats (PND28-30).

It is recognized that one of the multiple functions of PNN is to protect neurons from oxidative molecules in the neuronal microenvironment and to lower local oxidative stress[58]. This helps regulate neuronal activity, in particular that of the PV neurons[79]. Exposure to ELS has been associated with altered expression of genes coding for antioxydant enzymes in the hippocampus of PND9 rodents [70], but it is unclear whether these changes are correlated with changes in the density of PNNs and if brain regions of the corticolimbic circuit are affected. *Thus, the second aim of this project is to determine if ELS changes the oxidative state in the mPFC and BLA, and how changes in PNN might correlate with oxidative stress protection.* To address this aim, we will measure by qPCR the expression of specific antioxidant enzymes such as catalase (*Cat*) and superoxide dismutase 1 (*Sod1*) as well as the expression of fission proteins, *Fis1* and *Dnm1l* that participate in the regulation of the mitochondria pool within activated cells. We will

initially compare expression of these proteins in whole tissue extracts of the BLA and mPFC of juvenile rats, followed up by specific mitochondrial expression of these genes in continuing studies.

3. Methods

3.1. Animals

Pregnant female Sprague Dawley rats (Charles River, Kingston, USA) were received in our animal facility on GD14 and kept on a 12h reverse light cycle (lights off at 9:00, lights on at 21:00) with *ad libitum* access to rat chow and water. The day of parturition was considered postnatal day (PND) 0 and on PND1 litters were culled to 10 pups each with 5 males and 5 females if possible. Pups were weaned and housed with their same-sex littermates on PND21. Mothers and pups were weighted on PND1, 4, 10, 14, 21 and 28. 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. Limited bedding paradigm

Early life stress was induced in pups using the limited bedding (LB) paradigm, which has been documented to induce fragmented maternal care and lead to cognitive, social, and emotional deficits in pups[18]. From PND 1-10, litters were randomly assigned to either LB or normal bedding (NB) conditions. Litters in the LB condition were given half a piece of paper towel as bedding material and placed on a metal grid above one inch of sawdust, whereas NB litters are given one inch of sawdust as bedding material in addition to half a piece of paper towel. On PND10, all litters were returned to NB conditions. Maternal behaviour was recorded from PND5-6 to analyze characteristics of maternal care in NB and LB litters (i.e., nursing time, pup grooming, self-grooming, and fragmentation of maternal care). Trained raters scored maternal behaviour on the minute for 60 minutes at four timepoints during the light and dark phases (2 per phase).

3.3. Tissue collection and allocation

On PND28, a total of 41 pups from 12 different litters were sacrificed either by intracardiac perfusion with paraformaldehyde (PFA) for RNAscope/immunohistochemistry (IHC) imaging studies or rapid decapitation for quantitative PCR analysis. For the imaging studies, a total of 17 pups were anesthetized with a cocktail of ketamine-xylazine (150ul, sc) and perfused (under basal conditions) with 0.9% saline-heparin solution (5min) followed by 4% PFA (20min). Brains were extracted and placed in 4% PFA overnight. The following day, brains were placed in a 30% sucrose solution for dehydration (1-2 days at 4^{o}C) and then were kept frozen at -80^{o}C. For qPCR studies, the remaining 24 pups were sacrificed by rapid decapitation and fresh brains were rapidly collected and snap frozen in isopentane on dry ice before storage at -80^{o}C.

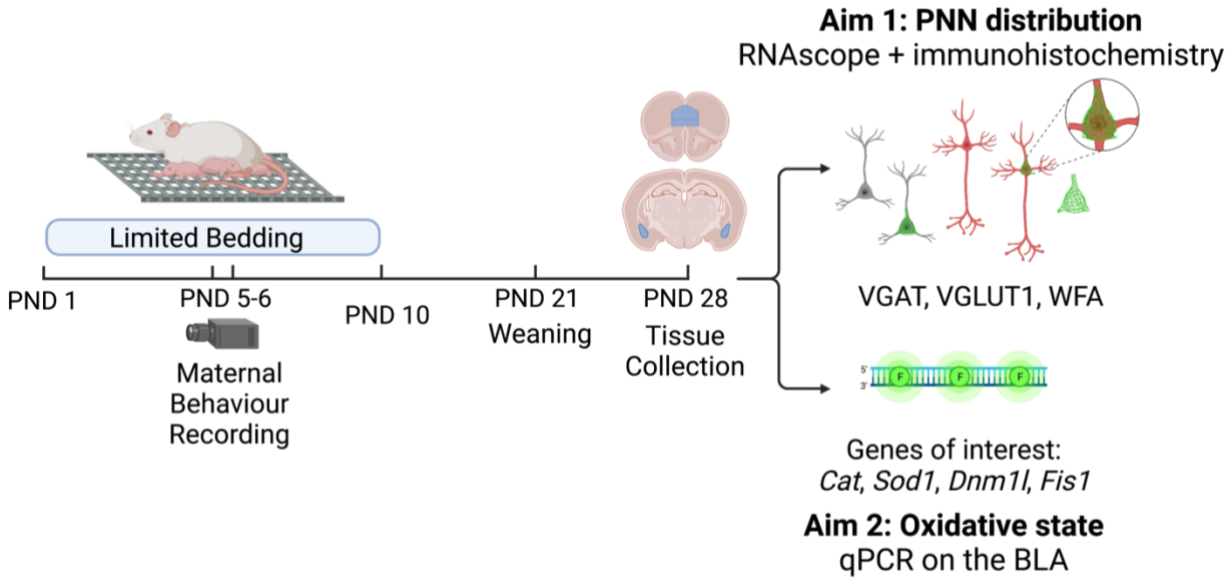

Figure 5. Experimental timeline. Litters were assigned to limited or normal bedding from postnatal day (PND) 1-10. Maternal behaviour was recorded from PND5-6 and pups were weaned on PND 21. On PND28, brains were collected and tissue from the mPFC and BLA were processed for experiments addressing our two aims. For aim 1 we used RNAscope/immunohistochemistry to label neurons containing the vesicular GABA transporter (VGAT), vesicular glutamate transporter 1 (VGLUT1) and PNN using *Wisteria floribunda* agglutinin (WFA). For aim 2, we used qPCR (quantitative polymerase chain reaction) to identify the expression of antioxidant (*Cat*, Sod1) and fission genes (*Dnm1l*, *Fis1*). Figure created with BioRender.com.

3.4. In-situ hybridization (RNAscope) and immunohistochemistry for PNN

Perfused brains were sliced on a cryostat at 20μm onto Superfrost charged slides. Sections containing the mPFC and BLA regions were collected and 5 high quality sections per animal were selected for RNAscope processing: 2 sections of the mPFC at Bregma level 3.72mm and 3.00mm, and 3 sections of the BLA at Bregma level -2.04mm, -2.52mm, and -3.12mm, according to the Paxinos & Watson atlas of the rat brain[80]. To ensure that glutamatergic and GABAergic cells are marked specifically, we used *in-situ* hybridization to identify cells expressing VGLUT1 (Slc17a7: sodium-dependent inorganic phosphate cotransporter member 7) and VGAT (Slc32a1: GABA vesicular transporter member 1), as opposed to immunostaining excitatory cells with CAMKII. Recent evidence suggests that certain inhibitory interneurons are also CAMKII+, which would result in erroneous marking of excitatory neurons[41]. The RNA *in-situ* hybridization was performed using the RNAscope Multiplex Fluorescent Reagent Kit v2 Assay probes and reagents (ACDBio, 323100) according to the manufacturer instructions with some modifications to the pretreatment. Slides were baked for 30 minutes at 60℃ in a circulating air oven to ensure that sections were dry and flat on the slide. Sections were post-fixed in 4% PFA for 30 minutes at 4℃ and subsequently dehydrated in increasing concentrations of ethanol at room temperature. To quench endogenous peroxidase activity, the sections were treated with 1% H_2O_2 for 10 minutes at room temperature. Tissues were permeabilized by incubation in RNAscope target retrieval reagent heated in a steamer at approximately 99℃, then dehydrated in 100% ethanol for 3 minutes and dried for 5 minutes at 60℃ in a circulating air oven. Protease application was omitted to maintain the integrity of PNNs. Probes for VGLUT1 (317001, Rn-Slc17a7, ACDBio) and VGAT (424541-C2, Rn-Slc32a1-C2, ACDBio) were hybridized for 2 hours in humidity-controlled oven (HybEZ II, ACDBio) at 40℃. Reagents provided in the Multiplex Fluorescent Reagent Kit for signal amplification and probe-specific HRP detectors were added with Opal dyes (Opal 570: OP-001003, Akoya Biosciences; Opal 690: FP1497A, Perkin Elmer) diluted 1:1000 to visualize the signal. For the subsequent IHC to visualize PNNs, slides were washed with PGT (1x PBS: 0.27mM KCl, 13.7mM NaCl, 1.19mM phosphates; 0.2% gelatin; 0.25% Triton X-100) and incubated with WFA (FL-

1351, Vector Laboratories) diluted 1:500 for 30 minutes at room temperature. After washing in PBS, slides were coverslipped using Fluoromount-G mounting medium, with DAPI (00495952, Invitrogen).

3.4.1. Microscopy

Images of the RNAscope/ IHC stained sections were captured as z-stacks (9 slices with 1.14μm spacing) at 20x magnification on an Olympus FV1200 confocal microscope. Multi-area time-lapse imaging was used to acquire tiled pictures of the entire PL, IL mPFC and BLA regions on both brain sides. Laser power and detection parameters were consistent across animals with laser intensities of 0.4% for 405 nm wavelength (DAPI), 2.0% for 488 nm (WFA), 0.1% for 543 nm (VGLUT1) and 1.9% for 635 nm (VGAT).

3.4.2. Cell Quantification

Using ImageJ software, z-stacks were converted to max intensity z-projections and all 4 channels were aligned and merged using the Big Warp plugin. Cell counting was done on QuPath software[81] using a machine learning object classifier trained to identify VGLUT1+ and VGAT+ cells through training on 48 images. Validation of the classifiers on 11 images showed an average of 35% error. Although this may be a large margin of error, the classifiers were used consistently across all images, which allows us to assess differences among groups accurately even if the absolute values are not precise. Thus, this limitation should not affect the validity of our results.

To quantify the cell types of each region, the IL and PL mPFC, and BLA regions were outlined and cells were identified using QuPath's built-in cell detection function. DAPI detection parameters were held constant across sections and were only occasionally adjusted to accommodate for slight imaging differences. The machine learning object classifier quantified the VGLUT1+ and VGAT+ cells. Further, cells expressing PNNs were counted manually in QuPath and were identified as being WFA+/VGLUT1+, WFA+/VGAT+ or WFA+ only.

3.5. RNA Extraction for qPCR

Brains were partially defrosted and placed on a rat metal matrix, then sliced 3mm thick at the mPFC level and 2mm thick at the BLA level. Punches (2mm diameter) were obtained from the mPFC and BLA,

and left and right hemispheres were pooled into one Eppendorff tube. RNA was extracted from the tissue samples using the Qiagen RNeasy Micro kit extraction protocol and reagents. Tissue samples were homogenized and incubated in lysis buffer for 3 minutes at room temperature, after which, homogenates were placed into columns and centrifuged for 30 seconds at 8000 × g and the flow-through was transferred to a new tube. A precipitation buffer was added and tubes were centrifuged for 3 minutes at 12000 × g to pellet the precipitate. The supernatant was transferred to a new tube with isopropanol and centrifuged again for 15 seconds at 8000 × g. RNA was cleaned 3 times with wash buffers and 80% ethanol before diluting with DEPC-treated water. A UV spectrophotometric analysis was used to confirm adequate RNA quantity and purity in the sample. cDNA was immediately synthesized from the extracted RNA with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) and stored at -20^{o}C until further use.

3.6. Quantitative PCR for oxidative stress expression

As described in Ruigrok et al. for mice brain homogenates[70], we aim to test whether ELS altered genes promoting or protecting against oxidative stress. Formation of PCR products was assessed using a 7500 Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 2 minutes of RNA predenaturation at 50^{o}C, 10 minutes polymerase activation at 95^{o}C and 40 cycles of replication (15 s at 95^{o}C, 60 s at 62^{o}C). The reaction mix consisted of SYBR green (Promega), 1.25ng/ul of cDNA template and 0.1uM of each forward and reverse primer. Primer sequences (table 1) were generated using the National Center for Biotechnology Information database to convert the mouse sequences outlined in Ruigrok et al. 2021 to rat sequences. Primers (Integrated DNA Technologies) had an efficiency of 95% to 101%. Relative gene expression was normalized to the reference gene glyceraldehyde-6-phosphate dehydrogenase (GAPDH) and calculated using the ΔΔCt method.

Table 1. Primer sequences for qPCR analysis (5' to 3')

Gene name	Gene symbol	Forward primer	Reverse primer
Catalase	*Cat*	CCCTCAGAAACCCGATGTCC	TGTGCCATCTCGTCGGTGAA
Dynamin 1-Like	*Dnm1l*	TGACCAAAGTACCTGTAGGTG	GCATCAGTACCCGCATCCAT
Fission, mitochondrial 1	*Fis1*	GGCAACTACCGGCTCAAGGA	GCCTACCAGTCCATCTTTCTTCA
Superoxide dismutase 1	*Sod1*	GCGGATGAAGAGAGGCATGTT	GTACGGCCAATGATGGAATGC
Glyceraldehyde-3- phosphate dehydrogenase	*GAPDH*	TGCCAAGTATGATGACATCAAGAAG	AGCCCAGGATGCCCTTTAGT

3.7. Statistical Analysis

Data was analyzed using GraphPad Prism version 9. To analyze maternal behaviour, two-way ANOVAs were performed with bedding as a between-subject variable and light phase as a within-subject variable. Subsequent simple effects tests were conducted to decompose significant interaction effects. For RNAscope experiments, three-factor ANOVAs were performed with bedding and sex as between-subject factors and brain hemisphere as a within-subject factor and interaction effects decomposed using simple effects tests. For PCR experiments, two-factor between-subjects ANOVAs were performed using bedding and sex as variables and subsequent simple effects tests were used to decompose significant interactions. Unpaired t-tests were also performed to compare bedding conditions in male and female animals. Significance was set at $p<0.05$. Data points two standard deviations above or below the mean were considered outliers and removed from the analyses.

4. Results

4.1. Maternal behaviour and mean pup weights

Several characteristics of maternal behaviour (nursing, pup grooming) and maternal self care (self-grooming) were analyzed to determine the effect of the LB paradigm on maternal care during the light and

dark phases. Fragmentation, a hallmark of the LB paradigm, was scored as the number of times maternal behavior changed from one sampling time to the next. The unpredictability of changes in maternal care can be a stressful experience for pups and result in altered sensory stimulation[82]. The total time spent nursing, pup grooming and self-grooming, and the total number of behavioral fragmentation were calculated for each 60-minute sampling period and averaged across light and dark timepoints. For all variables, two-way ANOVAs was performed using bedding as a between subject factor and light phase as a within subject factor.

A two-way ANOVA revealed that behaviour was more fragmented during the dark phase compared to the light phase ($F(1,10)=21.36$, $p<0.001$), but no main effect of bedding or bedding × light phase interaction were found (Fig. 3A). Furthermore, a two-factor ANOVA showed that mothers spent significantly more time nursing during the light phase ($F(1,10)=72.65$, $p<0.001$) compared to the dark phase, but there was no main effect of bedding or interaction effect. However, a Student's t-test performed within each light phase showed a significant increase in the time LB mothers spent nursing during the light phase compared to NB mothers ($t(10)=2.25$, $p=0.048$, Fig. 3B). Additionally, because sensory stimulation through pup grooming is important for brain development[82], we measured the total time mothers spent grooming their pups. As shown in Figure 3C, two-way ANOVA revealed a significant interaction of bedding and light phase ($F(1,10)=5.95$, $p=0.035$), where a simple effects test showed that LB mothers groomed their pups less compared to NB mothers during the light phase ($F(1,10)=6.97$, $p=0.016$). Lastly, we measured maternal self-grooming to approximate maternal stress and coping. Results from a two-way ANOVA, showed a significant main effect of light phase where mothers self-groomed more during the dark phase than the light phase ($F(1,10)=32.33$, $p<0.001$), but there was no main effect of bedding or interaction of bedding × light phase (Fig. 3D).

Mean pup weight was calculated per litter by dividing the total litter weight by the number of pups in the litter. Two-way ANOVA showed a significant interaction of bedding and age ($F(4,40)=4.86$, $p=0.003$). As depicted in Figure 3E, a simple effects test revealed that the mean pup weight was reduced in

LB rats compared to NB rats on PND21($F(1,32)=13.00$, $p=0.001$), with no significant differences observed between LB and NB rats on PND1 through 14.

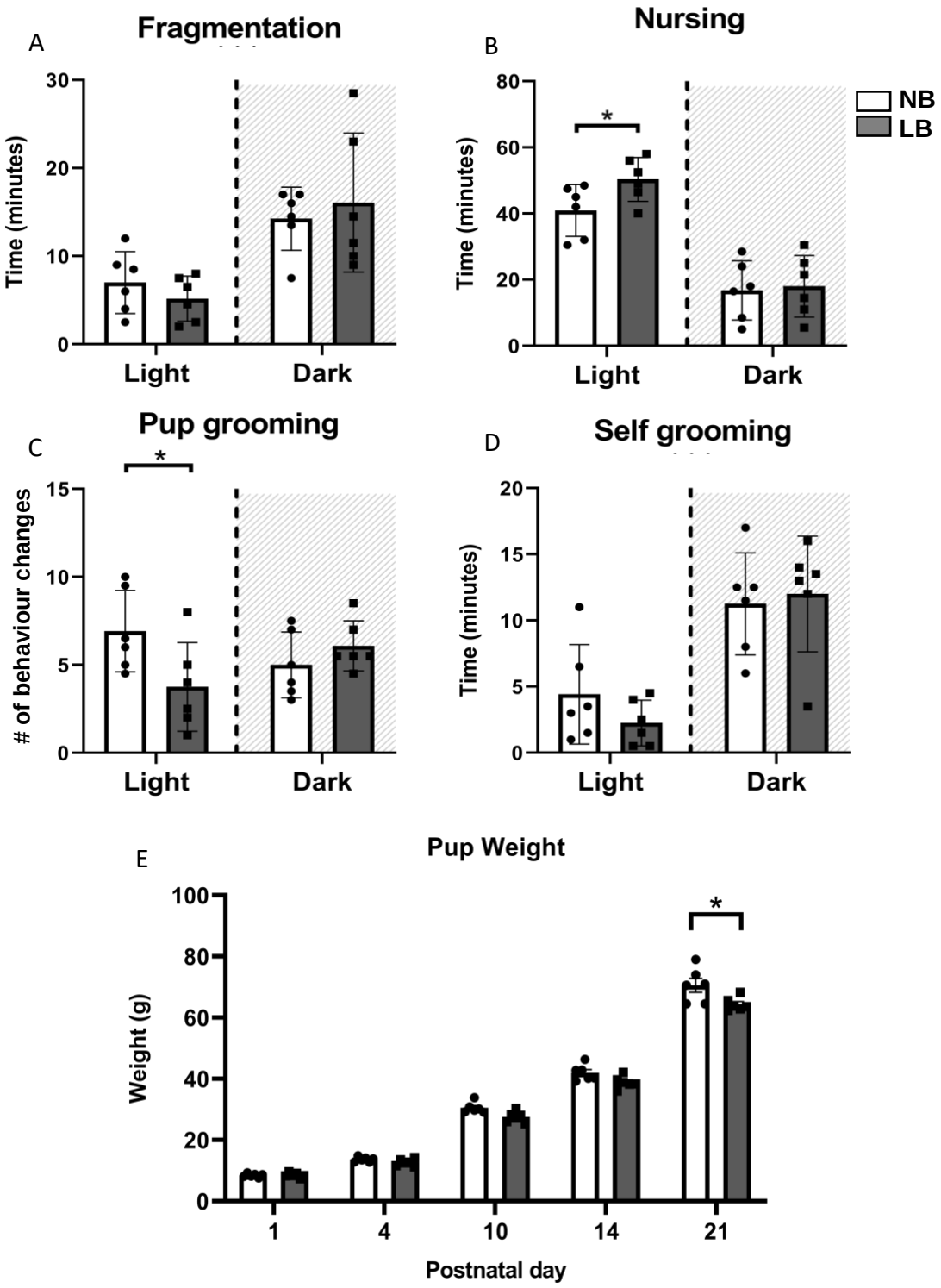

Figure 6. Maternal behaviour in light and dark phases and mean pup weights from PND1-21. **A:** LB mothers exhibited no change in fragmented behaviour compared to NB mothers, but fragmentation was significantly increased during the dark phase ($p<0.001$). **B:** LB mothers spent significantly more time nursing during the light phase as indicated by a Student's t-test ($p=0.048$). **C:** Time spent pup grooming was significantly reduced in LB mothers during the light phase ($p=0.016$). **D:** NB and LB mothers both spent more time self-grooming in the dark phase ($p<0.001$). **E:** The mean weight of LB pups was reduced at PND21 ($p=0.001$) compared to NB pups. Data are mean $\pm$ SEM of n = 6 litters/group. *$p<0.05$.

4.2. Effect of LB on the density of PNNs on inhibitory and excitatory cells in the BLA

An illustration of cells in the BLA and mPFC expressing VGAT, VGLUT1 and PNN is shown in Figure 7. Quantification of cell density of VGAT, VGLUT1, PNN, colocalization of VGAT+/WFA+ and VGLUT1+/WFA+, and percentages of VGAT+ and VGLUT1+ with PNN in the BLA is depicted in Figure 8. Cell density was calculated for VGLUT1+ cells, VGAT+ cells, WFA+ cells, VGLUT1+/WFA+ cells, and VGAT+/WFA+ cells by dividing the total number of positively stained cells by the area of the BLA or mPFC section considered. To analyze the cell densities, we conducted a three-factor ANOVA using bedding and sex as between-subject factors and brain hemisphere as a within-subject factor. Since the three-factor ANOVA revealed no hemispheric differences among any of the cell densities, we pooled data from both brain hemispheres by averaging the left and right sides and proceeded to analyze the data using a two-factor ANOVA with bedding and sex as between-subject variables.

In the BLA, a two-way between subjects ANOVA revealed a main effect of bedding on VGAT cell density where both LB males and females exhibited increased VGAT cell density ($F(1,12)=7.62$, $p=0.017$) compared to NB rats (Fig. 8A). There was no further main effect of sex or bedding × sex interaction for VGAT. For VGLUT1 cell density, two-way ANOVAs showed no significant main effects or interactions. However, a Student's t-test performed within each sex showed that VGLUT1 cell density was significantly reduced only in the BLA of LB females ($t(6)=-3.41$, $p=0.014$) and remained unchanged in males (Fig. 8B). As illustrated in Figure 8C, two-way ANOVAs did not reveal any significant main effects or interactions for density of PNNs. To identify if PNNs might favour formation around a specific cell type, we determined the density of VGAT or VGLUT cells harbouring PNN. We found no significant main effects of sex or bedding and no significant interactions (Fig. 8D-E).

To assess the ratio of inhibitory and excitatory cells encapsulated by PNN, we determined the percentage of total VGLUT1+ or VGAT+ cells also presenting as WFA+. Results from a two-factor between subjects ANOVA indicated no significant sex or interaction effects, but showed a main effect of bedding on the percentage of VGAT+/WFA+ cells in the BLA, where LB males and females displayed a

significant decrease in the percentage of total VGAT+ cells co-expressing WFA+ ($F(1,12)=13.19$, $p=0.003$) (Fig. 8F). This change is likely due to the increase in total VGAT cells, rather than a shift in the ratio of inhibitory and excitatory cells encapsulated by PNNs since no change in the percent of PNNs surrounding VGLUT1 cells was observed (Fig. 8G).

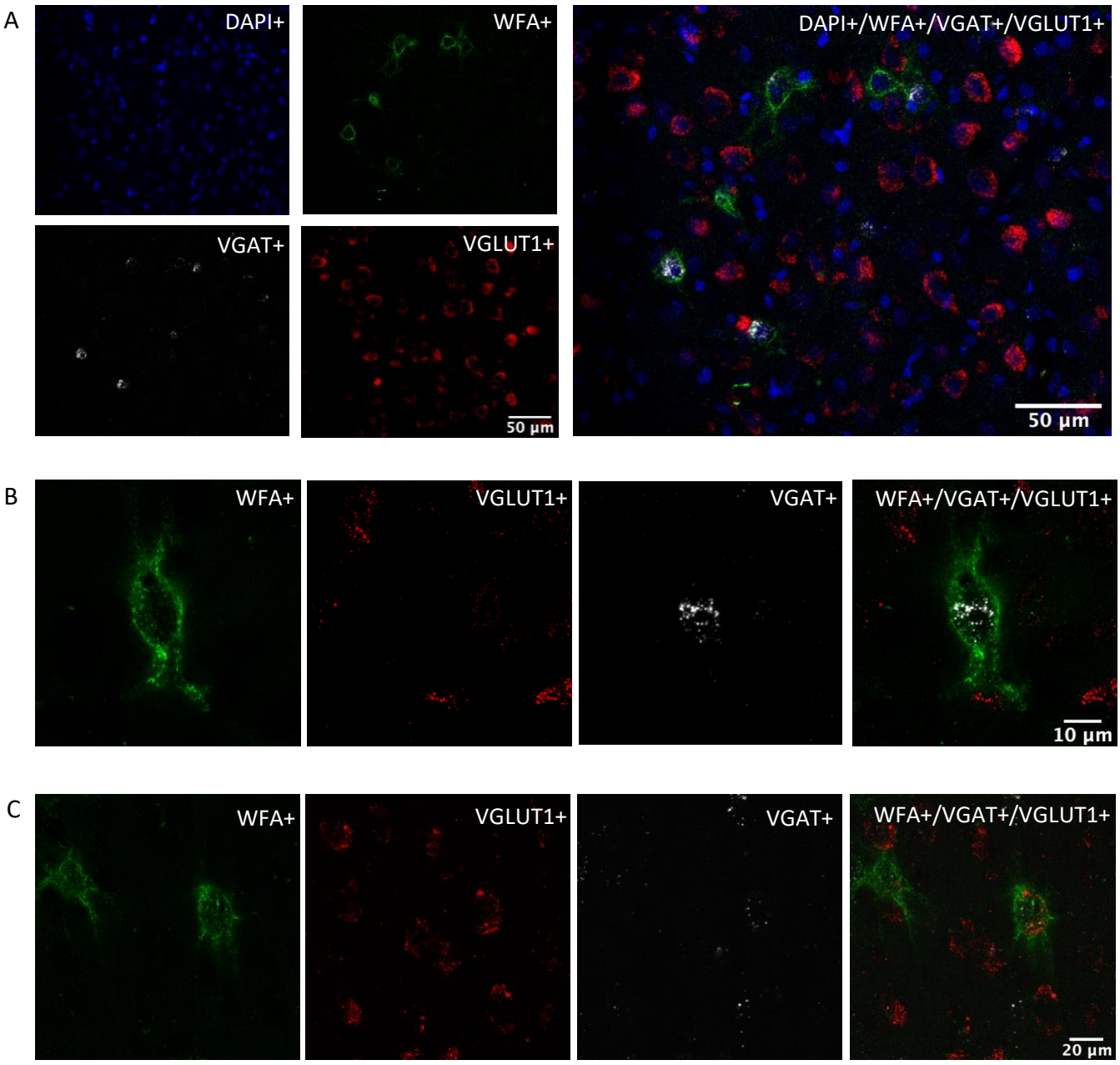

Figure 7. Representative images of RNAscope for VGLUT1 and VGAT and immunohistochemistry for PNNs. **A:** Individual and merged channels of VGLUT1 (red), VGAT (white) RNAscope and WFA (green) immunohistochemistry, taken at 20x in the BLA. **B:** Image of a cell co-expressing VGAT and WFA in the BLA, taken at 60x with 3x zoom. **C:** Image of a cell co-expressing VGlut1 and WFA in the PL mPFC, taken at 60x. Scale bar represents 50um, 10um, and 20um for images A-C, respectively.

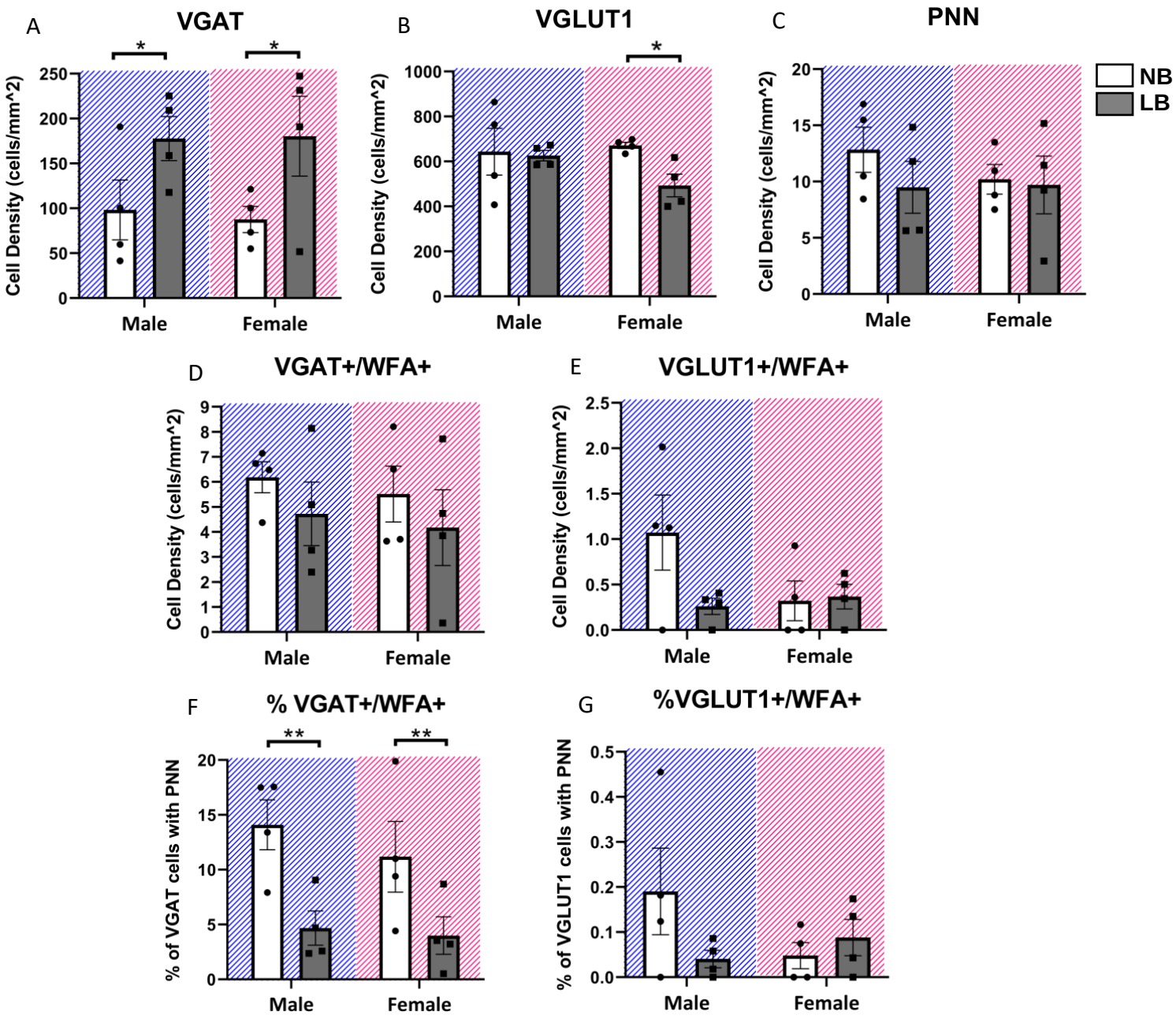

Figure 8. VGAT, VGLUT1, and PNN cell densities and percentages of co-expression in the BLA. **A:** In the BLA, LB conditions increased VGAT cell density in both males and females (p=0.017). **B:** VGLUT1 cell density was reduced in LB females (p=0.014), but not males in the BLA as shown by a Student's t-test. **C:** Bedding conditions and sex had no effect on PNN cell density in the BLA. **D:** The density of VGAT cells co-expressing PNNs was not altered in LB animals, nor was the density of VGLUT1 cells co-expression PNN **(E)**. **F:** LB males and females showed a significant decrease in the percent of VGAT cells co-expressing PNNs (p=0.003). **G:** The percent of VGLUT1 cells co-expressing PNN remained unchanged. Two-way ANOVAs with bedding and sex as between-subjects variables were performed unless otherwise stated. Data are mean ± SEM of n = 4 animals/group. *p<0.05; **p<0.01.

4.3. Effect of LB on the density of PNNs on inhibitory and excitatory cells in the mPFC

Similar analyses were conducted for the mPFC as the BLA for VGAT, VGLUT1, PNN, VGAT+/WFA+, VGLUT1+/WFA+ cell density and percentages of VGAT+ and VGLUT1+ with PNN as reported in Appendix A. Again, three-way ANOVAs revealed no main effect of hemisphere on cell densities, so data from the left and right brain sides were pooled.

Two-way between-subject ANOVAs revealed no main effect of sex or bedding and no significant interaction effects for VGAT, VGLUT1, PNN, VGAT+/WFA+, or VGLUT1+/WFA+ cell density in the PL or IL mPFC, as depicted in Figure 9. However, results from a Student's t-test performed within each sex indicated that the density of cells colocalizing VGLUT1+ and WFA+ is significantly increased in LB males compared to NB males in the IL mPFC ($t(6)=2.48$, $p=0.048$), but not the PL mPFC (Fig.9E). For the percent of VGLUT1 cells co-expressing PNN, a two-way ANOVA revealed a main effect of sex where females displayed a higher percentage compared to males ($F(1,12)=6.17$, $p=0.029$), but no main effect of bedding or interaction effect was found. Furthermore, no significant main effects or interactions for the percent of VGAT cells harbouring PNN were reported in the mPFC. To confirm the trends observed in the present data and reduce variability, we are replicating these experiments to increase the sample size for both the mPFC and BLA.

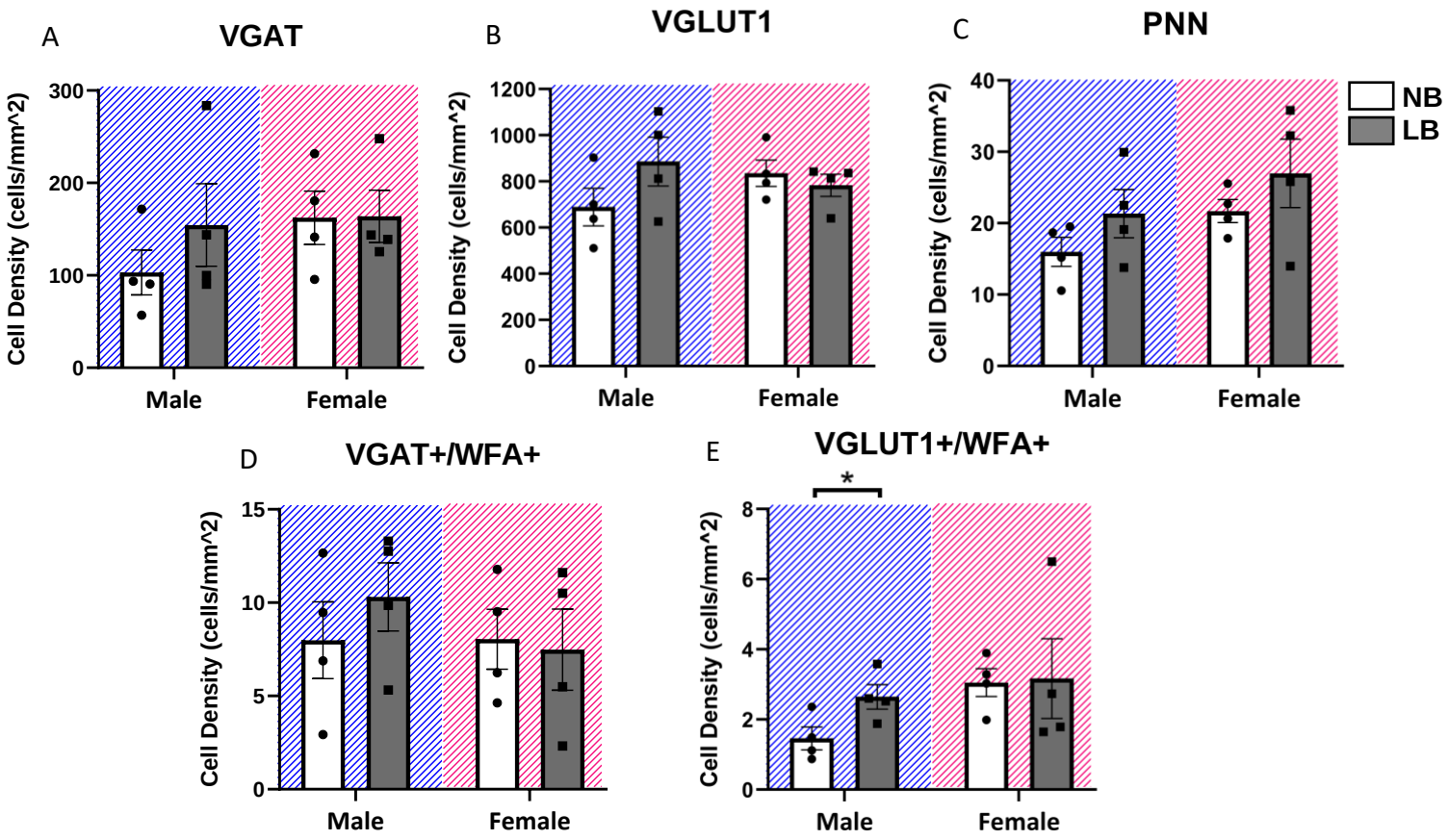

Figure 9. PNN and VGLUT1 cell densities and co-expression in the IL mPFC. **A:** LB conditions did not alter VGAT density and VGLUT1 cell density was also unchanged in the IL mPFC **(B)**. **C:** PNN density was not altered as a function of LB and the density of VGAT cells harbouring PNN also remained unchanged following LB **(D)**. **E:** The density of VGLUT1 cells co-expressing PNN was increased in LB males ($p=0.048$), but not females in the IL mPFC as shown by a Student's t-test. Two-way ANOVAs with bedding and sex as between-subjects variables were performed unless otherwise stated. Data are mean ± SEM of n = 4 animals/group. $^{*}p<0.05$ indicated by Student's t-test between LB and NB groups.

4.4. Expression of oxidative stress induced-genes in the mPFC and BLA after LB

The relative expression of oxidative stress-induced genes *Cat, Fis1, Dnm1l* and *Sod1* was analyzed using two-factor between-subject ANOVAs with bedding and sex as variables. Data analysis revealed that *Cat* gene expression was altered in both the mPFC and BLA. In the mPFC, the ANOVA showed no effect of sex or bedding, but a significant sex × bedding interaction ($F(1,20)=5.14$, $p=0.035$). A simple effects test revealed that LB males exhibited significantly higher expression of the *Cat* gene ($F(1,20)=5.32$, $p=0.032$) compared to NB males and females regardless of bedding condition (Fig. 10A). In the BLA, a two-way between-subject ANOVA revealed no main effect of sex or bedding, but found an interaction trending towards an effect opposite to the mPFC. Although the interaction of bedding and sex was not statistically significant ($F(1,19)=3.52$, $p=0.076$), the simple effects analysis notably indicated that *Cat* gene expression was reduced in LB males compared to NB males ($F(1,19)=4.02$, $p=0.060$), as shown in Figure 10B. Analyses of the females yielded no significant results or trends in either the mPFC or BLA.

Two-way ANOVAs did not show any main effects or interactions for *Sod1* or *Fis1* expression in the mPFC or BLA (Fig. 10C-F). *Dnm1l* expression was affected in the BLA by sex where males expressed higher levels of *Dnm1l* than females ($F(1,20)=4.41$, $p=0.049$), but unaffected by bedding or bedding × sex interactions (Fig. 10G). There was no effect of sex, bedding or sex × bedding interaction on *Dnm1l* expression in the mPFC (Fig. 10H).

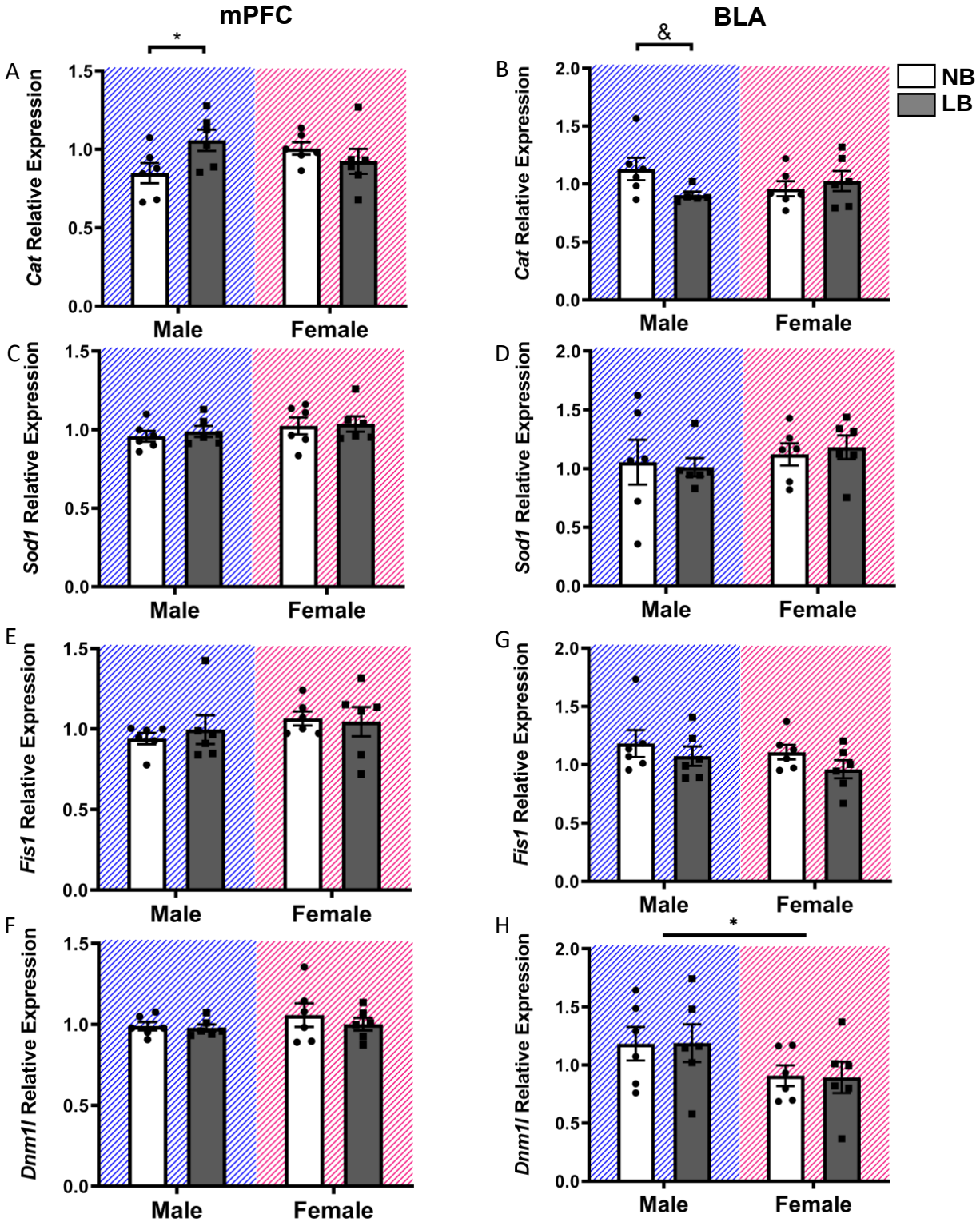

Figure 10. Relative expression of the *Cat* and *Sod1* gene in the mPFC (left) and BLA (right). **A:** Simple effects tests found that LB males showed significantly higher *Cat* expression than NB males in the mPFC ($p=0.032$). **B:** In the BLA, simple effects tests showed a trend where *Cat* expression was decreased in LB males compared to NB males ($p=0.060$). **C:** The expression of *Sod1* was not altered in the mPFC or BLA **(D). E:** Nor was the expression of *Fis1* altered in the mPFC or BLA **(F)**. **G:** In the mPFC, *Dnm1l* expression was unaltered, but in the BLA, *Dnm1l* expression was elevated in males compared to females **(H)**. Two-way ANOVAs with bedding and sex as between-subject factors were performed. Data are mean ± SEM of n = 6 animals/group. Note that for *Cat* data, one outlier was removed from LB males in the BLA. $^{*}p<0.05$; $^{\&}p<0.10$.

5. Discussion

In this project, we examined how early life stress, in the form of limited bedding access (LB) impacts PNN distribution around inhibitory and excitatory cells, and whether changes in PNNs could affect oxidative stress in two main structures of the corticolimbic system of juvenile rats, the mPFC and BLA. Particularly, we aimed to establish whether changes associated with ELS appear in a sex- and hemispheric-dependent manner as these have been documented to occur for the BLA in previous studies from our laboratory. Although we did not find a significant effect of LB on the density of PNNs in both structures, we observed an increase in the total number of VGAT+ cells in the BLA of males and females following LB, which translated to a lower percentage of VGAT+ neurons ensheathed by PNN in LB animals. Few glutamatergic cells were surrounded by PNNs, but the density of VGLUT1+ cells with PNN was increased in the IL mPFC of LB males only. Regarding the oxidative state, we reported an increase in the expression of catalase, an antioxidant enzyme, in the mPFC, but not the BLA of males following LB conditions. Overall, our results indicate that exposure to LB resulted in both regional and sex differences, where changes in the BLA were more prominent than in the mPFC, and male juveniles displayed more changes than females. We did not find inter-hemispheric differences in PNN distribution and cell densities.

In the BLA, our main finding was that LB increased VGAT cell density in both males and females without a corresponding increase in PNN cell density. This resulted in a lower percentage of VGAT cells colocalizing PNNs. This might suggest an increased number of immature GABAergic interneurons in the BLA as PNNs stabilize neuronal activity and reduce plasticity of mature cells[28]. Although neuron immaturity should be confirmed by cell staining with markers like doublecortin (DCX) or bromodeoxyuridine (BrdU), which label immature and proliferating cells[83,84], the increase in VGAT cell number and density was puzzling to us because it suggests that neurogenesis may occur in the BLA of juvenile rats exposed to early life stress. However, neurogenesis past the embryonic stage has not been confirmed in the rat BLA; studies have found that neurogenesis in the amygdala is completed before birth[85] and have not observed reliable changes in neuron number from the first week of life to 7 months

postnatally[86]. Nonetheless, other studies indicate that new neurons are produced in the amygdala of adult non-human primates[87] and voles[88] as demonstrated by the presence of BrdU+ cells, thus the possibility of neurogenesis in the BLA remains highly debated. After decades of controversy, adult neurogenesis has been confirmed in other brain regions of rodents, like the subventricular zone and dentate gyrus of the hippocampus[89].

In the rodent hippocampus, adult neural stem cells (NSCs) can differentiate into excitatory and inhibitory granule cells that will integrate into the existing circuitry over the course of several weeks[90,91]. When NSCs become activated, they divide to either produce more NSCs or progenitor cells that can differentiate into neurons and astrocytes[90]. Many newborn cells will die within 3 weeks of their birth, but cells selected for survival will remain stable parts of the dentate gyrus for the animal's lifespan[90]. Most NSC proliferation occurs during the first few months of life, where the mouse hippocampus increases in size until 6 months of age to make room for new neurons, and plateaus by 7-10 months of age in rodents[90]. Various intrinsic and extrinsic factors stimulate or reduce adult neurogenesis. For example, growth factors, physical exercise and antidepressants increase neurogenesis, whereas factors like ageing, using drugs of abuse, and fear are known to decrease neurogenesis[90]. Interestingly, acute stress tends to increase neurogenesis in the adult rat hippocampus[92], whereas chronic stress generally reduces neurogenesis in the rodent hippocampus[90,93].

Similarly, ELS generally decreases neurogenesis in the hippocampus of adult rodents[94,95], although studies have also reported no significant effect of ELS on hippocampal neurogenesis in adult female rats[96]. One study even found an increase in neurogenesis in the hippocampus of female rats following 2 weeks of maternal separation[97], which corresponds to the increase in VGAT that we observed if neurogenesis can be confirmed in the BLA. A study by Raineki et al. did find neurons expressing DCX in the BLA following a maltreatment model of ELS, and showed that maltreated rat pups on PND13 exhibited suppressed DCX in the BLA[22]. Although our current results do not indicate any sex differences, some evidence exists that hippocampal neurogenesis is altered in a sex-dependent manner following ELS. After 24 hours of maternal deprivation on PND3, males exhibited increased neurogenesis in the hippocampus on PND21, while

females exhibited decreased neurogenesis[98]. The differences observed in neurogenesis following ELS may be explained by changes throughout development or differences in the consequences of various ELS paradigms. The majority of studies investigating neurogenesis use maternal separation rather than limited bedding that we used and they do not investigate the juvenile period as we have done in the present study. Nevertheless, changes in neurogenesis following ELS suggests that stress may alter intrinsic factors that induce or reduce neuron proliferation.

Research has shown that ELS alters the expression of growth factors, like brain-derived neurotrophic factor[99–101], which may influence neuronal proliferation. Growth factors are known to stimulate neurogenesis and several groups have successfully reprogrammed glial progenitor cells (GPCs) into neurons *in vitro* and *in vivo* by overexpressing certain growth and transcription factors[29,102,103]. Although the neuronal subtype generated from GPCs varies according to the brain region in live rodents, GABAergic neurons are primarily produced in certain regions, like the striatum and midbrain[102,103]. Specifically, most of the reprogrammed GABAergic interneurons in these regions are PV INs, but the mechanism selecting the neuronal subtype remains unclear[103]. Some evidence suggests that the specific neurogenic gene selected to reprogram the GPC could affect the particular GABAergic interneuron subtype generated[103]. This evidence indicates that neurogenesis is possible in typically non-neurogenic regions under specific circumstances, which would consequently influence the activity and plasticity of that region, for example, the BLA.

A higher percentage of VGAT cells without PNN in the BLA might thus represent a functional "marker" of ELS in juvenile animals, and extend the period of plasticity and vulnerability of GABAergic interneurons and circuitry development. GABAergic transmission modulates NSC activation and initially depolarizes immature glutamatergic granule cells born during adult neurogenesis. Functionally different from mature neurons, immature PNs have a low threshold of excitation and also maintain a heightened state of plasticity into the juvenile period[90]. Thus, ELS-induced changes in GABAergic INs might have long term consequences on brain circuitry once newborn neurons become integrated into functional networks.

We previously documented higher BLA plasticity and excitability of PNs in the BLA of juvenile males after LB[24], which was evidenced by higher LTP after high frequency stimulation. This might be a consequence of an increased number of immature VGAT neurons and an overall inhibitory tone that might be insufficient to dampen PN activity in response to LB in males. In females, we previously found that, in contrast to males, LB had no effect on LTP in the BLA, so it is unlikely that changes in immature VGAT cell density is a major contributor to cell activity in females. Our current data indicate that this might be due to a reduction in VGLUT1 cell density in the juvenile BLA compared to NB controls. Together, these results suggest that ELS leads to an E/I imbalance that may contribute to aberrant neuronal activity. However, whether these alterations in inhibitory and excitatory cell densities cause aberrant firing activity in the BLA is unknown since we did not measure any concomitant electrophysiological properties of BLA principal neurons. Regardless, these cell density changes suggest that ELS influences synapse formation during the critical period. Thus, at the end of the critical period, PNN will stabilize aberrant synaptic inputs and possibly strengthen altered neurotransmission following ELS.

Although we found a lower percentage of VGAT cells harbouring PNN, this change surprisingly did not translate to a reduction in the absolute number of PNNs or VGAT cells with PNN. Previous work from our lab using double immunocytochemistry showed an increase in PNN surrounding PV INs in the BLA following ELS[24], which was not reflected in the present study measuring gene expression in the entire GABAergic cell population. Thus, our present result might indicate a decrease in PNNs around populations of inhibitory interneurons other than PV INs. However, this previous work also showed that PNN colocalization with neurons expressing GAD67, a marker of GABAergic neurons, increased in the male BLA[24]. This discrepancy might be explained by differences in methodology where this project identified inhibitory cells by marking mRNA transcripts coding for GABA transporters, while the previous work identified immunostained GAD67 proteins using a threshold of immunofluorescence detection. Furthermore, we found no differences in the effect of ELS on the right and left hemispheres, which is also contrary to previous work showing an increase in PNN colocalizing PV IN in the male BLA on the right side of the brain only[24]. Since few studies have investigated hemispheric differences regarding PNNs, the

sample size of the current experiment should be increased to improve robustness and reduce variability before drawing conclusions about hemispheric alterations following ELS.

Although our results showed that PNN density was unexpectedly unchanged in the BLA, PNNs exhibited the anticipated pattern of cell distribution across the BLA and mPFC where most PNNs surrounded VGAT+ cells, but some PNNs also encapsulated glutamatergic neurons. Approximately 5-15% of all VGAT cells were surrounded by PNNs in the mPFC and BLA of LB and NB animals, whereas less than 1% of VGLUT cells harboured PNNs in both structures. In the mPFC, about 0.2-0.5% of excitatory neurons were encapsulated by PNNs, while only 0.05-0.2% of glutamatergic cells in the BLA were PNN+. Different regions of the cerebral cortex have also been shown to express varying levels of PNN-associated principal neurons[40]. For example, the average density of principal neurons with PNN in the primary visual cortex is 6.59 cells per mm^2, whereas the density of excitatory cells expressing PNN is only 1.27 cells per mm^2 in the secondary motor cortex[40]. The function of this small population of cells has not been widely studied, but principal neurons with PNN in the rat cerebral cortex and cerebellum are known to project to intra- and sub- cortical regions[39,40]. Interestingly, Morikawa et al. found that following fear conditioning, c-Fos was co-expressed with more excitatory cells harbouring PNNs than those without PNNs, which suggests that excitatory neurons with PNNs in the amygdala may be specifically recruited during fear conditioning[38].

Our results showed that the density of VGLUT1+ cells colocalizing with PNNs was altered following ELS. VGLUT1 and PNN co-expression was almost doubled in LB male rats compared to NB males in the IL mPFC specifically; no additional changes were observed in the PL mPFC or in females. PNNs around excitatory cells are not widely studied in the mPFC, so the consequences of this increased cell density remain elusive. Nevertheless, the presence of PNNs around glutamatergic cells in juvenile males could reflect accelerated maturation of excitatory neurons in the IL mPFC because PNN have previously only been seen around PNs in adult animals. Accelerated accumulation of PNNs may better protect the encapsulated neuron from harmful environmental effects, like ROS. Although glutamatergic cells are also negatively affected by ROS, the consequences of oxidative stress are more pertinent to

GABAergic interneurons, particularly PV INs, because of their high firing activity and requirement for high energy supply[104]. Following oxidative stress induced by brain ischemia in the cortex, about 10-15% more PV INs were lost than glutamatergic neurons[104]. Furthermore, PV IN exposure to elevated levels of ROS has been shown to lead to decreased PV expression in the mPFC associated with cognitive impairments[105]. Because oxidative stress induced by ELS can damage neurons unprotected by PNNs, we investigated whether LB modified the expression of some key enzymes that regulate the oxidative state of the mPFC and BLA.

By examining mitochondrial gene expression in whole tissue extracts, we found that LB males exhibited increased *Cat* gene expression in the mPFC, suggesting an increased antioxidant capacity. The *Cat* gene codes for the antioxidant enzyme catalase, which converts hydrogen peroxide to water and oxygen, thereby preventing a toxic reaction that induces oxidative stress. This result indicates that the mPFC is perhaps more resilient to oxidative stress, but the mechanism remains unknown since we did not observe an associated change in PNNs on highly vulnerable PV INs. Conversely, the juvenile BLA showed the opposite trend where *Cat* expression tended to be reduced in males after ELS. This result indicates that the BLA possibly remains susceptible to oxidative damage induced by ELS, especially on immature VGAT neurons. Together, these results suggest that the BLA is more susceptible to suffering negative consequences from ELS regarding increased oxidative stress and altered connectivity. The BLA might be more sensitive because it reaches adult maturation before the mPFC and the critical period of sensitivity is more closely aligned with the timing of LB in our study. In ELS animals, the BLA might develop more quickly to cope with the stressor of poor caregiving, but consequently strengthen connections that promote anxious behaviour.

Our results suggest that LB might have a greater impact on antioxidant enzymes than fission proteins on PND28, although this result still needs to be confirmed by increasing our sample size. We only observed altered gene expression of one antioxidant enzyme, *Cat*, in the mPFC and BLA, whereas Ruigrok and colleagues previously reported a change in fission protein expression in addition to antioxidant enzymes in the neonatal hippocampus after ELS in mice[70]. This difference might reflect the differential vulnerability

of various corticolimbic regions to ELS, or might indicate changes over the lifespan. Ruigrok et al. observed the changes in fission and antioxidant enzymes at PND9, directly following LB, while they only observed a change in one fission protein, *Fis1*, in adult animals[70], indicating that the oxidative state may fluctuate throughout development. Other studies also report changes in the oxidative state across development, for example, Eagleson et al. found that activity of the mitochondrial complex I and respiration were increased in PND21 mice, but reduced in adults[68]. Considering these results, they suggest that adaptive changes occurring early in life can become maladaptive in adulthood, which could be the case for the protective effects of *Cat* upregulation in the mPFC at PND28. Indeed, research has shown that superoxide dismutase 1 and catalase enzyme activity is reduced in the adult mPFC following ELS in rats[106]. Although these studies either used all male animals or reported no sex differences, other studies have noted sex differences regarding indicators of oxidative stress.

Our results indicate that the antioxidant defense system is more affected in males experiencing ELS than females. Males and females exhibit sexual dimorphisms in the mitochondria and sex steroids interact with redox homeostasis mechanisms to produce sex differences[107]. At baseline, adult females are shown to express higher levels of antioxidants like superoxide dismutase and glutathione peroxidase[108], whereas males display increased lipid peroxidation in the brain[109]. However, following ELS, some studies report increased mitochondrial vulnerability of females. In the mPFC, mitochondrial energy metabolism, reflected by cytochrome c oxidase activity, was decreased in females, but not males after maternal separation[66]. Further, mitochondrial proteins were altered in the female, but not male, hippocampus of LB animals[68]. Males or females may be more affected depending on the mitochondrial endpoints investigated and future work should integrate several variables in the analyses.

There are several limitations to the current studies that need to be taken into account. First, we used WFA to label PNNs in the mPFC and BLA, but studies in the mouse cortex have identified a subset of PNNs that are not WFA reactive[110,111]. As the most common marker of PNN, we chose WFA to label PNNs because it binds to a major PNN component, chondroitin sulfate, and is shown to bind the majority of

PNNs[110]. However, in both the hippocampus and cortex, PNNs have been identified that are only WFA positive, WFA and aggrecan positive, in addition to PNNs that are only aggrecan positive[110,112], indicating heterogeneity in PNN composition. The composition of PNNs is also region-specific where hyaluronic acid binding protein-positive PNNs were observed in layer 1 of the mouse cortex that is devoid of WFA+ PNNs[111]. Thus, we may have overlooked a certain population of PNNs by using WFA as the only marker of PNNs. Future work should consider using other PNN markers in addition to WFA, and could also investigate if ELS affects PNN composition.

Second, we investigated the gene expression of mitochondrial proteins in whole tissue extracts, but performing an enzyme activity assay of catalase and superoxide dismutase, specifically in the mitochondrial fraction of the BLA and mPFC, could improve sensitivity and specificity of the effects produced by ELS. In the present experiment, we measured the oxidative state of the mPFC and BLA of whole tissue extracts, which limits the conclusions we can make regarding the association of PNNs with the oxidative state of a cell. Even though we do not see any significant associations at the regional level, this may change if the oxidative state of PV neurons with PNNs is specifically compared to those without PNNs. Future work using in situ laser dissection followed by qPCR or RNAseq techniques will allow to specifically phenotype the INs that are surrounded by PNNs and examine the effects of ELS on this phenotype. Furthermore, future work in our lab aims to characterize inhibitory cell types surrounded by PNNs other than the majority of those expressing PV to clarify if a specific cell type is rendered vulnerable to ELS.

6. Conclusion

Overall, we found that exposure to LB conditions early in life led to regional and sex differences in neuronal cell density, oxidative enzyme expression as well as the percentage of cells expressing PNNs. The BLA was more susceptible to changes in inhibitory and excitatory cell density and oxidative stress, whereas the mPFC was more protected from oxidative stress by an increase antioxidant gene expression. Although we did not observe the expected changes in PNN density in the BLA, VGAT+ cell density was

increased after LB, suggesting that LB might have increased the presence of immature inhibitory interneurons in the BLA.

This work extends previous research investigating the impact of the limited bedding paradigm as a model of ELS on PNN development. Other groups investigating the effect of ELS on PNN often use different models of early adversity, like maternal separation[47,49] or the scarcity adversity model (LB from P8-P12)[57], thus this project also contributes to understanding the effect of different forms of ELS. Notably, different ELS paradigms may distinctly affect males and females, either by the nature of their consequences or the timing at which ELS is applied relative to sexually dimorphic brain development. Understanding the specific outcomes of different models is crucial for comparing results and drawing conclusions for future research. Furthermore, using RNAscope technology, we were able to specifically identify glutamatergic neurons and analyze how PNNs around excitatory cells are affected by ELS. Since PNNs are known to primarily encapsulate PV INs and inhibitory interneurons, few studies to our knowledge have examined the effect of ELS on PNNs encapsulating glutamatergic neurons.

This project will contribute to the understanding of ELS consequences in the hopes of better preventing, recognizing, and treating neuropsychiatric illnesses associated with childhood trauma. In particular, PNNs are potential targets for future therapies to restore plasticity and reduce anxiety. However, restoring plasticity would involve PNN degradation, which can also increase vulnerability to oxidative stress. Therefore, the mechanisms underlying PNN alterations due to ELS and the subsequent effect on neuronal circuitry and cellular damage need to be further explored. The current project will improve our understanding of how ELS causes the corticolimbic circuit to deviate from normal development, help us recognize some early markers of ELS, and identify the most vulnerable brain areas in males and females.

7. Bibliography

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Appendix A. Quantification of cell density and percentages in the PL and IL mPFC

		PL mPFC		IL mPFC	
	Bedding	Male	Female	Male	Female
VGAT+ cells/mm^2	NB	114.88 (± 18.18)	154.78 (± 24.62)	103.19 (± 24.30)	162.29 (± 28.89)
	LB	151.69 (± 43.34)	169.03 (± 27.75)	154.44 (± 44.58)	163.98 (± 28.23)
VGLUT1+ cells/mm^2	NB	854.65 (± 164.39)	830.00 (± 28.50)	689.01 (± 81.69)	835.14 (± 57.13)
	LB	875.11 (± 152.68)	799.60 (± 37.11)	886.19 (± 105.35)	783.15 (± 48.08)
PNN+ cells/mm^2	NB	26.91 (± 5.03)	27.83 (± 1.93)	15.98 (± 2.04)	21.70 (± 1.62)
	LB	29.06 (± 3.61)	31.59 (± 5.86)	21.34 (± 3.39)	26.98 (± 4.80)
VGAT+/PNN+ cells/mm^2	NB	14.15 (± 4.07)	11.85 (± 1.57)	7.99 (± 2.06)	8.04 (± 1.61)
	LB	13.81 (± 2.45)	10.77 (± 2.21)	10.31 (± 1.82)	7.48 (± 2.18)
VGLUT1+/PNN+ cells/mm^2	NB	2.29 (± 0.33)	3.26 (± 0.61)	1.46 (± 0.33)	3.05 (± 0.40)
	LB	2.78 (± 0.73)	4.16 (± 0.85)	2.65 (± 0.35)*	3.17 (± 1.14)
%VGAT+/ PNN+	NB	14.30% (± 3.54%)	9.75% (± 1.82%)	10.84% (± 4.57%)	8.69% (± 2.69%)
	LB	12.24% (± 2.25%)	7.41% (± 1.34%)	10.31% (± 1.64%)	5.87% (± 1.66%)
%VGLUT1+/ PNN+	NB	0.28% (± 0.03%)	0.41% (± 0.09%)	0.24% (± 0.04%)	0.37% (± 0.05%)
	LB	0.31% (± 0.04%)	0.53% (± 0.10%)$^{\#}$	0.32% (± 0.03%)	0.42% (± 0.13%)

Cell counts were acquired from RNAscope/immunohistochemistry experiments using QuPath automated counting for the *in-situ* hybridization signal and manually for the immunohistochemical staining of PNNs. Two-way ANOVAs with bedding and sex as between-subject factors were performed to identify significant differences. Data are mean ± SEM n = 4 animals/group. *Significant difference between NB and LB groups, $p<0.05$. $^{\#}$Significant difference between males and females, $p<0.05$.

November 10, 2021

Animal Certificate

This is to certify that **Dr. Claire Dominique Walker, Department of Psychiatry, Douglas Mental Health University Institute,** currently holds an approved **Animal Use Protocol # 2020-8179** 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, 2021

Expiration date: September 30, 2022

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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