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Region-specific Effects of Maternal Separation on Perineuronal Net and Parvalbumin-expressing Interneuron Formation in Male and Female Rats

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Abstract—Early life experiences play a vital role in contributing to healthy brain development. Adverse experiences have a lasting impact on the prefrontal cortex (PFC) and basolateral amygdala (BLA), brain regions associated with emotion regulation. Early life adversity via maternal separation (MS) has sex-specific effects on expression of parvalbumin (PV), which is expressed in fast-spiking GABAergic interneurons that are preferentially enwrapped by perineuronal nets (PNNs). Importantly, PNN formation coincides with the closure of developmental critical periods and regulates PV-expressing interneuron activity. Since aberrant PNN organization has been reported following adverse experiences in adolescent and adult rats, we investigated the impact of adversity early in life in the form of MS on the developing brain. Rat pups were separated from their dams for 4 h per day from postnatal day (P) 2–20. Tissue sections from juvenile (P20), adolescent (P40), and early adult (P70) animals containing the PFC and BLA were fluorescently stained to visualize *Wisteria floribunda* agglutinin+ PNNs and PV-expressing interneurons, and density and intensity was quantified. Our results confirm past reports that PFC PNNs form gradually throughout development; however, PNN density plateaus in adolescence, while intensity continues to increase into adulthood. Importantly, MS delays PNN formation in the prelimbic PFC and results in sex-specific aberrations in PNN structural integrity that do not appear until adulthood. The present findings reveal sex-, age-, and region-specific effects of early life adversity on PNN and PV maturation, implicating neuroplastic alterations following early life adversity that may be associated with sex differences in psychopathology and resilience.

Key words: early life stress, perineuronal nets, parvalbumin, development, prefrontal cortex, basolateral amygdala.

INTRODUCTION

For decades, researchers have examined the effects of early life experiences on development, investigating the modulation of basic brain functions throughout the lifetime (Hensch, 2004), as well as developmental plasticity as it relates to complex neural functions (Danese and McEwen, 2012; Rook et al., 2015). Growing evidence suggests that exposure to adversity early in life can increase vulnerability to a multitude of neuropsychiatric disorders, including depression, anxiety, drug abuse, and schizophrenia (Enoch, 2011; Pace et al., 2006; Scheller-Gilkey et al., 2004). Clinical research further demonstrates that early life adversity disrupts neural maturation and leads to altered brain development (Heim and Binder, 2012; Pechtel and Pizzagalli, 2011; Teicher et al., 2003). Indeed, there is evidence that specific brain regions are impacted following adversity, including the prefrontal cortex (PFC; Brenhouse and Andersen, 2011) and amygdala (Hanson et al., 2015; Malter Cohen et al., 2013), which are implicated in emotion regulation (Eden et al., 2015).

One animal model of early life adversity is the maternal separation (MS) paradigm, which is a well-characterized analog to childhood neglect in humans (Lehmann and Feldon, 2000; Lupien et al., 2009; Pryce et al., 2005). Research demonstrates that MS alters behavioral development in rodents, leading to deficits in cognition (Aisa et al., 2007; do Prado et al., 2016), social interaction (Farrell et al., 2016; Holland et al., 2014), and emotion regulation (Daniels et al., 2004; Eiland and McEwen, 2012), which may be mediated by adversity-induced disruptions in GABAergic maturation (Grassi-Oliveira et al., 2016; Lewis and Moghaddam, 2006). There are several subtypes of GABAergic interneurons with discrete morphological and electrophysiological properties (DeFelipe et al., 2013; Kim et al., 2017). One

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Abbreviations: BLA, basolateral amygdala; Con, control; ECM, extracellular matrix; IL, infralimbic; MS, maternal separation; P, postnatal day; PFC, prefrontal cortex; PL, prelimbic; PNN, perineuronal net; PV, parvalbumin; WFA, wisteria floribunda agglutinin.

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such subtype includes parvalbumin (PV)-expressing interneurons, which play an integral role in the regulation of inhibitory/excitatory microcircuits throughout the brain via their fast-spiking inhibitory inputs onto pyramidal neurons (Ferguson and Gao, 2018; Sohal et al., 2009). The proper development of PV-expressing neurons is vital for neural and behavioral development (Brown et al., 2015; Powell et al., 2012). Indeed, early life adversity leads to altered PV expression in the PFC (Brenhouse and Andersen, 2011; do Prado et al., 2016; Grassi-Oliveira et al., 2016) and basolateral amygdala (BLA; Seidel et al., 2008). These alterations are linked to behavioral disruption in the form of reduced cognitive function (Wieck et al., 2013), decreased social interaction (Holland et al., 2014), and increased anxiety-like behavior (Ganguly et al., 2015), making these PV interneurons a target for further investigation in adversity-induced dysregulation.

An important component of the regulation of PV maturation is the formation of specialized extracellular matrix (ECM) structures called perineuronal nets (PNNs) – lattice-like structures that preferentially surround PV-expressing interneurons (Enwright et al., 2016; Härtig et al., 1992). Many proteins and ECM molecules (Wang and Fawcett, 2012) create the back bone (Carulli et al., 2010; Kwok et al., 2010) and scaffold (Deepa et al., 2006) of PNNs. Link proteins bind these molecules together (Kwok et al., 2010), generating a polyanionic barrier around neurons that may serve as neuroprotection for fast-spiking PV interneurons that are susceptible to oxidative stress (Cabungcal et al., 2013; Brenhouse and Schwarz, 2016). In addition to protecting enwrapped neurons, PNN development may also lead to reduced plasticity in the central nervous system (Pizzorusso et al., 2002), as the formation of PNNs corresponds with the closure of critical periods throughout the brain (Mauney et al., 2013) and PNNs in the BLA protect fear memories from erasure (Gogolla et al., 2009). Thus, PNNs are thought to play an important role in regulating the activity of the neurons they ensheath, and alterations in PNN structure or density may therefore have consequences for neural functioning (Reichelt et al., 2019; Sorg et al., 2016). Exposure to adverse experiences has been linked to aberrant PNN density and intensity in the PFC (Page and Coutellier, 2018), BLA (Santiago et al., 2018), and hippocampus (Riga et al., 2017), suggesting that stress affects PNN organization throughout the brain. Notably, adverse early life experiences may alter the initial formation of PNNs. Early postnatal administration of an oxidative agent resulted in the reduction of PNNs surrounding PV-expressing interneurons in the PFC (Cabungcal et al., 2013), potentially leaving the increased proportion of PV cells lacking PNNs to be exposed to further oxidative damage. Recent work also demonstrates that MS paired with early weaning alters the structural integrity of PNNs enwrapping PV neurons in adult males (Murthy et al., 2019); however, age- and sex-specific effects of MS has yet to be investigated.

Converging evidence suggests that there are sex-dependent consequences of early life adversity for the brain and the development of neuropsychiatric illness (Gobinath et al., 2017; Goel and Bale, 2009; Kessler and McLeod, 1984; McEwen, 2010; Teicher et al., 2003). Animal studies offer corroboration, demonstrating that males and females are differentially affected by adverse experiences (Andersen and Teicher, 2008; Grassi-Oliveira et al., 2016; Luine et al., 2017). Males and females appear to have different long-term behavioral (Kalinichev et al., 2002; Luine et al., 2017) and neurobiological (Bangasser and Valentino, 2014) responses to early life adversity. Indeed, reduction in PV levels following neonatal MS occurred when assessed at juvenility in females, with no effects seen in males until adolescence (Holland et al., 2014), suggesting that the consequences of early life adversity may appear at different stages of development in males and females. Few studies, however, have investigated how aberrant PNN formation may underlie adversity-induced effects on PV-expressing interneurons. Page and Coutellier (2018) found that adolescent stress resulted in sex- and age-specific alterations in PNNs surrounding PV cells in the PFC. While ECM disorganization following adolescent adversity has been characterized (Page and Coutellier, 2018), the effect of such adversity during the early postnatal period on PNN formation remains unknown.

Thus, the current study sought to elucidate the sex-dependent developmental effects of early life adversity on ECM formation and GABAergic maturation by investigating how MS alters the developmental formation of PNNs enwrapping PV-expressing interneurons in the PFC and BLA throughout development in males and females.

EXPERIMENTAL PROCEDURES

Subjects

Gestational day 15 pregnant Sprague-Dawley rats were ordered from Charles River Laboratories (Wilmington, MA). The day of birth was designated as postnatal day (P) 0. Litters were culled to 10 pups with as close to five males and five females as possible on P1. All animals were housed in a temperature (22–23 °C)- and humidity-controlled environment with a 12-h light/dark cycle (light period 0700–1900, approximately 332 lux) in standard polycarbonate wire-top cages with pine shaving bedding. Food (ProLab 5P00) and water were available *ad libitum* throughout gestation and development. All subjects for the present study were weaned on P21 and double-housed with same-sex littermates, matched for age and experimental condition, until experimentation. All experiments were performed in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (NIH) with approval from the Institutional Animal Care and Use Committee at Northeastern University.

Maternal separation

Entire litters (male and female offspring) were assigned in a random fashion to either a MS or control (Con) rearing condition. A maximum of two animals per experimental group were used per litter to avoid litter effects (n = 5–8/group). Pups in the MS group were isolated

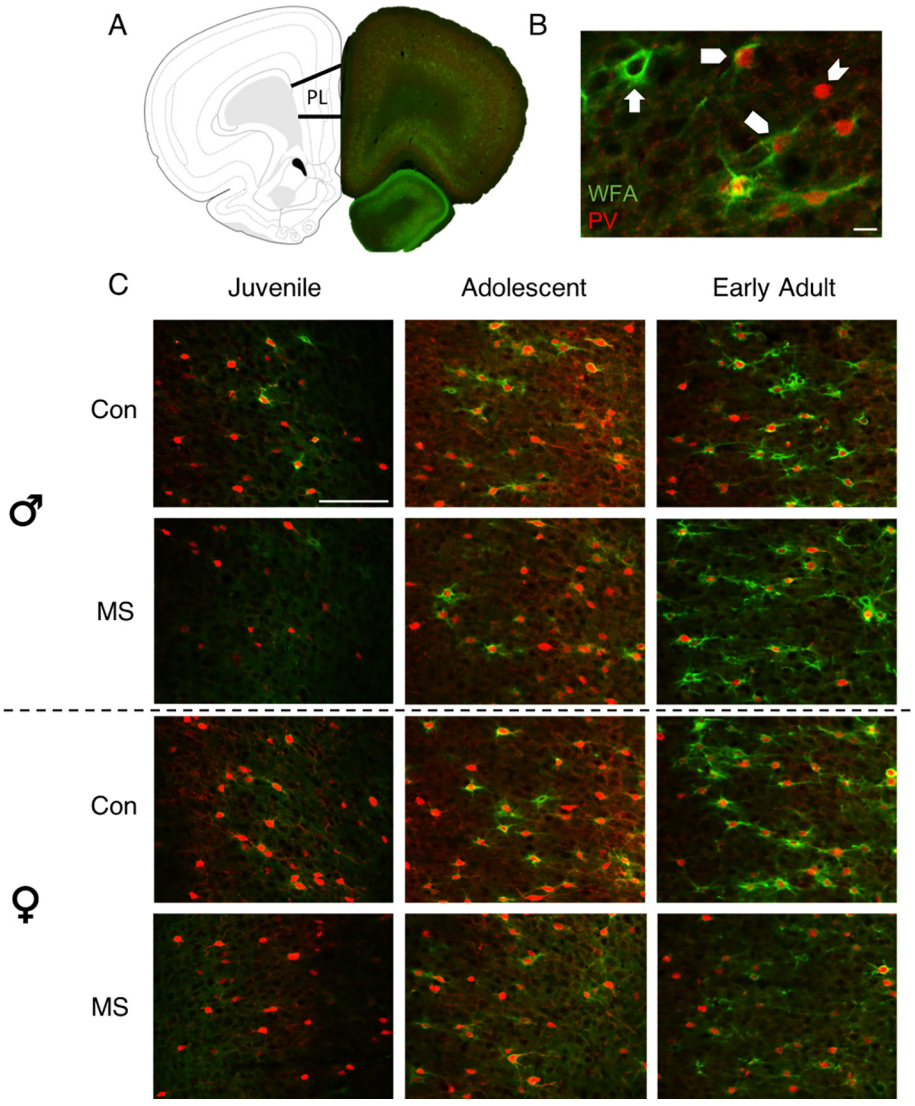

Fig. 1. Wisteria floribunda agglutinin (WFA; green) + perineuronal nets (PNNs) surrounding parvalbumin (PV; red)-expressing interneurons in the prelimbic (PL) prefrontal cortex (PFC). (**A**) Representative diagram (left) and stitched fluorescence image (right) of the PL. (**B**) Double-stained fluorescent photomicrograph of WFA + PNNs surrounding non-PV cells (white arrow), PNNs surrounding PV cells (white pentagonal arrow), and PV cells lacking PNNs (white chevron); scale bar = 10 μm. (**C**) Representative photomicrographs of the PL of male and female rats exposed to maternal separation (MS) or control (Con) rearing sacrificed at juvenility (P20), adolescence (P40), and early adulthood (P70); scale bar = 100 μm.

for four hours per day (0900–1300) between P2 and P20, as previously described (Andersen et al., 1999; Grassi-Oliveira et al., 2016). From P2 to P10, pups were kept in thermoneutral individual containers at 37 °C with bedding from their home cage. From P11 to P20, when pups can adequately thermoregulate, they were individually separated in small cages, also containing home cage bedding. Pups in the Con group were left undisturbed after P1 culling, except for routine weekly changes in cage bedding and weighing on P9, P15, and P20 (approximately five min of handling).

Immunohistochemistry

At the designated developmental time points (P20, P40, and P70), males and females were euthanized with CO_2 and intracardially perfused with ice-cold 0.9% physiological saline followed by 4% paraformaldehyde (PFA) solution. Brains were harvested and stored in PFA for three days with subsequent cryoprotection in 30% sucrose solution until ready for sectioning. All perfusions were performed between 0900 and 1200 to avoid diurnal changes in PNN and PV expression. Brains were sectioned with a freezing microtome (Leica) at a thickness of 40 μm through the PFC (between bregma 4.2 and 2.52 mm) and BLA (between bregma −1.72 and −3.36 mm), and free-floating sections were stored in freezing solution at −20 °C until immunohistochemical staining. To visualize PNNs and PV-expressing interneurons, free-floating sections were first washed in 1X phosphate buffered saline (PBS) and then blocked in 5% normal donkey serum and 1% bovine serum albumin. Sections were then incubated in primary conjugate lectin from *Wisteria floribunda* agglutinin (WFA; 1:250, L1516, MilliporeSigma) and primary rabbit anti-PV antibody (1:10,000, PA1-933, Thermofisher) at 4 °C for 48 h on an agitator, followed by washes in PBS containing 0.3% Triton™X-100 (PBST; Fisher Scientific) and a three-hour incubation in secondary antibody solution composed of streptavidin conjugate 488 (1:3,000, S32354, Thermofisher) and donkey anti-rabbit Alexa Fluor® 594 (1:500, A21207, Thermofisher) in 0.3% PBST. Sections were washed in PBS, mounted on positively-charged glass slides, and coverslipped with ProLong Gold antifade mounting reagent (P36930, Invitrogen). As immunohistochemical staining occurred over several runs, tissue from each experimental group was included in every run of stained subjects in order to control for spurious differences between batches.

Microscopy and image quantification

WFA + PNNs and PV + interneurons were imaged using a Keyence BZ-X701 All-in-One fluorescent microscope. Z-stacks were captured at 20× magnification in four serial sections of the prelimbic (PL) and infralimbic (IL) PFC and the BLA, with each quantified section separated by approximately 240 μm. Regions of interest

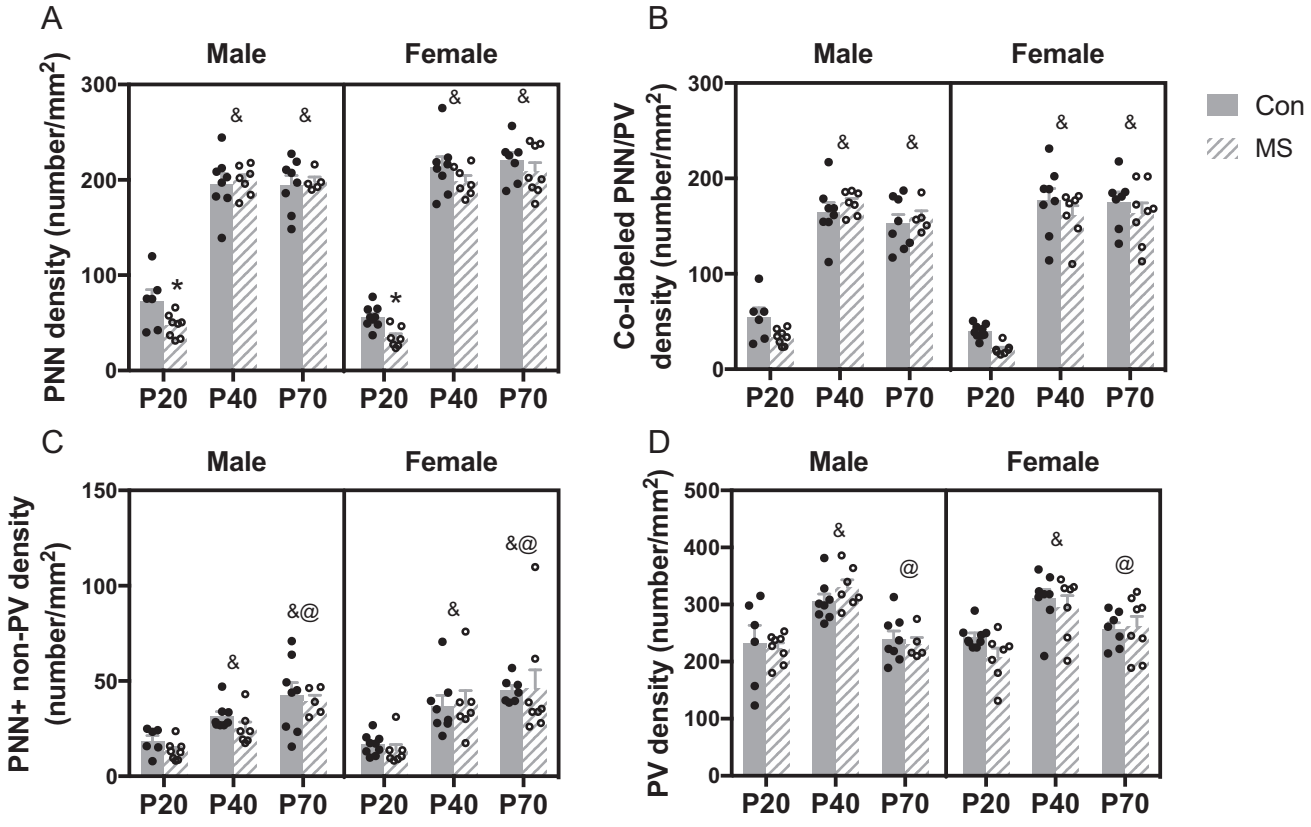

Fig. 2. Maternal separation (MS) delays the formation of perineuronal nets (PNNs) in the prelimbic (PL) prefrontal cortex (PFC). (**A**) MS reduced PNN density in both males and females in juvenility. These effects did not persist through adolescence and adulthood. (**B**) MS did not appear to specifically alter the density of PNNs surrounding parvalbumin (PV)-expressing interneurons in the PL. (**C**) The density of PNNs surrounding non-PV cells was not affected by MS in either males nor females throughout development. (**D**) MS did not change the density of PV-expressing interneurons in either sex at any age, $^{*}p < 0.05$; $^{\&}p < 0.001$ of age group compared to P20; $^{@}p < 0.001$ of age group compared to P40; $n = 5$–8/group.

were delineated using clearly visible landmarks and predefined boundaries according to a rat brain atlas (Paxinos and Watson, 2009). Three z-stacks were captured for the PL, one per IL, and one per BLA, bilaterally (64 stacks per animal; see Figs. 1A, 4A, and 7A for representative neuroanatomical regions).

After image acquisition, each set of TIFFs was uploaded to ImageJ (Schneider et al., 2012), where the number of WFA + PNNs, PV + interneurons, and PNNs colocalized with PV were counted through the entirety of the stained tissue section (average 21 μm per section) by an experimenter blind to experimental condition, and density was quantified according to the area of each counted region. For PFC images, the area was identical for each image (PL: 4.296 mm^2; IL: 1.432 mm^2); however, BLA volume was quantified via the freehand trace tool in ImageJ and density was calculated (average BLA volume: 1.19 mm^2). There was no difference in BLA volume between experimental group, sex, or age (data not shown).

Intensity analysis was accomplished using the "Perineuronal net Intensity Program for the Standardization and Quantification of ECM Analysis" (PIPSQUEAK; Slaker et al., 2016) version 3.021, a FIJI (Schindelin et al., 2012) plugin developed for the detection and quantification of PNNs and PV cells. Images were analyzed in PIPSQUEAK using predetermined parameters for the detection of WFA and PV staining. All analysis in both the PFC and BLA was conducted in semi-autonomous mode by an experimenter blind to experimental condition to correct misidentification of PNNs and PV neurons. The low background subtraction setting was used to better detect faint staining. After identification of WFA and PV labeling, double labeling of PNNs and PV cells was identified by PIPSQUEAK, with at least 80% overlap needed to be considered colocalized.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism 7 software or IBM SPSS Statistics V.25. Before analyses were performed, groups were tested for outliers using Grubbs' Test, homogeneity of variances was assessed via Levene's Test of Equality of Error Variances, and Normality of residuals was assessed via the Shapiro–Wilk Test of Normality. Levels of skewness and kurtosis were also assessed. Group analyses were then run using three-way ANOVAs (Sex × Rearing × Age). Since our key questions involved sex- and experience-dependent development of PNNs, significant interactions and main effects of

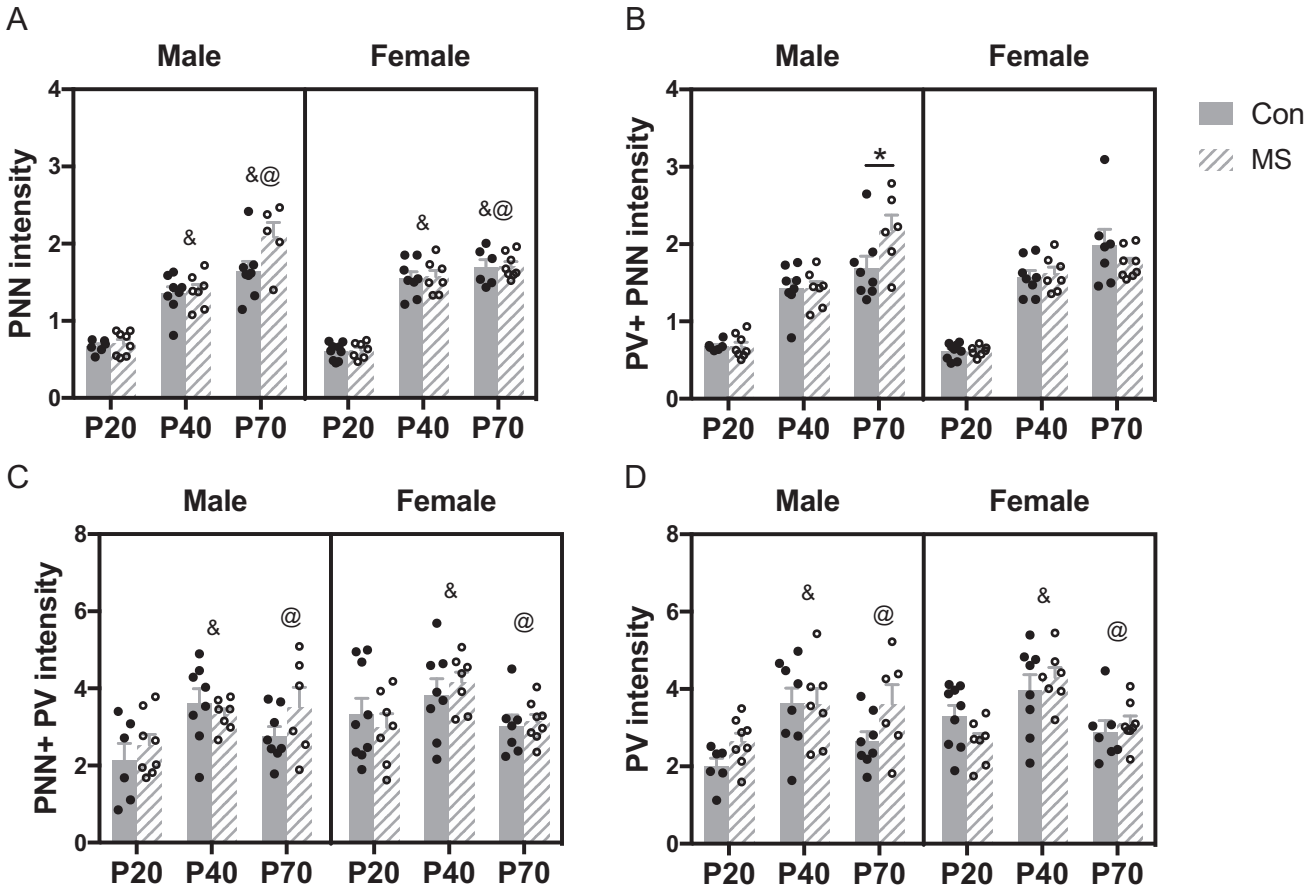

Fig. 3. Maternal separation (MS) alters later-life intensity of perineuronal nets (PNNs) surrounding parvalbumin (PV)-expressing interneurons in the prelimbic (PL) prefrontal cortex (PFC). (**A**) Overall PNN intensity was not affected by rearing at any age. (**B**) Rearing altered the intensity of PNNs surrounding PV neurons in a sex- and age-specific manner, where males exposed to MS demonstrated increased intensity in adulthood. (**C**) There was no effect of rearing on male or female intensity of PV neurons surrounded by PNNs throughout development. (**D**) PV intensity was also not altered by MS at any age. $^{*}p < 0.05$; $^{\&}p < 0.001$ of age group (Con and MS pooled) compared to P20; $^{@}p < 0.001$ of age group (Con and MS pooled) compared to P40; n = 5–8/group.

rearing were followed up with Sidak post-hoc tests to correct for multiple comparisons between rearing groups at each age, and significant main effects of age were followed up with Fisher's LSD to probe pair-wise differences between three ages. Since ANOVA-generated p-values are not sufficient as metrics of magnitude of the effect of a manipulation (Sullivan and Feinn, 2012), effect size was measured using SPSS and presented as partial eta squared (partial η^2) for ANOVAs. As described in Richardson (2011), small, medium, and large effect sizes were categorized using values of partial η^2 = 0.01, 0.06, and 0.14, respectively. Additionally, pairwise comparisons where p values were at trend level were reported as such when Hedge's g was $\geq$0.8.

RESULTS

We investigated the effects of early life adversity via MS, as compared to Con, on PNN formation and colocalization with PV neurons in the PFC and BLA of male and female rats at juvenility (P20), adolescence (P40), and early adulthood (P70).

Effects of MS in the prelimbic PFC throughout development

Density. First, the effects of MS on overall PNN density in the PL PFC were assessed throughout development in male and female rats (see Fig. 1 for representative photomicrographs). Three-way ANOVA revealed a significant interaction between Sex and Age ($F_{2,76}$ = 4.185, p = 0.019, η_p^2 = 0.099) and a main effect of Age ($F_{2,76}$ = 462.872, $p < 0.0001$, η_p^2 = 0.924), with both males and females expressing significantly fewer PNNs at P20 compared to P40 ($p < 0.0001$) and P70 ($p < 0.001$). Notably, MS reduced the density of PNNs (main effect of Rearing $F_{1,76}$ = 5.492, p = 0.022, η_p^2 = 0.067), where subjects exposed to MS had fewer PNNs in the PL compared to Con, driven by a difference only in juvenility (p = 0.03; Fig. 2A).

Next, we investigated how age, rearing and sex affect different sub-populations of PNNs, specifically PNNs that enwrap PV-expressing interneurons versus PNNs that surround other neurons. Additionally, the overall density of cells expressing PV was quantified within each region

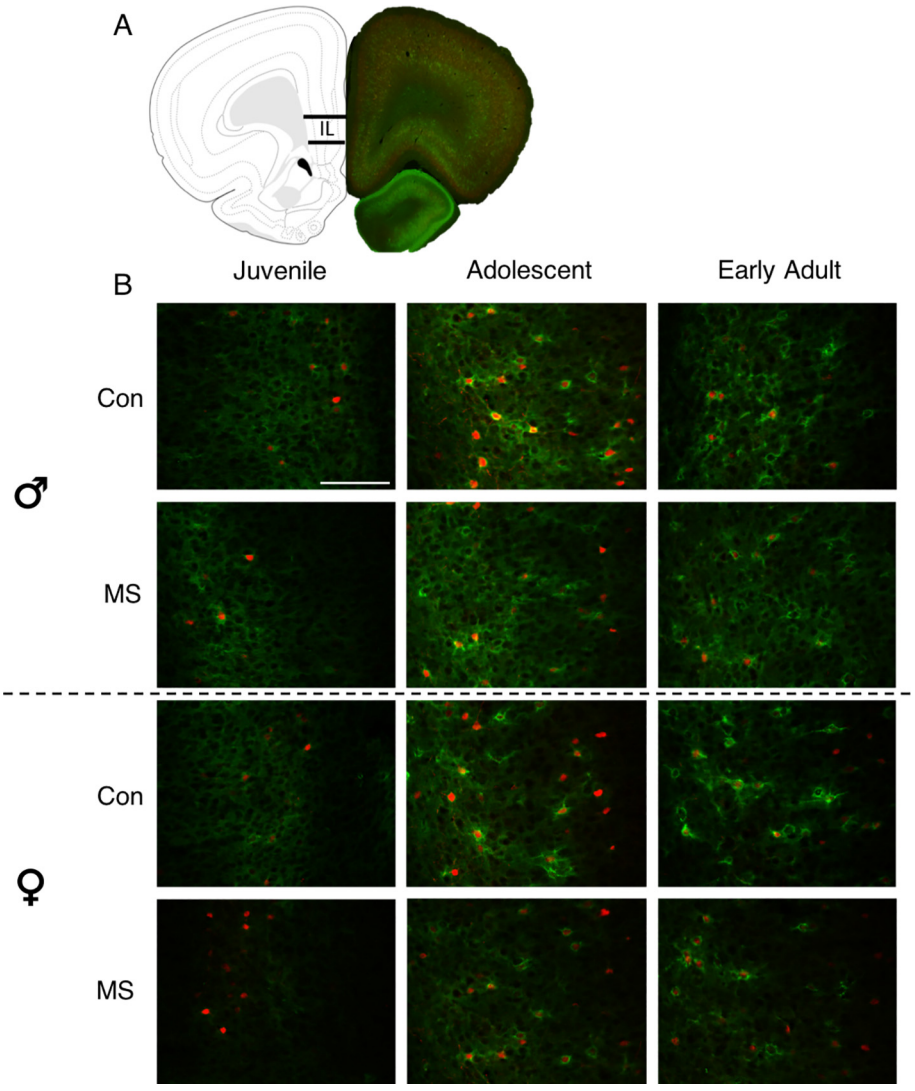

Fig. 4. Wisteria floribunda agglutinin (WFA; green) + perineuronal nets (PNNs) surrounding parvalbumin (PV; red)-expressing interneurons in the infralimbic (IL) prefrontal cortex (PFC). (**A**) Representative diagram (left) and stitched fluorescence image (right) of the IL. (**B**) Representative photomicrographs of the IL of male and female rats exposed to maternal separation (MS) or control (Con) rearing sacrificed at juvenility (P20), adolescence (P40), and early adulthood (P70); scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of interest. The density of PNNs surrounding PV-expressing interneurons increased throughout development (main effect of Age $F_{2,76} = 291.295$, $p < 0.0001$, $\eta_p^2 = 0.885$), with significantly fewer PNN expressed at P20 compared to P40 ($p < 0.001$) and P70 ($p < 0.001$). No significant effect of rearing was observed (main effect of Rearing $F_{1,76} = 2.691$, $p = 0.105$, $\eta_p^2 = 0.034$; Fig. 2B). Similarly, there was an effect of Age ($F_{2,76} = 37.402$, $p < 0.0001$, $\eta_p^2 = 0.499$) – but not Rearing (main effect of Rearing $F_{1,75} = 1.268$, $p = 0.192$, $\eta_p^2 = 0.022$) – on PNNs surrounding non-PV-expressing cells (Fig. 2C). Lastly, there was a main effect of Age on PV-interneuron density ($F_{2,76} = 31.560$, $p < 0.0001$, $\eta_p^2 = 0.454$), with peak expression at P40 revealed by a difference between P20–P40 ($p < 0.001$) and P40–P70 ($p < 0.001$). MS did not affect the density of PV-expressing neurons in the PL at any age (main effect of Rearing $F_{1,76} = 0.528$, $p = 0.470$, $\eta_p^2 = 0.007$; Fig. 2D).

Intensity. We also quantified intensity measures to determine how MS affects the structural integrity of PNNs as well as the protein expression of the PV cells that they enwrap. In contrast to PL density measures, overall PNN intensity was not affected by Rearing condition (main effect of Rearing $F_{1,77} = 1.090$, $p = 0.30$, $\eta_p^2 = 0.014$); however, the intensity of PNNs in the PL increased throughout development into adulthood (main effect of Age $F_{2,76} = 152.636$, $p < 0.0001$, $\eta_p^2 = 0.799$; Fig. 3A), with $p < 0.001$ difference between all ages.

When the intensity of PNNs surrounding PV neurons was assessed, there was a significant three-way interaction (Rearing × Sex × Age $F_{2,76} = 3.985$, $p = 0.023$, $\eta_p^2 = 0.094$), as well as an increase in PV + PNN intensity throughout the lifetime (main effect of Age $F_{2,76} = 140.763$, $p < 0.0001$, $\eta_p^2 = 0.785$). Pair-wise comparisons show that MS increased the intensity of PNNs enwrapping PV neurons in adult males, compared to controls ($p = 0.0193$). Assessing the intensity of the PV cells that were surrounded by PNNs yielded main effects of Sex ($F_{1,76} = 4.821$, $p = 0.031$, $\eta_p^2 = 0.059$) and Age ($F_{2,76} = 8.742$, $p < 0.0001$, $\eta_p^2 = 0.185$), where females overall demonstrated more intensely stained cells and peak expression at P40 (P20-P40 $p < 0.0001$; P40-P70 $p = 0.008$). Similarly to the density measure, the intensity of PV neurons was not affected by Rearing or Sex, but was altered by Age ($F_{2,76} = 15.699$, $p < 0.0001$, $\eta_p^2 = 0.290$) with peak intensity in adolescence (P20-P40 $p < 0.0001$; P40-P70 $p = 0.001$).

Effects of MS in the infralimbic PFC throughout development

Density. We also evaluated potential aberrations in PNN development in the IL PFC (see Fig. 4 for representative photomicrographs). PNN density was significantly altered by both Rearing ($F_{1,76} = 7.791$,

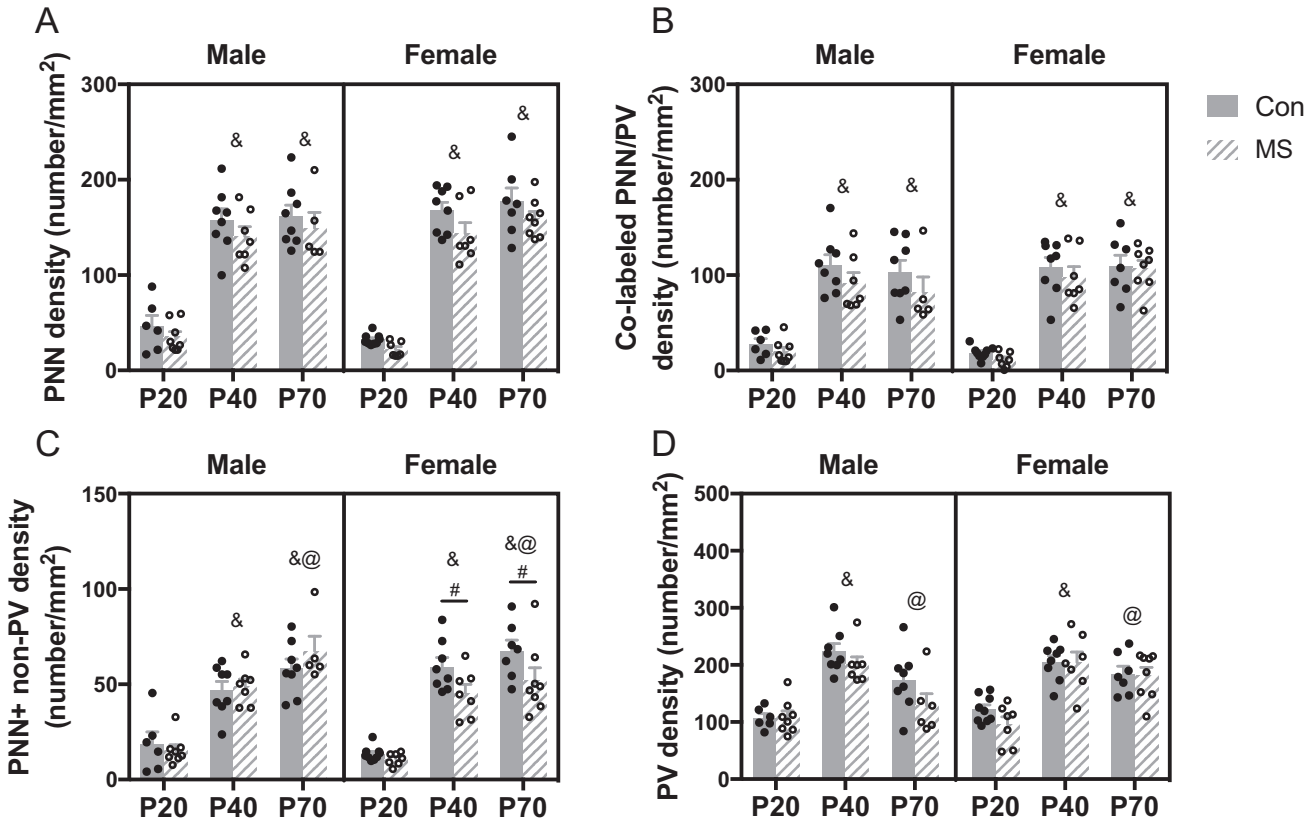

Fig. 5. Maternal separation (MS) alters later-life perineuronal net (PNN) density in the infralimbic (IL) prefrontal cortex (PFC). (**A**) PNN density was altered by MS (p = 0.0007), showing an overall reduction. (**B**) There was also a main effect of rearing on the density of PNNs surrounding parvalbumin (PV) neurons (p = 0.034), where MS reduced the density of co-labeled PNNs. (**C**) A rearing × sex interaction ($p = 0.015$) was observed on the density of PNNs surrounding non-PV cells. This interaction was driven by females demonstrating significantly fewer PNNs surrounding non-PV cells in adolescence and adulthood following MS. (**D**) There was no effect of rearing on the density of PV neurons in the IL, $^{\#}p < 0.07$; $^{\&}p < 0.001$ of age group compared to P20; $^{@}p < 0.001$ of age group compared to P40; n = 5–8/group.

$p = 0.0007$, $\eta_p^2 = 0.093$) and Age ($F_{2,76} = 215.964$, $p < 0.0001$, $\eta_p^2 = 0.850$), where PNN density was reduced by MS while overall density increased with age from P20 to P40 ($p < 0.001$; Fig. 5A).

Analysis of PNN subpopulations in the IL revealed that MS reduced the density of PNNs enwrapping PV-expressing interneurons (main effect of Rearing $F_{1,76} = 4.662$, $p = 0.034$, $\eta_p^2 = 0.058$), while age increased density (main effect of Age $F_{2,76} = 104.863$, $p < 0.0001$, $\eta_p^2 = 0.734$; Fig. 5B). Density of co-labeled PNNs and PV cells was higher at P40 ($p < 0.001$) and P70 ($p < 0.001$), compared to P20. Interestingly, MS also reduced the density of PNNs surrounding non-PV-expressing cells in a sex-specific manner (Rearing × Sex interaction $F_{1,76} = 6.150$, $p = 0.015$, $\eta_p^2 = 0.075$), as well as an overall increase in density throughout development (main effect of Age $F_{2,76} = 107.686$, $p < 0.0001$, $\eta_p^2 = 0.739$; Fig. 5C). Post-hoc comparisons revealed increases at all three ages ($p < 0.001$), as well as a trend-level reduction in PNNs enwrapping non-PV cells in females at adolescence ($p = 0.065$; Hedge's $g = 1.39$) and early adulthood ($p = 0.057$; Hedge's $g = 0.960$). Density measurement of PV neurons revealed only a main effect of Age ($F_{2,76} = 51.245$, $p < 0.0001$, $\eta_p^2 = 0.574$; Fig. 5D), with a peak in adolescence (P20–P40: $p < 0.001$; P40–P70: $p < 0.003$).

Intensity. When intensity was quantified for IL PNNs, no effect of either Rearing or Sex was apparent, while PNN intensity increased significantly throughout the lifetime (main effect of Age $F_{1,76} = 72.130$, $p < 0.0001$, $\eta_p^2 = 0.652$; Fig. 6A), with increases at all ages ($p < 0.001$).

Similarly, there was only a main effect of Age ($F_{2,76} = 66.912$, $p < 0.0001$, $\eta_p^2 = 0.635$) on the intensity of PNNs enwrapping PV neurons (Fig. 6B), with increases at all ages ($p \leq 0.001$). Females overall had higher intensity of PV cells surrounded by PNNs (main effect of Sex $F_{1,76} = 4.537$, $p = 0.036$, $\eta_p^2 = 0.056$), which was also altered throughout development (main effect of Age $F_{2,76} = 13.620$, $p < 0.0001$, $\eta_p^2 = 0.261$; Fig. 6C). PV intensity was also only affected by Age ($F_{2,76} = 19.455$, $p < 0.0001$, $\eta_p^2 = 0.336$; Fig. 6D), with peak intensity in adolescence (P20–P40 $p < 0.0001$; P40–P70 $p < 0.0001$).

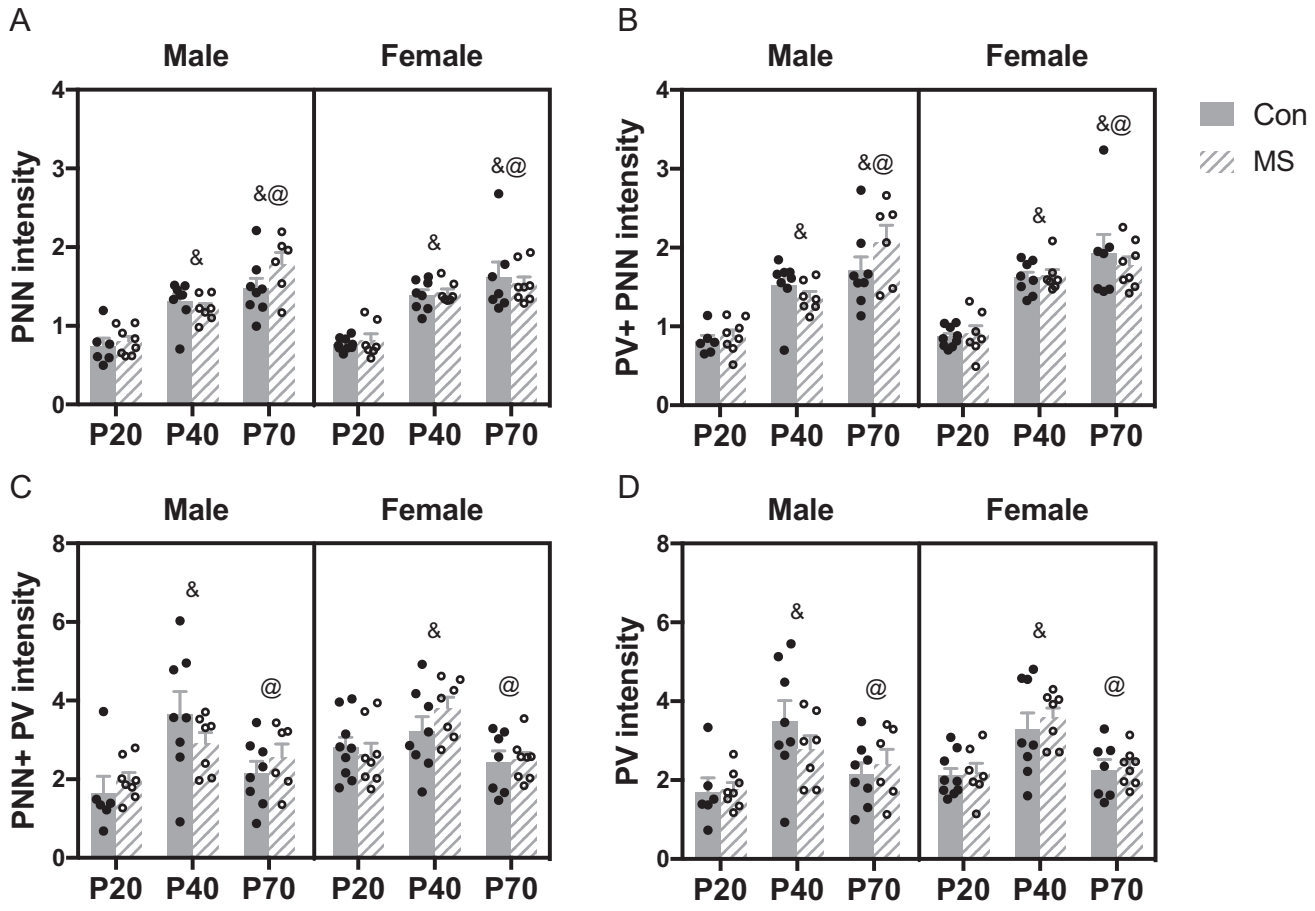

Fig. 6. Maternal separation (MS) does not alter the intensity of perineuronal nets (PNNs) in the infralimbic (IL) prefrontal cortex (PFC). There was no effect of rearing on PNN intensity (**A**), intensity of PNNs surrounding parvalbumin (PV) neurons (**B**), intensity of PV neurons enwrapped by PNNs (**C**), or overall PV intensity (**D**) in the IL of males or females throughout development, $^{\&}p < 0.001$ of age group compared to P20; $^{@}p < 0.001$ of age group compared to P40; $n = 5$–8/group.

Effects of MS in the BLA throughout development

Density. We also analyzed neurostructural development following early life adversity in the BLA of males and females (see Fig. 7 for representative photomicrographs). Evaluation of overall PNN density in the BLA revealed a Rearing × Sex × Age interaction ($F_{2,63} = 6.988$, $p = 0.002$, $\eta_p^2 = 0.182$; Fig. 8A). Pair-wise comparisons revealed a trend-level increase in adolescent male PNN density in the BLA following MS, compared to Con males (p = 0.078) with a moderate effect size (Hedge's $g = 1.48$), but Rearing did not significantly affect PNN density at any other age in males or females. Similar to our findings in the PFC, PNN density also increased throughout development (main effect of Age $F_{2,63} = 42.841$, $p < 0.001$, $\eta_p^2 = 0.576$).

Analysis of both the density of PNNs surrounding PV neurons ($F_{2,63} = 16.065$, $p < 0.0001$, $\eta_p^2 = 0.338$; Fig. 8B) and PNNs enwrapping non-PV neurons ($F_{2,63} = 4.671$, $p = 0.013$, $\eta_p^2 = 0.129$; Fig. 8C) revealed only a main effect of Age. Overall PV density, however, was altered by MS throughout development in a sex-specific manner (Rearing × Sex × Age interaction $F_{2,63} = 3.894$, $p = 0.025$, $\eta_p^2 = 0.110$; Rearing × Age interaction $F_{2,63} = 3.436$, $p = 0.038$, $\eta_p^2 = 0.098$; Sex × Age interaction $F_{2,63} = 3.737$, $p = 0.029$, $\eta_p^2 = 0.106$; main effect of Rearing $F_{1,63} = 6.508$, $p = 0.013$, $\eta_p^2 = 0.094$; main effect of Sex $F_{1,63} = 7.969$, $p = 0.006$, $\eta_p^2 = 0.112$; main effect of Age $F_{2,63} = 24.110$, $p < 0.0001$, $\eta_p^2 = 0.434$; Fig. 8D). MS exposure resulted in increased PV density in male adolescents ($p = 0.0055$) with no pair-wise effects in females at any age.

Intensity. Intensity analyses in the BLA revealed Rearing × Sex ($F_{1,63} = 5.891$, $p = 0.018$, $\eta_p^2 = 0.086$) and Sex × Age ($F_{2,63} = 3.423$, $p = 0.039$, $\eta_p^2 = 0.098$) interactions (Fig. 9A). Female MS animals demonstrated increased PNN intensity in adulthood at trend-level ($p = 0.07$; Hedge's $g = 1.67$). Overall PNN intensity also increased throughout development ($F_{2,63} = 10.615$, $p < 0.001$, $\eta_p^2 = 0.252$), with significant increases between P40 and P70 ($p = 0.005$).

While MS did not alter population-specific intensity measures, there was a consistent main effect of Age on the intensity of PNNs surrounding PV neurons ($F_{2,63} = 13.022$, $p = 0.006$, $\eta_p^2 = 0.152$; Fig. 9B), PV

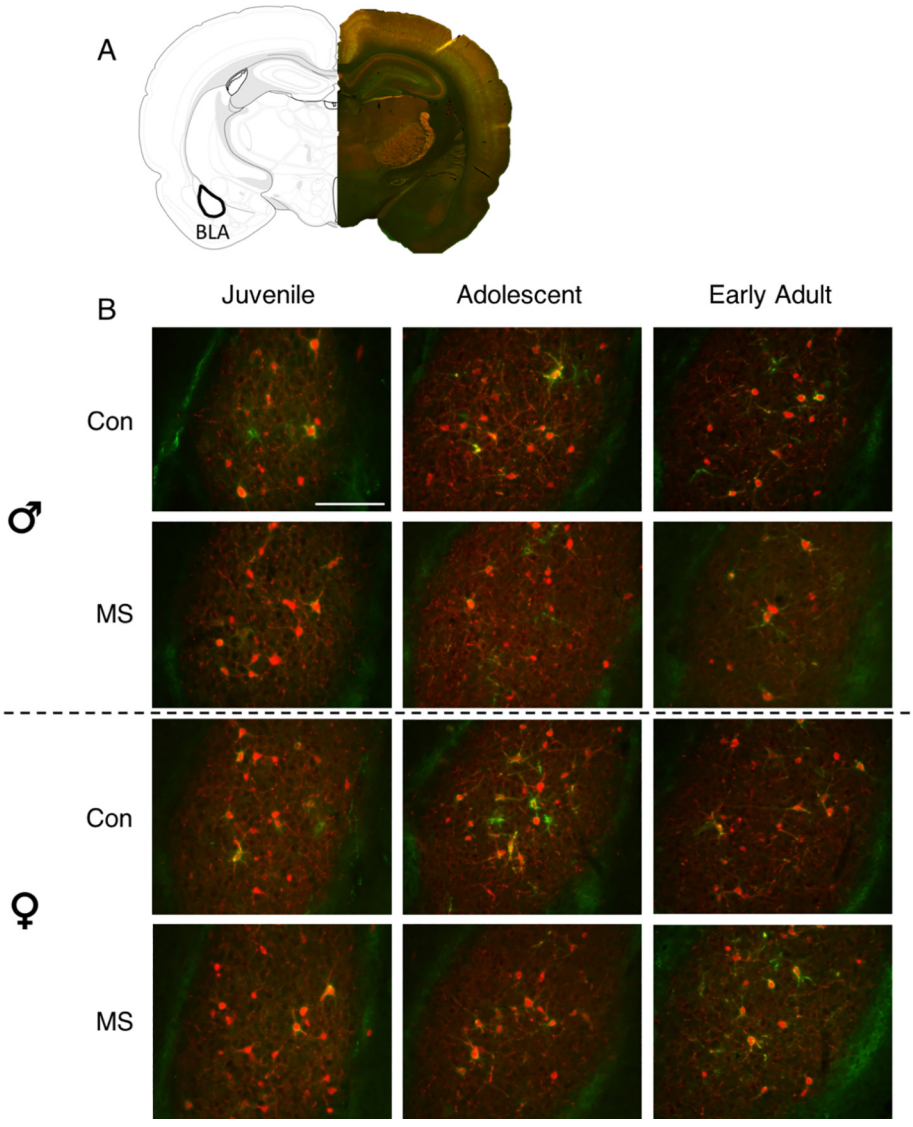

Fig. 7. Wisteria floribunda agglutinin (WFA; green) + perineuronal nets (PNNs) surrounding parvalbumin (PV; red)-expressing interneurons in the basolateral amygdala (BLA). (**A**) Representative diagram (left) and stitched fluorescence image (right) of the BLA. (**B**) Representative photomicrographs of the BLA of male and female rats exposed to maternal separation (MS) or control (Con) rearing sacrificed at juvenility (P20), adolescence (P40), and early adulthood (P70); scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neurons surrounded by PNNs ($F_{2,63}$ = 3.941, p = 0.024, η_p^2 = 0.111; Fig. 9C), and overall PV intensity ($F_{2,63}$ = 5.635, p = 0.006, η_p^2 = 0.152; Fig. 9D) in the BLA. These age effects were consistently observed between P40 and P70 (for PV + PNNs: p = 0.001; for PNN + PV: p = 0.023; for overall PV: p = 0.002).

DISCUSSION

Evidence suggests that early life adversity causes GABAergic dysfunction throughout the brain (Grassi-Oliveira et al., 2016; Riga et al., 2017) and that PV interneuron maturation is regulated by ECM development (Enwright et al., 2016; Härtig et al., 1992). There is, however, a dearth of information regarding sex-dependent adversity-induced disruption of ECM formation. Therefore, the current study sought to investigate region-specific effects of early life adversity in the form of MS by quantifying PNN formation throughout development in males and females. To do this, we measured the density of WFA + PNNs and PV neurons, as well as two subsets of PNN populations consisting of PNNs surrounding PV interneurons and PNNs ensheathing non-PV neurons. We also quantified the intensity of PNNs, PV neurons, PNNs surrounding PV neurons, and PV neurons enwrapped by PNNs in the PFC and BLA at three different developmental time points.

In the PFC, we observed that overall PNN density increased through adolescence, but plateaued after adolescence into early adulthood. This developmental effect was apparent in the subpopulation of PNNs surrounding PV neurons, while PNNs surrounding non-PV cells continued to increase in number after adolescence. Notably, PNN intensity also increased through adulthood in both the PL and IL PFC. PV density and intensity, however, peaked in adolescence and decreased back to juvenile levels in adulthood. Rearing also affected PNN and PV maturation. Early postnatal MS resulted in reduced amount of PNNs in the PL of juvenile animals. This delay in the formation of PNNs was not specific to PNNs surrounding PV neurons or non-PV neurons. Importantly, rearing, sex, and age all interacted to affect the intensity of PNNs surrounding PV neurons in the PL, where MS resulted in higher intensity of adult male – but not female – PV + PNNs. MS also moderately decreased the density of PNNs enwrapping non-PV neurons in adolescent and adult females. Analysis in the BLA also revealed that density and intensity of PNNs increased throughout development. While MS did not affect PNN density in the BLA, adolescent males exposed to MS had more PV neurons. Additionally, MS increased the intensity of

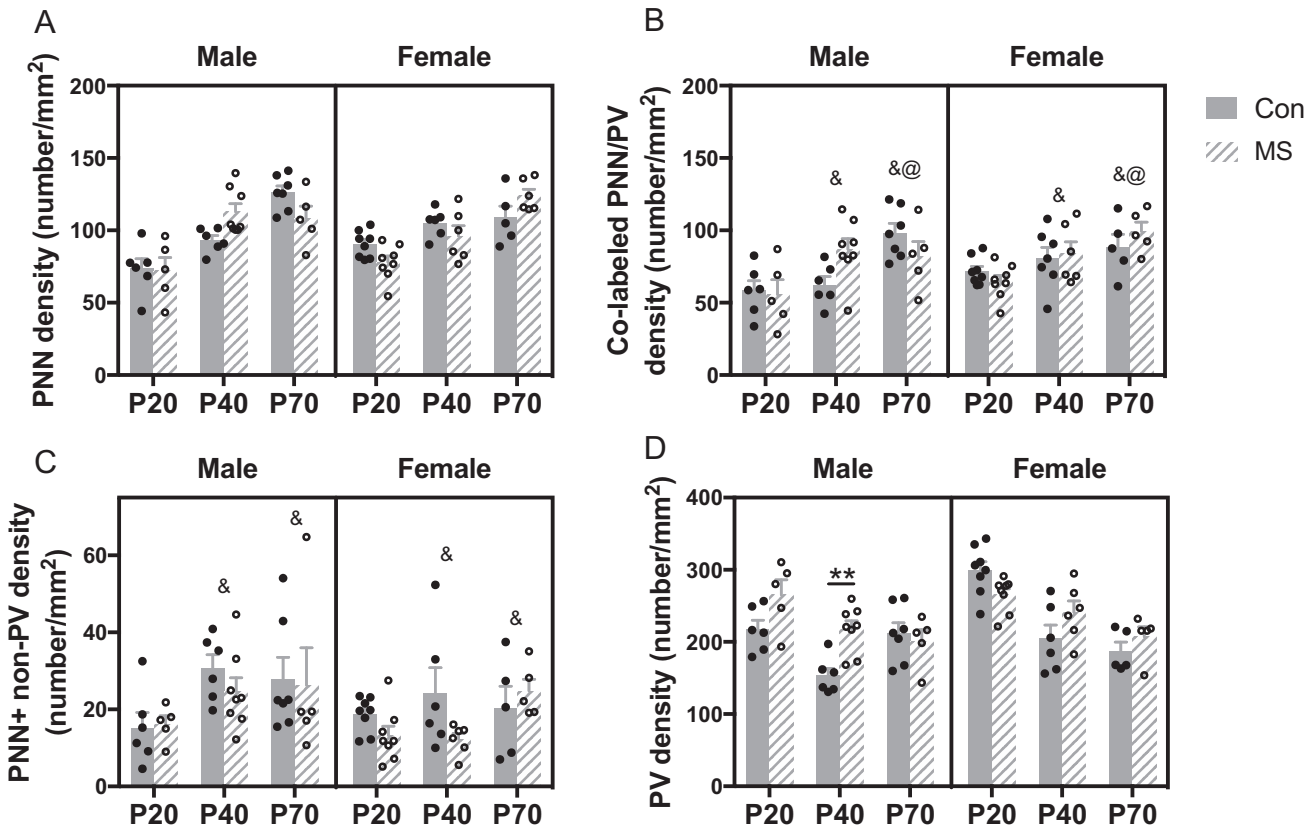

Fig. 8. Maternal separation (MS) alters overall perineuronal net (PNN) and parvalbumin (PV)-expressing interneuron density in the basolateral amygdala (BLA). (**A**) Overall PNN density was affected by MS in a sex- and age-specific manner, where males exposed to MS had more PNNs in adolescence. (**B**) There was no effect of rearing on the density of PNNs enwrapping PV neurons. (**C**) The density of PNNs surrounding non-PV neurons was also not affected by MS. (**D**) MS had sex- and age-specific effects on PV density in the BLA. MS increased the density of PV neurons in adolescent males – but not females, $^{**}p < 0.01$; $^{\&}p < 0.001$ of age group compared to P20; $^{@}p < 0.001$ of age group compared to P40; $n = 5$–8/group.

female – but not male – PNNs in adulthood. Taken together, the present findings reveal sex-, age-, and region-specific effects of early life adversity on PNN and PV maturation (see Fig. 10 for summary).

The literature consistently reports that GABAergic and extracellular maturation corresponds with major changes in neurocircuitry and plasticity (see review: Reichelt et al., 2019). Similar to past studies (Baker et al., 2017; Mauney et al., 2013), the present work indicates an increase in PNN density – both surrounding PV and non-PV neurons – throughout development in the PFC and BLA. This maturational increase occurred earlier in the PFC than the BLA, where PFC PNN density peaked at adolescence and plateaued into adulthood. This pattern is similar to PNN development in the visual cortex (Ye and Miao, 2013). Interestingly, we found that PNN intensity increased after adolescence in both the PFC and BLA, suggesting that the composition of PNNs is still being strengthened after overall construction has ceased. Adolescence is often referred to as a transitional developmental period, during which substantial behavioral and neural modification occurs (Andersen and Teicher, 2008; Semple et al., 2013). The continued compositional development of PNNs throughout adolescence and into early adulthood may underlie functional maturation during this stage of development. Additionally, while past work suggests that PV cell count moderately increases throughout development in the PFC and BLA (Baker et al., 2017), we found that both PV density and intensity peak in adolescence and decrease into adulthood. Correspondingly, work by Ye and Miao (2013) also report a peak in cortical PV density in adolescence followed by a reduction in adulthood. Research indicates substantial age- and region-specific modulation of PV (Honeycutt et al., 2016), denoting an important role for PV maturation in the organization of cortical circuits.

PNN density is reportedly reduced in the PFC (Mauney et al., 2013) and amygdala (Pantazopoulos et al., 2010a,b) of individuals with schizophrenia, implicating PNN dysfunction in human psychopathology. PNN formation plays an integral role in the regulation of activity-dependent neuronal plasticity by modifying the activation patterns of PV interneurons (Favuzzi et al., 2017). While the functional repercussions of MS on PV interneurons were not investigated here, research suggests that the electrophysiological properties of these cells may be altered by stress (Perova et al., 2015). Indeed, social defeat-induced stress was found to alter PNN organization in the CA1 of the hippocampus and disrupt hippocampal inhibitory neurotransmission (Riga et al., 2017). Given

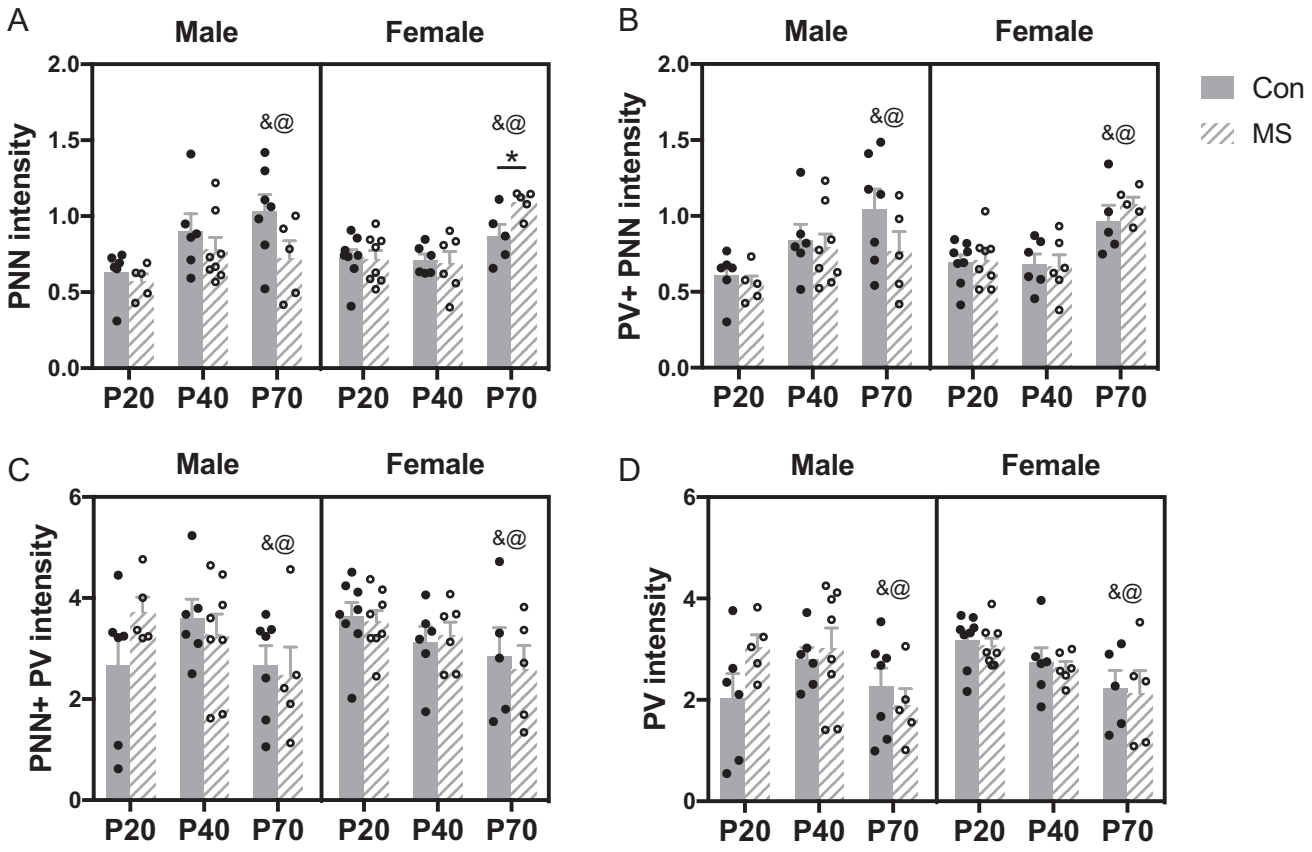

Fig. 9. Maternal separation (MS) alters perineuronal net (PNN) intensity in the basolateral amygdala (BLA). (**A**) PNN intensity was altered by MS in a sex-specific manner. Specifically, females demonstrated increased PNN intensity following MS. There was no effect of Rearing on the intensity of PNNs surrounding PV neurons (**B**), intensity of PV cells enwrapped by PNNs (**C**), or PV intensity (**D**), $^{*}p < 0.05$ of rearing group; $^{\&}p < 0.05$ of age group compared to P20; $^{@}p < 0.05$ of age group compared to P40; $n = 5–8$/group.

past research investigating the role of PNN regulation on the inhibitory/excitatory activity of PV-expressing neurons throughout the brain (Dityatev et al., 2007), the transient decrease in the density of PNNs following MS seen in the current study may affect the physiological properties of PV interneurons that underlie behavioral disruption. Importantly, PNNs are dynamic structures that continue to develop and change throughout the lifetime according to experience (Foscarin et al., 2011), and prior research supports our assertion that MS-induced aberrant PNN formation in the PL PFC normalized after juvenility. Page and Coutellier (2018) demonstrated that PNNs enwrapping PV neurons were altered by adversity when measured at adolescence; however, PNNs returned to normal levels by adulthood. While PNN organization may normalize throughout development following initial exposure to adverse experiences – suggesting a transient effect of adversity on neurostructural aberrations – anxiety-like behaviors endured into adulthood (Page and Coutellier, 2018).

There is evidence that alterations in PNN intensity occur without changes in PNN density (Enwright et al., 2016; Santiago et al., 2018), suggesting that PNN structural integrity can be compromised without reduction in PNN number. It was hypothesized here that MS would result in reduced PNN staining intensity; however, female adults had higher intensity of PNNs surrounding PV neurons in the PL following MS. Similar to findings from Santiago et al. (2018), we found no effect of adversity on PNN density in the BLA; however, there was a sex-dependent effect of MS, where PNN intensity was increased in female adults. Indeed, Murthy et al. (2019) found no stress-induced change in adult hippocampal PNN density following MS with early weaning, but reported a significant increase in PNN intensity in adulthood. Increased PNN intensity in the hippocampus was also demonstrated following adult social defeat-induced persistent stress (Riga et al., 2018). Research suggests that decreased structural integrity of PNNs impacts neuroplasticity in the brain (Wang and Fawcett, 2012), suggesting that the altered PNN intensity seen here could impact long-term neural functioning. Taken together, there is clear evidence across studies that adversity influences later-life PNN intensity, and future studies will need to investigate how MS affects the structural integrity of PNNs and whether alterations in PNN intensity following early life adversity have long-term behavioral consequences.

Evidence from our lab and others suggests that PV expression is reduced in animals exposed to MS (Grassi-Oliveira et al., 2016; Holland et al., 2014; Wieck et al., 2013); however, the current study did not reveal

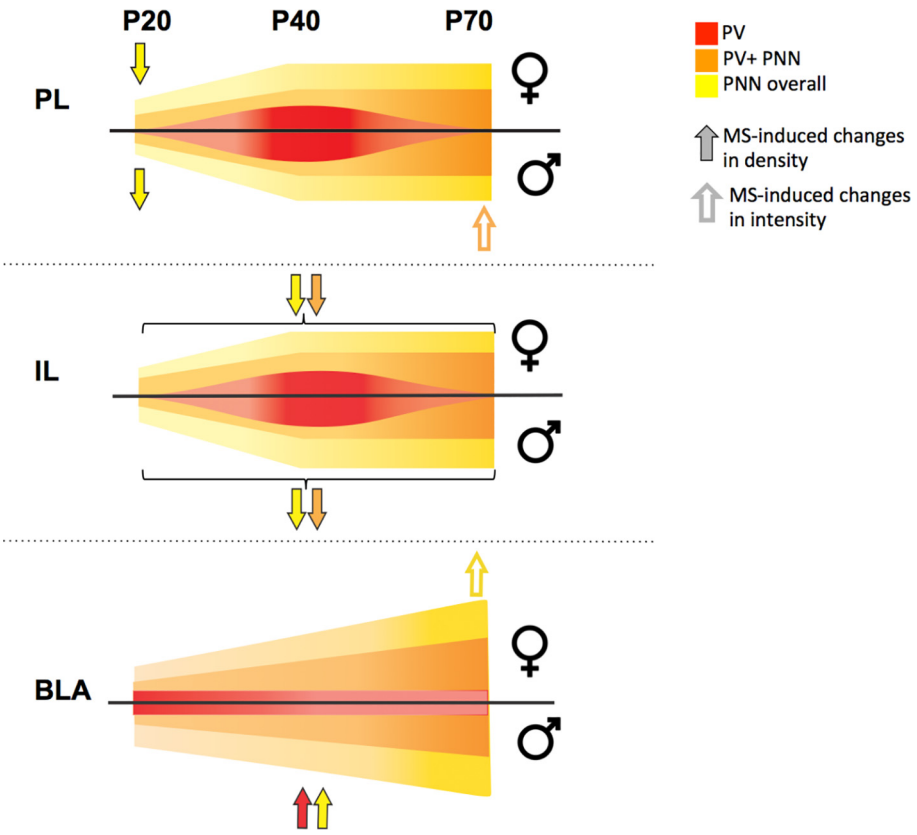

Fig. 10. Schematic representing sex-, age-, and rearing-specific effects on overall perineuronal net (PNN), parvalbumin (PV) neuron, and PV + PNN density and intensity in the prelimbic (PL) and infralimbic (IL) prefrontal cortex (PFC), and basolateral amygdala (BLA). For each brain region, the x-axis represents age with females above and males below the line. Colored arrows with a black border represent MS-induced changes in density and unfilled arrows with a colored border represent changes in intensity following MS. Width of color band represents change in density throughout development, while the gradient of color bands denotes changes in intensity. Color of arrows and bands signify PV cells (red), PV + PNNs (orange), or overall PNNs (yellow).

alterations in PFC PV interneuron density following MS. By adolescence, however, MS increased the density of PV cells in the BLA of males. Here, we see varied effects of both sex and rearing condition at different developmental time points, suggesting sex-dependent alterations in interneuron microcircuitry. These sex differences in PV maturation following MS in the BLA may underlie the divergent impact of early life adversity on the onset of symptoms of neuropsychiatric disorders in males and females. It has been hypothesized that adversity-induced reductions in PV interneuron expression results not from death of PV interneurons, but from deviations in their maturation (Powell et al., 2012). While we were surprised by the lack of effect of MS on PV density in the PFC, this finding may speak to individual differences in PV maturation and response to stress, as well as the complex experience-dependent neuronal programming in the PFC (Kolb et al., 2012). Our findings may also indicate between-study disparities in threshold PV detection and identification. For example, the current study's threshold for PV detection was very low, identifying cells that had lower intensities that may differ from other studies' operationalizations. This difference may underlie the contrasting results in the current study, as well as discrepancies throughout the literature. Also, it is possible that while PV neurons were not different between groups in the current study, MS may still differentially affect PV neuron function and subsequent behavioral development. Thus, more research is needed to understand how MS affects PV development.

The findings from this study should be interpreted in light of certain limitations and considerations. First, the current study used timed-pregnant dams that were shipped to our facility on gestational day 15, potentially exposing the offspring to prenatal stress that may have interacted with our MS paradigm. While not a limitation per se, it is also important to note that we manually counted PNNs, PV interneurons, and PNN-enwrapped PV interneurons instead of utilizing unbiased stereological methods. This decision was made because both PNNs and PV interneurons are not uniformly distributed throughout cortical layers (Mauney et al., 2013; Melchitzky et al., 1999); stereological estimates rely on uniform distribution of cells for accurate results (Gardi et al., 2008; Gundersen et al., 1988). Therefore, we chose to pseudo-randomly acquire z-stacks across all layers that extended through all focal planes of the stained tissue, allowing us to avoid potential estimation errors caused by stereological random sampling that may miss certain sections of the tissue, as well as retaining 3D visualization of the PNN/PV colocalization. Layer-specific aberrations in PNN organization have also been reported (Enwright et al., 2016; Ueno et al., 2017). Although we analyzed MS-induced alterations in PNN formation across all cortical layers, the current study does not report layer-specific effects of adversity. While we observed effects of adversity on the formation of PNNs in the PFC, the current study may have missed nuanced differences between layers in their response to MS. We also acknowledge that only WFA + PNNs were assessed in the current study. There are many subsets of PNNs with varying neuroanatomical distributions and likely also differing functional properties (Berretta et al., 2015). It will be important going forward to untangle the effects of early life adversity on different subsets of PNNs. Lastly, it is important to note that past findings reveal that female hormonal fluctuations play a role in regulating neuroplasticity (Chen et al., 2009; Rasia-Filho et al., 2004; Warren et al., 1995), as well as PV interneuron activity (Clemens et al.,

2019). Contrarily, however, it has also been found that estrous cycle phase and estrogen levels do not affect PV number (Torres-Reveron et al., 2009). Thus, while we cannot rule out the effect of hormone cycles on PNN or PV density in the adult PFC or BLA, hormonal cycle may not directly regulate PNN or PV density.

The findings presented here provide compelling evidence that early postnatal adversity disrupts PNN and PV maturation in a region-specific manner. Importantly, we demonstrate that alterations in neonatal care result in organizational changes that do not appear until adulthood. These data suggest that aberrant PNN maturation during critical and sensitive periods of development may be linked to altered composition and structural integrity later in life. The importance of PNN formation in healthy neurodevelopment is still being investigated; however, these findings help unravel the nuanced developmental effects of early life adversity in different regions of the brain, as well as the potential neurobiological underpinnings of sex differences in response to adversity.

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