

A two-hit adversity model in developing rats reveals sex-specific impacts on prefrontal cortex structure and behavior

Kelsea R. Gildawie, Lilly M. Ryll, Jessica C. Hexter, Shayna Peterzell, Alissa A. Valentine, Heather C. Brenhouse*

Department of Psychology, Northeastern University, Boston, MA, USA

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ABSTRACT

Adversity early in life substantially impacts prefrontal cortex (PFC) development and vulnerability to later-life psychopathology. Importantly, repeated adverse experiences throughout childhood increase the risk for PFC-mediated behavioral deficits more commonly in women. Evidence from animal models points to effects of adversity on later-life neural and behavioral dysfunction; however, few studies have investigated the neurobiological underpinnings of sex-specific, long-term consequences of multiple developmental stressors. We modeled early life adversity in rats via maternal separation (postnatal day (P)2-20) and juvenile social isolation (P21-35). In adulthood, anxiety-like behavior was assessed in the elevated zero maze and the presence and structural integrity of PFC perineuronal nets (PNNs) enwrapping parvalbumin (PV)-expressing interneurons was quantified. PNNs are extracellular matrix structures formed during critical periods in postnatal development that play a key role in the plasticity of PV cells. We observed a female-specific effect of adversity on hyperactivity and risk-assessment behavior. Moreover, females – but not males – exposed to multiple hits of adversity demonstrated a reduction in PFC PV cells in adulthood. We also observed a sex-specific, potentiated reduction in PV + PNN structural integrity. These findings suggest a sex-specific impact of repeated adversity on neurostructural development and implicate PNNs as a contributor to associated behavioral dysfunction.

1. Introduction

Adverse environmental influences during sensitive periods of development can disrupt prefrontal cortex (PFC) maturation (Chocyk et al., 2010; Stevenson et al., 2008) and increase vulnerability to later-life onset of neuropsychiatric disorders, including schizophrenia, post-traumatic stress disorder, and anxiety disorders (Agid et al., 1999; Heim et al., 2008, 2009). Importantly, evidence suggests that individuals that experience childhood adversity are more likely to be exposed to further adverse experiences throughout development (Manyema et al., 2018). Clinical studies demonstrate enhanced vulnerability to a second “hit” of adversity (McLaughlin et al., 2010), where repeated stressful life events throughout childhood result in more severe neuropsychiatric symptoms, particularly in women (Bale and Epperson, 2015; Manyema et al., 2018; Suliman et al., 2009). There is, therefore, a critical need to understand the sex-specific neuromolecular

underpinnings of developmental responses to adversity, and how this may relate to dysfunction later in life.

Several paradigms exist to model early life adversity in rodents (Molet et al., 2014; Schmidt et al., 2011), improving our understanding of neural mediators driving the onset of behavioral disruption (Sarkar et al., 2019). The maternal separation (MS) paradigm is a well-characterized analogue to childhood neglect in humans (Lehmann and Feldon, 2000). Repeated isolation from the dam throughout postnatal development results in increased anxiety-like behavior (Daniels et al., 2004; Ganguly et al., 2015), which has been seen to affect females more than males in adolescence (Honeycutt et al., 2020). Isolation paradigms have also been utilized after postnatal development to elicit behavioral disruption later in life (see review: Fone and Porkess, 2008). While social isolation (SI) has often been employed to induce adversity throughout life after weaning (Lapiz et al., 2003; Liu et al., 2019; Möller et al., 2013a; Schiavone et al., 2009), few studies report SI during

Abbreviations: PFC, prefrontal cortex; PL, prelimbic; IL, infralimbic; PNN, perineuronal net; WFA, *Wisteria floribunda* agglutinin; MS, maternal separation; Con, control; SI, social isolation; PH, pair-housed; P, postnatal day; PBS, phosphate buffered saline; PFA, paraformaldehyde; EZM, elevated zero maze.

* Corresponding author at: 325 Nightingale Hall, Northeastern University, 360 Huntington Ave, Boston, MA, 02115, USA.

E-mail address: h.brenhouse@northeastern.edu (H.C. Brenhouse).

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discrete developmental time points, such as juvenility (Bicks et al., 2020; Pietropaolo et al., 2008) or different periods of adolescence (Jaric et al., 2019; Li et al., 2018). Evidence regarding sex-dependent effects of SI is also sparse, with inconsistent effects on anxiety-like behavior in males and females (Pietropaolo et al., 2008; Rodgers and Cole, 1993). Notably, early life adversity paradigms are often combined to assess the additive effects of multiple adverse experiences throughout development (Castillo-Gómez et al., 2017; Deslauriers et al., 2013; Jaric et al., 2019; Monte et al., 2017; Rincel et al., 2019). Some evidence suggests that there are sex-specific effects of multiple hits of developmental stressors on later-life anxiety-like behavior, working memory, and social behavior (Hudson et al., 2014; Monte et al., 2017; Rincel et al., 2019); however, a dearth of information remains regarding the potential neuroplastic mediators underlying sex-specific effects of multiple behavioral stressors on anxiety-like behavior.

The neonatal (Greenhill et al., 2015) and juvenile (Bicks et al., 2020) periods are implicated as stages of increased plasticity and susceptibility to disrupted environment. These windows of susceptibility have been linked to the maturation of cortical excitatory/inhibitory balance (Hensch, 2004, 2005). GABAergic machinery maturation is dynamic throughout early life (Minelli et al., 2003); however, the structural development of GABAergic circuitry occurs chiefly during early life, concluding towards the end of juvenility (Caballero and Tseng, 2016). Notably, social experience during juvenility has been found to be necessary for the development and function of fast-spiking, GABAergic parvalbumin (PV)-positive interneurons (Bicks et al., 2020). It is therefore likely that altered experience during early postnatal life may disrupt typical neural development, resulting in aberrant adult behavior. Evidence suggests that chronic adversity early in life affects the development of PFC PV interneurons (Schiavone et al., 2009). Both neonatal MS (Grassi-Oliveira et al., 2016; Holland et al., 2014) and juvenile SI (Bicks et al., 2020; Lukkes et al., 2012) have been found to decrease PFC PV levels. PV cells are preferentially enwrapped by perineuronal nets (PNNs; Enwright et al., 2016), specialized extracellular matrix structures that provide functional and physical protection, and regulation of cells that they enwrap (Cabungcal et al., 2013; Härtig et al., 1999). PFC PNN formation is protracted throughout development (Mauney et al., 2013), experiencing a spike in number during juvenility (Ueno et al., 2017a, 2017b) and maturing structurally until early adulthood (Gildawie et al., 2020a). Compounding evidence indicates that delayed PNN maturation underlies critical period formation and susceptibility to adversity early in life (see review: Reichelt et al., 2019). Early life adversity impacts the development of PNNs in the PFC; animals exposed to post-weaning SI displayed reduced PNN intensity in the PFC (Ueno et al., 2017a, 2017b). It is also important to highlight the burgeoning exploration of sex-specific effects of adversity on PV + PNNs. Neonatal MS was found to affect later-life PFC PV + PNN structural integrity in a sex-dependent manner (Gildawie et al., 2020a) and adolescent unpredictable chronic mild stress had sex- and age-specific effects on PNN/PV colocalization that correlated with increased anxiety-like behavior in females, but not males (Page and Coutellier, 2018). Together, these results implicate PNN/PV maturation in proper behavioral development, while the moderating effect of sex has yet to be fully understood.

Work in humans demonstrates that women exposed to multiple stressors during development have higher rates of depression and posttraumatic stress disorder in adolescence, compared to men (Suliman et al., 2009). Additionally, findings from human postmortem studies suggest that adults with affective disorders have fewer PFC PNNs, compared to controls (Alcaide et al., 2019; Mauney et al., 2013), suggesting that PNN aberrations may underlie later-life susceptibility to neuropathology. Importantly, research suggests that multiple hits of adversity throughout the lifetime have additive effects on behavioral and GABAergic function later in life (Avital and Richter-Levin, 2005; Deslauriers et al., 2013; Monte et al., 2017). To our knowledge, no studies have investigated whether multiple hits of adversity have a

synergistic effect on altered PFC PNN number and structural integrity. Additionally, it is unknown whether such changes occur specifically in female rats, mimicking increased vulnerability of young girls to multiple traumatic events and the subsequent onset of neuropsychiatric disorders. Here, we aimed to determine whether neonatal MS and juvenile SI results in female-specific compounding effects on adult PFC PNNs enwrapping PV-expressing interneurons that underlie susceptibility to anxiety-like behavior.

2. Materials and methods

2.1. Subjects

All subjects were bred in an in-house colony with Sprague-Dawley rats originally obtained from Charles River Laboratories (Wilmington, MA). On postnatal day (P)1, litters were culled to 10 pups (5 males and 5 females). Only one rat per litter was assigned to each experimental group to avoid potential litter effects. Animals were housed in standard polycarbonate wire-top cages with pine shaving bedding in a facility controlled for temperature (22–23 °C) and humidity with a 12-h light/dark cycle (light period 0700–1900, approximately 332 lx). Food (Pro-Lab 5P00) and water (glass bottles) were available *ad libitum* to dams and weaned subjects. Experiments were carried out 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.

2.2. Developmental adversity

2.2.1. Maternal separation

Beginning at P2, male and female pups were isolated from the dam and littermates for four hours per day (0900–1300) until P20, as previously described (Gildawie et al., 2020a; Grassi-Oliveira et al., 2016; Holland et al., 2014; Honeycutt et al., 2020). Between P2 and P10, pups were separated in individual containers with bedding and droppings from the home cage – to assuage potential stress responses from altered olfactory cues – in a warm water bath (37 °C). When pups were able to thermoregulate, separations were conducted in individual small cages containing home cage bedding (P11 to P20). All separations took place in a different room from where dams were located. Pups in control (Con) litters were handled for five minutes twice per week to alleviate handling disparities between groups due to separations. All litters underwent routine weekly husbandry and weighing on P9, P15, and P20 (approximately five min of handling).

2.2.2. Juvenile social isolation

At weaning (P21), pups were separated from their dams and randomly assigned to be housed individually (SI) or pair-housed (PH) with a same-sex, condition-matched conspecific. Rats undergoing SI were housed in the same room as PH rats to have visual, auditory, and olfactory contact without any form of social interaction with littermates or conspecifics post-weaning (Möller et al., 2013b; Schiavone et al., 2009). At P35, all SI rats were housed with a condition- and sex-matched conspecific until experimentation.

2.3. Behavioral testing

Rats were transported into a dimly lit testing room and left undisturbed to acclimate for 10–20 min. Anxiety-like behavior was tested in the elevated zero maze (EZM), a circular plexiglass alley elevated 70 cm off the ground. The alleyway was divided into four equal quadrants. Two of the quadrants (situated across the maze from one another) were walled with black plexiglass. The two remaining quadrants were unwallled and open to the surrounding testing environment. Each rat was placed in the closed area of the maze and left to explore the maze for five minutes. Maze exploration was recorded by an Ausdom HD 1080p

Webcam and behavior was scored separately by an experimenter blind to experimental condition. Scored behavior included measures of locomotion-related behavior (time spent in open area (s), frequency to open, number of crossings) and risk assessment behavior (number of head pokes, and head poke duration (s)), as previously described as discrete types of anxiety-like behavior (Roy and Chapillon, 2004; Sidor et al., 2010). The maze was cleaned with 50 % ethanol solution between each animal.

2.4. Immunohistochemistry

Fifteen days after behavioral testing (P85), animals were deeply anesthetized with CO₂ and intracardially perfused with ice-cold 0.9 % physiological saline followed by 4% paraformaldehyde (PFA) solution (see Fig. 1A for experimental timeline). Perfusions were performed

between 0900 and 1200 to mitigate variability in PV and PNN expression throughout the day. After tissue collection, brains were post-fixed in PFA for three days and cryoprotected in 30 % sucrose solution. Brains were sliced on a freezing microtome (Leica) to 40 μ m sections and stored at -20°C in freezing solution until fluorescent staining.

Free-floating sections containing the PFC (between bregma 4.2 and 2.52 mm) were first washed in 1X phosphate buffered saline (PBS). The tissue was blocked in 5% normal donkey serum and 1% bovine serum albumin, then incubated in primary conjugate lectin from *Wisteria floribunda* agglutinin (WFA; 1:250, L1516, MilliporeSigma) and primary rabbit anti-PV antibody (1:1000, NB-120-11427, Novus Biologicals) at 4 $^{\circ}\text{C}$ for 48 h in PBS containing 0.3 % TritonTM X-100 (PBST; Fisher Scientific). Sections were washed in PBST, followed by a three-hour incubation in secondary antibody solution composed of streptavidin conjugate 488 (1:3000, S32354, ThermoFisher) and donkey anti-rabbit

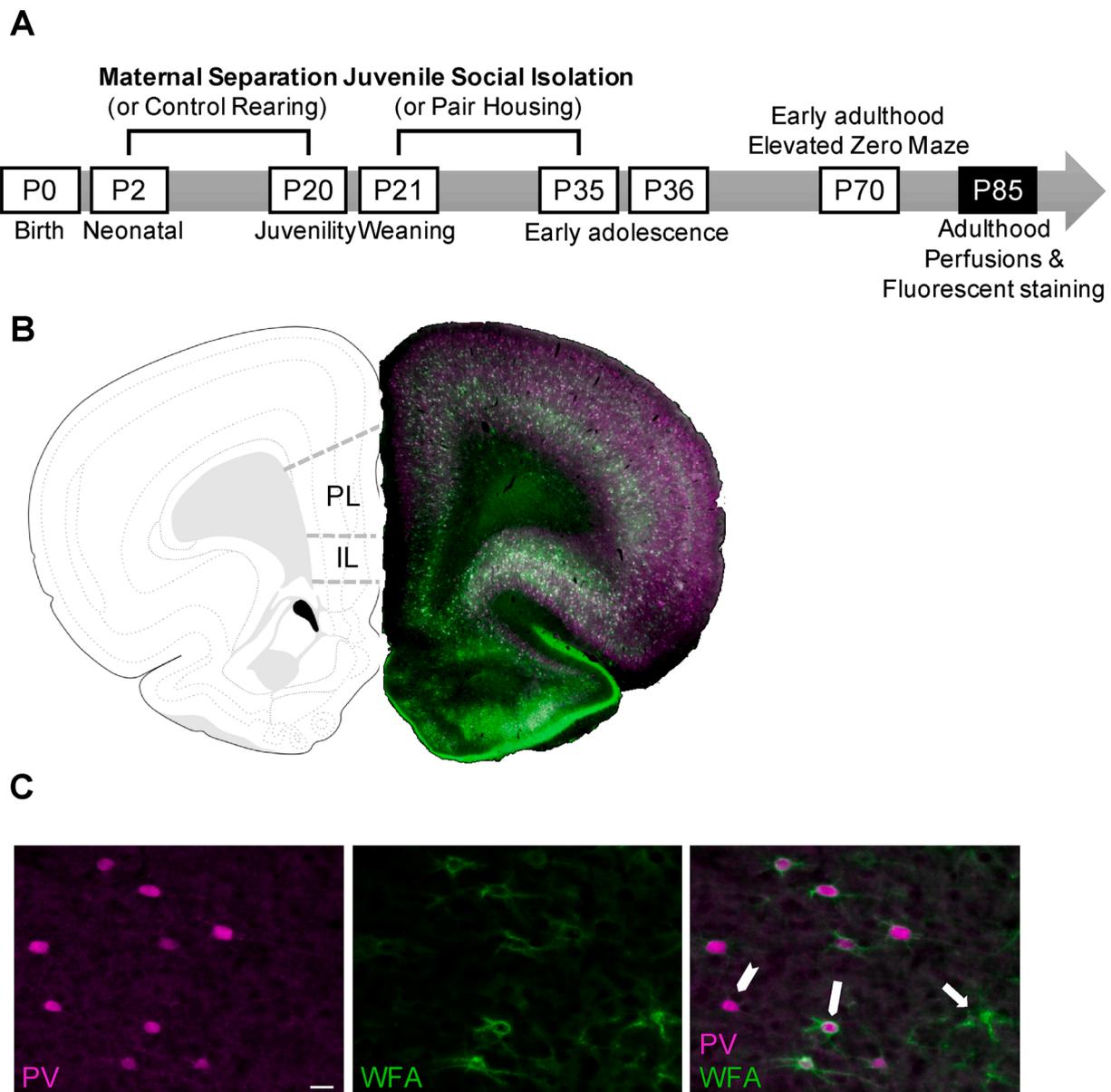


Fig. 1. Experimental timeline and neuroanatomical representations. (A) Pups were exposed to maternal separation (or control rearing) postnatal day (P)2 to 20, followed by social isolation (or standard pair housing) P21-35. Rats were then pair housed until testing in the elevated zero maze on P70 and perfusions on P85. (B) Representative diagram (left) and stitched full-brain image (right) of the prelimbic (PL) and infralimbic (IL) prefrontal cortex. (C) Representative photomicrographs of parvalbumin (PV)-expressing neurons (magenta), *Wisteria floribunda* agglutinin (WFA)+ perineuronal nets (PNNs; green), and a merged image with WFA + PNNs (green) and PV (magenta). White arrows point to PNNs surrounding PV cells, white chevrons point to PV cells lacking PNNs, and white pentagons point to PNNs surrounding PV cells. Scale bar = 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Alexa Fluor® 350 (1:500, A10039, Invitrogen) in 0.3 % PBST. Sections were washed in PBS and incubated in NeuroTrace™ 530/615 (1:200, N21482, Invitrogen) in PBS for one hour to visualize fluorescent Nissl staining. Sections underwent a final round of washes in PBS, then were mounted on positively-charged glass slides and coverslipped with ProLong Gold antifade mounting reagent (P36930, Invitrogen). Every round of staining included subjects from each experimental group to obviate batch-specific differences.

2.5. 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 20x magnification (image size: 724.69µm × 543.52µm) in three serial sections of the prelimbic (PL) and infralimbic (IL) cortices (see Fig. 1B for neuroanatomical diagram). The “Quick Full Focus” feature was used to yield one TIFF containing all images within the stack, encompassing all focal planes captured. Regions of interest were delineated using clearly visible landmarks and pre-defined boundaries according to a rat brain atlas (Paxinos and Watson, 2007) clearly visible via NeuroTrace™. Two sets of z-stacks were captured for the PL and one per IL, bilaterally (18 stacks per animal).

After image acquisition, each set of TIFFs was quantified as previously reported (Gildawie et al., 2020a) using the “Perineuronal net Intensity Program for the Standardization and Quantification of ECM Analysis” (PIPSQUEAK; Slaker et al., 2016), a FIJI (Schindelin et al., 2012) macro plugin developed specifically to quantify PNNs and PV cells. The number and intensity of WFA + PNNs, PV + interneurons, and PNNs colocalized with PV were semi-automatically counted through the entirety of the stained tissue section (average 21 µm per section) by an experimenter blind to experimental condition. Images were first analyzed in PIPSQUEAK using predetermined parameters for the detection of WFA and PV staining. To better detect faint staining, the low background subtraction setting was used. An experimenter blind to experimental condition corrected for misidentification of PNNs and PV neurons to achieve the most accurate identification for each image. Double labeling of PNNs and PV neurons was then semi-automatically identified by PIPSQUEAK, with at least 80 % overlap needed to be considered colocalized. A final approval step allowed the experimenter to correct any misidentified colocalization. Measures acquired were PV cell count and intensity, PNN count and intensity, colocalized PNN/PV count, intensity of PNNs surrounding PV cells, and intensity of PV cells surrounded by PNNs (see Fig. 1C for examples of PV and WFA staining).

2.6. Statistical analyses

Statistical analyses were conducted using GraphPad Prism 7 software or IBM SPSS Statistics V.25. Prior to analysis, data were tested for outliers using Grubbs’ Test, homogeneity of variances was assessed with Levene’s Test of Equality of Error Variances, and Normality of residuals was evaluated via the Shapiro-Wilk Test of Normality (with assessment of skewness and kurtosis). Three-way ANOVAs were then performed to assess experimental effects of Sex, Rearing, and Housing in the EZM and immunohistochemical analysis. Main effects of Rearing or Housing, as well as interactions between Rearing and Housing or Sex and Rearing or Housing, were followed up with two-way ANOVAs (Rearing x Housing) separated by Sex to further assess the impact of multiple hits of developmental adversity on males and females. Effect size (η_p^2) was calculated in SPSS and categorized as small ($\eta_p^2 = 0.01$), medium ($\eta_p^2 = 0.06$), or large ($\eta_p^2 = 0.14$; Richardson, 2011). Significant main effects and interactions were further assessed with Tukey’s HSD post-hoc tests to compare between groups, while correcting for multiple comparisons.

3. Results

3.1. Neonatal maternal separation and juvenile social isolation have opposing effects on adult risk assessment and hyperactivity in females

Anxiety-like behavior was measured in both males and females using the EZM following neonatal MS and juvenile SI. A three-way interaction between Rearing, Housing, and Sex was not apparent when the duration spent in the open area of the maze was assessed ($F_{1,82} = 0.119$, $p = 0.731$, $\eta_p^2 = 0.001$; Fig. 2A). There were also no significant main effects of Rearing ($F_{1,82} = 0.380$, $p = 0.539$, $\eta_p^2 = 0.005$), Housing ($F_{1,82} = 3.101$, $p = 0.082$, $\eta_p^2 = 0.036$), or Sex ($F_{1,82} = 0.111$, $p = 0.740$, $\eta_p^2 = 0.001$). Follow-up analyses were, therefore, not performed. As shown in Fig. 2B, however, the frequency of entrances to the open area was significantly impacted by juvenile SI (main effect of Housing: $F_{1,82} =$

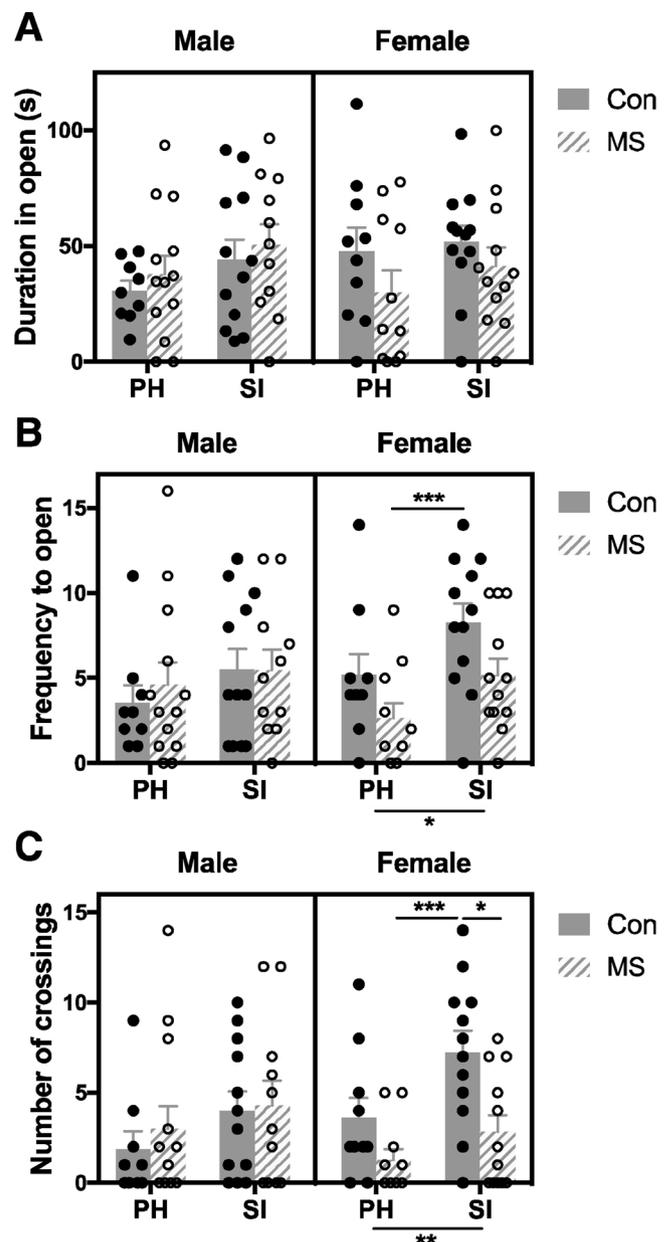


Fig. 2. Effects of maternal separation (MS) and social isolation (SI) on adult anxiety-like behavior and locomotion in the elevated zero maze. (A) No effects of MS or SI on duration in the open area. (B) SI increased the frequency to the open area and (C) number of crossings in females that was opposed by MS. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; $n = 9-13$ /group.

6.505, $p = 0.013$, $\eta_p^2 = 0.073$) and was mildly affected by Rearing in a sex-dependent manner (Rearing x Sex interaction $F_{1,82} = 4.126$, $p = 0.045$, $\eta_p^2 = 0.048$). Follow-up two-way ANOVAs separated by Sex revealed that effects of Rearing (females: $F_{1,41} = 7.085$, $p = 0.011$, $\eta_p^2 = 0.147$; males: $F_{1,41} = 0.164$, $p = 0.687$, $\eta_p^2 = 0.004$) and Housing (females: $F_{1,41} = 6.919$, $p = 0.012$, $\eta_p^2 = 0.144$; males: $F_{1,41} = 1.237$, $p = 0.273$, $\eta_p^2 = 0.029$) were driven by females. Specifically, females exposed to MS entered the open area of the maze less than Con animals. Contrarily, SI females entered the open area more than PH counterparts. Tukey post-hoc comparisons show that females exposed to SI alone entered the open area significantly more than females exposed to MS alone ($p = 0.0028$). We also measured the number of times animals crossed from one enclosed area to the other, as a measure of locomotion (Fig. 2C). Similar to the frequency of entrances to the open area of the maze, there was a main effect of Housing ($F_{1,82} = 8.576$, $p = 0.004$, $\eta_p^2 = 0.095$) and an interaction between Rearing and Sex ($F_{1,82} = 8.576$, $p = 0.007$, $\eta_p^2 = 0.086$) on the number crossings from one closed area to the other. Again, when two-way ANOVAs were performed, females seemed to drive the decrease in crossings following MS (main effect of Rearing; females: $F_{1,41} = 11.679$, $p = 0.001$, $\eta_p^2 = 0.222$; males: $F_{1,41} = 0.829$, $p = 0.368$, $\eta_p^2 = 0.020$) and SI (main effect of Housing; females: $F_{1,41} = 6.972$, $p = 0.012$, $\eta_p^2 = 0.145$; males: $F_{1,41} = 2.802$, $p = 0.102$, $\eta_p^2 = 0.064$) exposure. Post-hoc analyses comparing each group revealed that the number of crossings was significantly higher in females exposed to only SI compared to those with only MS ($p = 0.0005$) or those exposed to both manipulations (Con, SI vs. MS, SI: $p = 0.0110$). These data demonstrate hyperactivity following juvenile SI. In contrast, MS showed an opposing effect, decreasing locomotion in adult females, but not males.

The number and duration of head pokes into the open area (without subsequent exit out of the closed area) was evaluated to gauge risk assessment, which has been previously shown to be a more sensitive measure of anxiety-like behavior (Roy and Chapillon, 2004; Sidor et al., 2010). As shown in Fig. 3A, we observed that the number of head pokes depended on Rearing, Housing, and Sex ($F_{1,82} = 6.948$, $p = 0.010$, $\eta_p^2 = 0.078$). Further analyses separated by Sex revealed that female (Rearing x Housing interaction $F_{1,41} = 4.807$, $p = 0.034$, $\eta_p^2 = 0.105$), but not male ($F_{1,41} = 2.216$, $p = 0.144$, $\eta_p^2 = 0.051$) head poke frequency depended on both Rearing and Housing. Follow-up post-hoc analysis revealed no group differences. When the duration of head pokes was assessed, a small – but significant – three-way interaction was observed ($F_{1,82} = 4.390$, $p = 0.039$, $\eta_p^2 = 0.051$). There was also an overall decrease following SI (main effect of Housing $F_{1,82} = 4.590$, $p = 0.035$, $\eta_p^2 = 0.053$) that was driven by a non-significant, but moderate decrease head poke duration in females (main effect of Housing; females: $F_{1,41} = 4.026$, $p = 0.051$, $\eta_p^2 = 0.089$; males: $F_{1,41} = 2.416$, $p = 0.128$, $\eta_p^2 = 0.056$; Fig. 3B). Follow-up two-way ANOVAs also showed a main effect of Rearing in females ($F_{1,41} = 5.667$, $p = 0.022$, $\eta_p^2 = 0.121$), but not males ($F_{1,41} = 0.072$, $p = 0.789$, $\eta_p^2 = 0.002$). Tukey post-hoc comparisons revealed that SI decreased the time spent head poking (MS, PH vs. Con,

SI: $p = 0.0158$) and both hits of early life adversity prevented this decrease (Con, SI vs. MS, SI: $p = 0.0400$) in females. These findings suggest that – in females, but not males – juvenile SI results in decreased risk assessment behavior in females, while MS has the opposite effect.

3.2. Early life adversity has compounding effects on female prefrontal cortex parvalbumin-expressing interneurons and perineuronal net structural integrity

3.2.1. Prelimbic prefrontal cortex

3.2.1.1. Parvalbumin-expressing neuron density and intensity. The number and intensity of PV-expressing interneurons and the PNNs that enwrap them were assessed to determine whether there were sex-dependent effects of multiple adverse experiences throughout early development on the adult PFC. Three-way ANOVA revealed an interaction between Rearing, Housing, and Sex ($F_{1,75} = 4.180$, $p = 0.044$, $\eta_p^2 = 0.053$), as well as an overall decrease in PV count in the PL following MS (main effect of Rearing $F_{1,75} = 7.409$, $p = 0.008$, $\eta_p^2 = 0.090$; Fig. 4A). When males and females were analyzed separately, the effect of Rearing was found to be primarily driven by females ($F_{1,36} = 4.191$, $p = 0.048$, $\eta_p^2 = 0.104$), although males also demonstrated a non-significant, moderate decrease in PV neuron count ($F_{1,39} = 3.165$, $p = 0.083$, $\eta_p^2 = 0.075$). Tukey post-hoc comparisons reveal that females exposed to both MS and SI had fewer PV cells in the PL compared to females exposed to SI alone ($p = 0.042$), which was not apparent in males ($p = 0.9542$). When PV intensity was assessed, however, no effects were observed (Rearing x Housing x Sex interaction $F_{1,75} = 1.513$, $p = 0.223$, $\eta_p^2 = 0.020$; Fig. 4B).

3.2.1.2. Perineuronal net density and intensity. We also quantified the number and intensity of PNNs following MS and SI. While Rearing, Housing, and Sex did not change the number of PL PNNs ($F_{1,75} = 0.560$, $p = 0.457$, $\eta_p^2 = 0.007$; Fig. 4C), PNN intensity was significantly altered (Rearing x Housing x Sex interaction $F_{1,75} = 6.722$, $p = 0.011$, $\eta_p^2 = 0.082$; Fig. 4D). Both MS (main effect of Rearing $F_{1,75} = 5.940$, $p = 0.017$, $\eta_p^2 = 0.073$) and SI (main effect of Housing $F_{1,75} = 6.434$, $p = 0.013$, $\eta_p^2 = 0.079$) decreased overall PNN intensity. Separate two-way ANOVAs in males and females revealed an interaction between Rearing and Housing ($F_{1,36} = 5.214$, $p = 0.028$, $\eta_p^2 = 0.127$) and a main effect of Housing ($F_{1,36} = 5.312$, $p = 0.027$, $\eta_p^2 = 0.129$) in females. Meanwhile, males showed no Rearing x Housing interaction ($F_{1,39} = 1.593$, $p = 0.214$, $\eta_p^2 = 0.039$) and a modest, nonsignificant decrease in PNN intensity following MS (main effect of Rearing $F_{1,39} = 0.3456$, $p = 0.071$, $\eta_p^2 = 0.081$), suggesting that the overall Rearing x Housing interaction was driven by females. Moreover, Tukey post-hoc comparisons show that PNN intensity in females exposed to both MS and SI was significantly lower than Con, SI ($p = 0.0365$); MS, PH ($p = 0.0159$); and Con, PH ($p = 0.0410$) females. These findings demonstrate an additive effect of MS and SI on PL PNN intensity, exclusively in females.

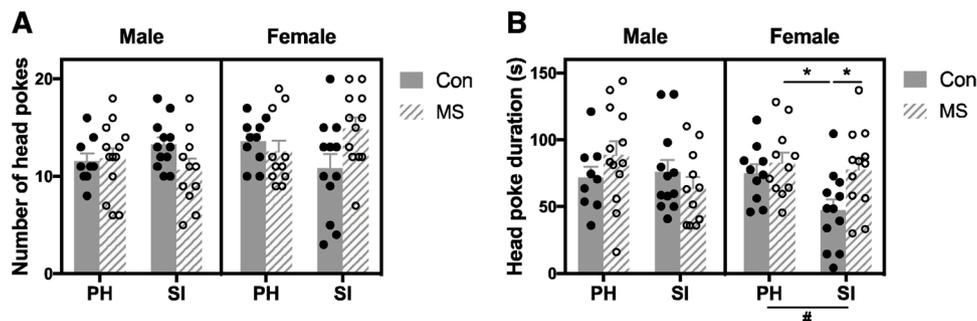


Fig. 3. Effects of maternal separation (MS) and social isolation (SI) on adult risk assessment in the elevated zero maze. (A) The number of head pokes into – without entering – the open area depended on both MS and SI, which was driven by females. No group differences were observed. (B) Head poke duration was significantly decreased by SI in females with an opposing effect of MS. #: $p < 0.1$; *: $p < 0.05$; $n = 9-13$ /group.

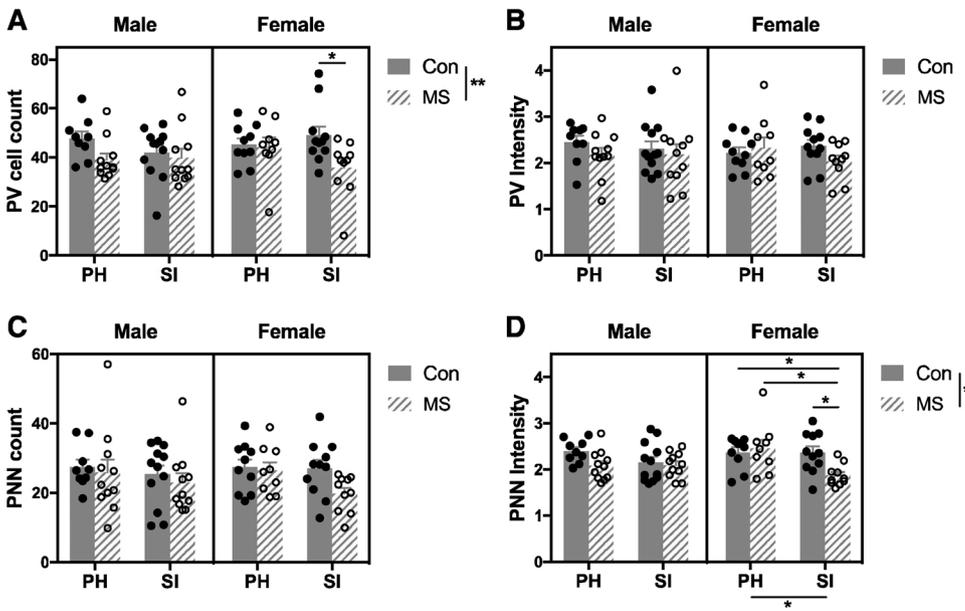


Fig. 4. Effects of maternal separation (MS) paired with social isolation (SI) on adult parvalbumin (PV)-expressing interneurons and perineuronal nets (PNNs) in the prelimbic (PL) prefrontal cortex. (A) A three-way interaction between Rearing, Housing, and Sex was revealed, where females exposed to both MS and SI had fewer PV cells in the PL than females exposed to SI alone. (B) Intensity of PV immunofluorescence and (C) PNN number were not altered. (D) An additive effect of MS and SI was apparent in female PNN intensity, where females exposed to MS and SI had PNNs that fluoresced less than all other female groups. *: $p < 0.05$; **: $p < 0.01$; $n = 9-13/\text{group}$.

3.2.1.3. Colocalized parvalbumin and perineuronal net density and intensity. Analysis of the number of PL PNNs specifically enwrapping PV neurons revealed an overall decrease following MS (main effect of Rearing $F_{1,75} = 7.632$ $p = 0.007$, $\eta_p^2 = 0.092$), as well as a three-way

interaction ($F_{1,75} = 5.085$, $p = 0.027$, $\eta_p^2 = 0.063$; Fig. 5A). Two-way ANOVAs split by Sex revealed a significant interaction between Rearing and Housing in females ($F_{1,36} = 4.503$, $p = 0.041$, $\eta_p^2 = 0.111$), but not males ($F_{1,39} = 1.116$, $p = 0.297$, $\eta_p^2 = 0.028$). Both males and females

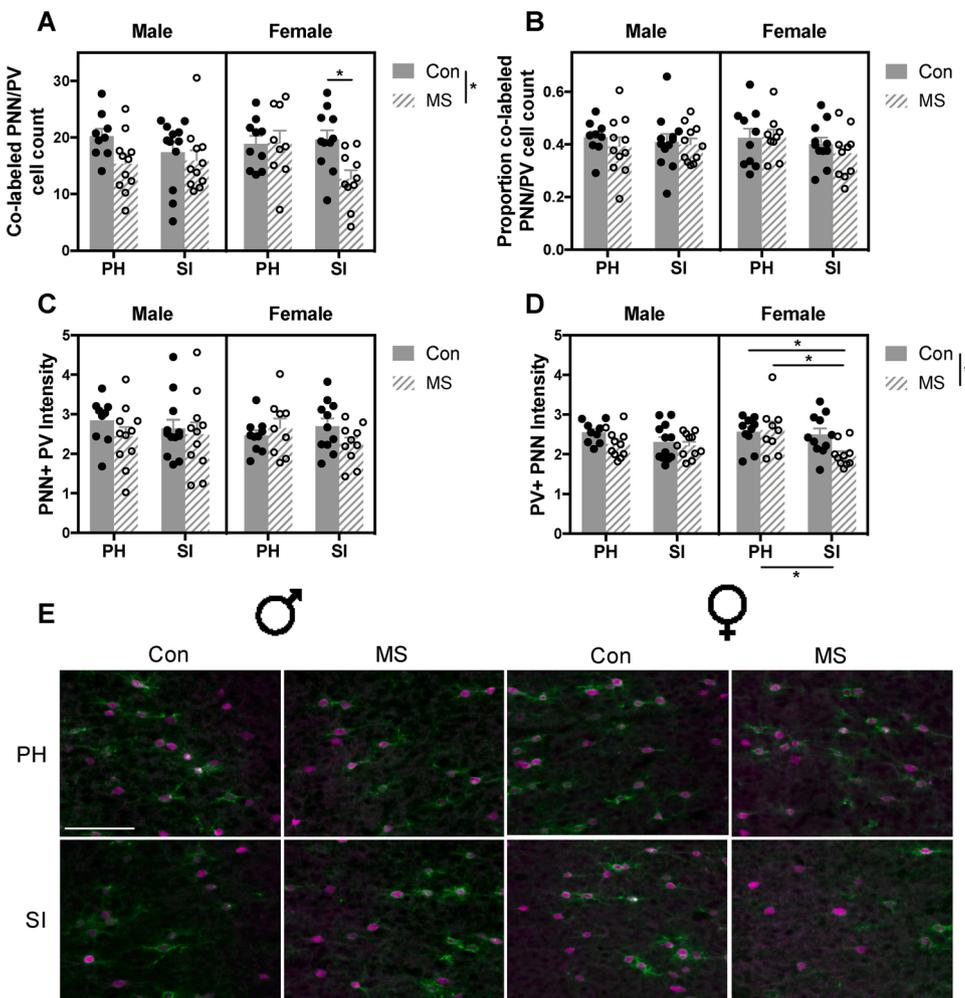


Fig. 5. Effects of maternal separation (MS) paired with social isolation (SI) on co-labeled parvalbumin (PV)-expressing neurons and perineuronal nets (PNNs) in the prelimbic (PL) prefrontal cortex. (A) The number of PNN + PV cells was decreased in both males and females in the PL, but only females showed a significant reduction in PNN + PV number following both MS and SI exposure. (B) Neither the proportion nor (C) intensity of PV cells enwrapped by PNNs was affected by Rearing or Housing condition. (D) An additive effect of MS and SI on the intensity of PNNs surrounding PV cells in the PL was observed only females. (E) Representative photomicrographs of adult *Wisteria floribunda* agglutinin (WFA; green)+ PNNs and PV cells (magenta) in the PL. Scale bar = 50 μm ; *: $p < 0.05$; $n = 9-13/\text{group}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

showed a moderate, but nonsignificant MS-induced decrease in PNN + PV cell count (main effect of Rearing; females: $F_{1,36} = 4.012$, $p = 0.053$, $\eta_p^2 = 0.10$; males: $F_{1,39} = 3.627$, $p = 0.064$, $\eta_p^2 = 0.085$). Planned assessment of group differences showed that females exposed to both MS and SI had significantly fewer PV neurons surrounded by PNNs, compared to females exposed to SI alone ($p = 0.0245$). This difference was not apparent in males ($p = 0.9240$). The proportion of PV cells surrounded by PNNs, however, did not change depending on differences in Rearing, Housing, or Sex ($F_{1,75} = 0.621$, $p = 0.443$, $\eta_p^2 = 0.008$; Fig. 5B). Analysis of intensity measures for colocalized PV cells and PNNs revealed that the intensity of PV neurons surrounded by PNNs was also unaltered ($F_{1,75} = 2.118$, $p = 0.150$, $\eta_p^2 = 0.027$; Fig. 5C). No main effects (Rearing $F_{1,75} = 1.597$, $p = 0.220$, $\eta_p^2 = 0.021$; Housing $F_{1,75} = 0.268$, $p = 0.606$, $\eta_p^2 = 0.004$) were apparent. When the intensity of PNNs enwrapping PV neurons was assessed, a small three-way interaction was observed ($F_{1,75} = 4.567$, $p = 0.036$, $\eta_p^2 = 0.057$). Additionally, MS (main effect of Housing $F_{1,75} = 5.001$, $p = 0.028$, $\eta_p^2 = 0.063$) and SI (main effect of Housing $F_{1,75} = 7.331$, $p = 0.008$, $\eta_p^2 = 0.089$) significantly decreased PV + PNN intensity (Fig. 5D). Follow-up analysis in males and females separately showed that the effect of Housing (females: $F_{1,36} = 5.639$, $p = 0.023$, $\eta_p^2 = 0.135$; males: $F_{1,39} = 1.70$, $p = 0.20$, $\eta_p^2 = 0.042$) was driven by females. Additionally, an interaction between Rearing and Housing was non-significant, but moderate in females ($F_{1,36} = 3.784$, $p = 0.060$, $\eta_p^2 = 0.095$), with no effect in males ($F_{1,39} = 0.873$, $p = 0.356$, $\eta_p^2 = 0.022$). Multiple comparisons showed that PNNs surrounding PV cells in animals exposed to both MS and SI fluoresced significantly less than those that underwent MS alone (females: $p = 0.0252$; males: $p = 0.9933$) and Con Rearing and PH (females: $p = 0.0410$; males: $p = 0.2040$) in females, but not males.

3.2.2. Infralimbic prefrontal cortex

3.2.2.1. Parvalbumin-expressing neuron density and intensity. To investigate potential region-specific effects of multiple adverse experiences, we also assessed density and intensity measures in the IL. Three-way ANOVA revealed that PV density was decreased by MS (main effect of Rearing $F_{1,75} = 9.475$, $p = 0.003$, $\eta_p^2 = 0.112$), but no interaction between Rearing, Housing, and Sex was observed ($F_{1,75} = 0.386$, $p = 0.536$, $\eta_p^2 = 0.005$; Fig. 6A). Follow-up two-way ANOVAs revealed a decrease in PV number following MS in both females (main effect of Rearing $F_{1,36} = 5.799$, $p = 0.021$, $\eta_p^2 = 0.139$) and males ($F_{1,39} = 3.816$, $p = 0.058$, $\eta_p^2 = 0.089$), although the effect in males was non-significant, but moderate. Multiple comparisons revealed no group differences. When we assessed PV intensity in the IL, no three-way interaction was observed ($F_{1,75} = 0.584$, $p = 0.447$, $\eta_p^2 = 0.008$; Fig. 6B).

3.2.2.2. Perineuronal net density and intensity. Further analysis of PNNs in the IL showed no alterations in PNN number due to Rearing, Housing, and Sex ($F_{1,75} = 0.203$, $p = 0.653$, $\eta_p^2 = 0.003$; Fig. 6C); however, a three-way interaction was observed when PNN intensity was assessed ($F_{1,75} = 10.237$, $p = 0.002$, $\eta_p^2 = 0.120$; Fig. 6D). Follow-up two-way ANOVAs separated by Sex showed a strong interaction between Rearing and Housing (females: $F_{1,36} = 8.364$, $p = 0.006$, $\eta_p^2 = 0.189$; males: $F_{1,39} = 2.142$, $p = 0.151$, $\eta_p^2 = 0.052$), as well as decrease in PNN intensity following SI (main effect of Housing; females: $F_{1,36} = 5.467$, $p = 0.025$, $\eta_p^2 = 0.132$; males: $F_{1,39} = 0.345$, $p = 0.560$, $\eta_p^2 = 0.009$) only in females. Tukey post-hoc comparisons revealed that females exposed to both MS and SI showed lower fluorescent intensity than females exposed to either MS ($p = 0.0049$) or SI ($p = 0.0267$) alone. These group differences were not apparent in males ($p = 0.9217$; $p = 0.9979$).

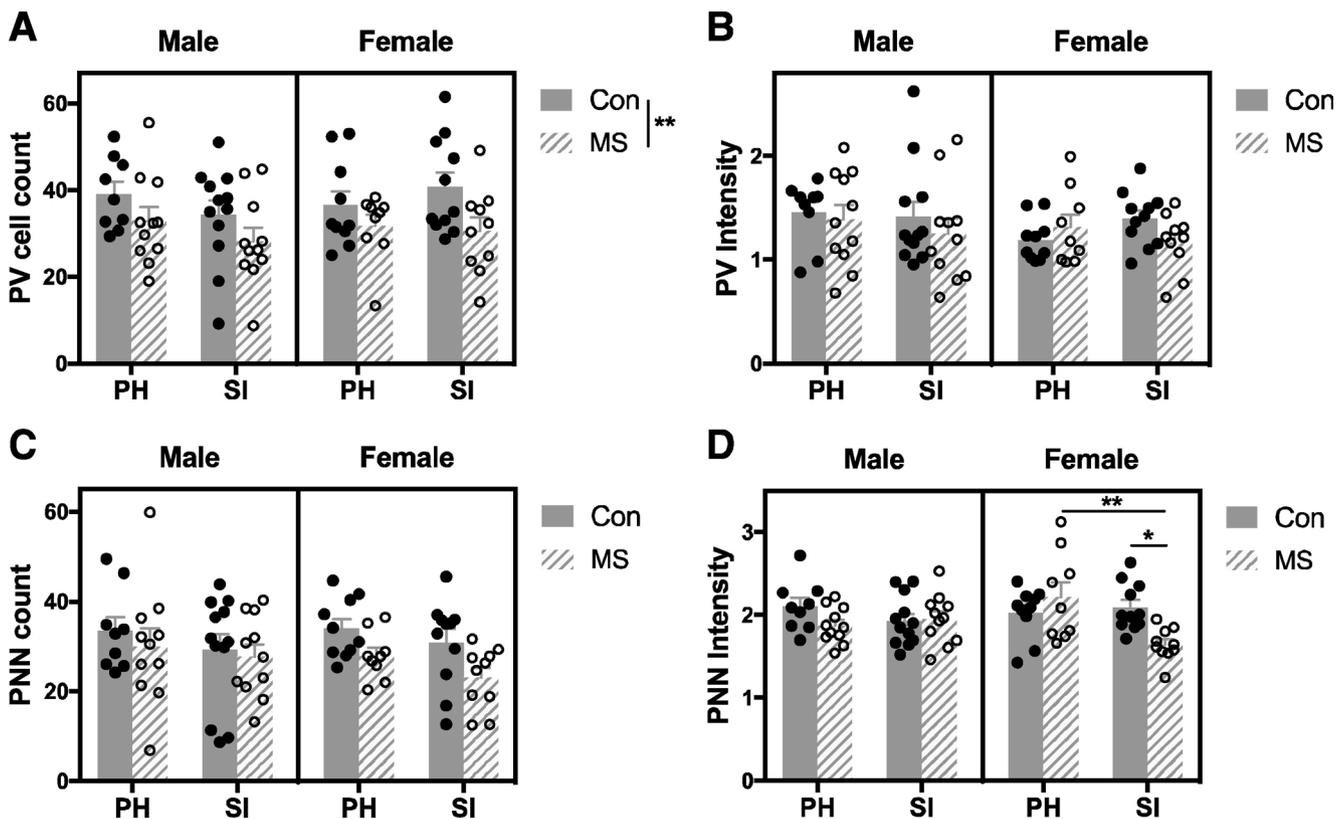


Fig. 6. Effects of maternal separation (MS) paired with social isolation (SI) on adult parvalbumin (PV)-expressing interneurons and perineuronal nets (PNNs) in the infralimbic (IL) prefrontal cortex. (A) PV cell count was decreased in both males and females. (B) Intensity of PV immunofluorescence and (C) PNN number were not changed by Rearing or Housing. (D) A three-way interaction between Rearing, Housing, and Sex was revealed, where an additive effect of MS and SI occurred in females only. *: $p < 0.05$; **: $p < 0.01$; $n = 9-13$ /group.

3.2.2.3. Colocalized parvalbumin and perineuronal net density and intensity. MS significantly decreased the overall number of PV neurons enwrapped by PNNs in the IL (main effect of Rearing $F_{1,75} = 9.775, p = 0.003, \eta_p^2 = 0.115$); however, there was no three-way interaction between Rearing, Housing, and Sex ($F_{1,75} = 0.942, p = 0.335, \eta_p^2 = 0.012$; Fig. 7A). Follow-up two-way ANOVAs showed a main effect of Rearing in females ($F_{1,36} = 7.861, p = 0.008, \eta_p^2 = 0.179$), with a non-significant, but moderate, effect in males ($F_{1,39} = 2.956, p = 0.093, \eta_p^2 = 0.070$). Tukey post-hoc comparisons revealed no group differences, however. The proportion ($F_{1,75} = 0.912, p = 0.343, \eta_p^2 = 0.012$; Fig. 7B) and intensity ($F_{1,75} = 0.645, p = 0.424, \eta_p^2 = 0.009$; Fig. 7C) of PV cells enwrapped by PNNs were not altered by Rearing, Housing, or Sex. When the intensity of PNNs surrounding PV neurons was assessed, a three-way interaction was observed ($F_{1,75} = 7.671, p = 0.007, \eta_p^2 = 0.093$) and SI decreased PV + PNN intensity ($F_{1,75} = 5.416, p = 0.023, \eta_p^2 = 0.067$; Fig. 7D). Separate two-way ANOVAs demonstrate an SI-induced decrease in PV + PNN intensity (main effect of Housing; females: $F_{1,36} = 4.178, p = 0.048, \eta_p^2 = 0.104$; males: $F_{1,40} = 1.201, p = 0.280, \eta_p^2 = 0.030$), exclusively in females. Additionally, there was a significant interaction between Rearing and Housing in females ($F_{1,36} = 6.292, p = 0.017, \eta_p^2 = 0.149$), but not males ($F_{1,40} = 1.432, p = 0.239, \eta_p^2 = 0.035$). Analysis of group differences showed that females – but not males – exposed to MS and SI had PNNs enwrapping PV cells that fluoresced significantly less than those exposed to SI alone (females: $p = 0.0419$; males: $p = 0.9562$) and MS alone females: $p = 0.0169$; males: $p = 0.9999$), suggesting a sex-specific additive effect of multiple hits of adversity throughout development.

4. Discussion

A vast literature of clinical and preclinical research provides evidence that adversity throughout early development has long-term consequences in the brain that impact behavior (see reviews: Andersen and Teicher, 2008; Ganguly and Brenhouse, 2015; Heim and Binder, 2012; Pechtel and Pizzagalli, 2011). Multiple episodes of such adversity throughout childhood have been further associated with increased neuropsychiatric symptoms, particularly in women (Manyema et al., 2018; Suliman et al., 2009). However, there is a paucity of plausible biological mechanisms to explain sex-specific disruptions to affect-regulating brain circuitry following one or more early life stressors. In the current study, we modeled adversity during two windows of susceptibility (neonatal and juvenile stages) in the form of MS and SI to determine whether there is a compounding effect of multiple hits of adversity on adult anxiety-like behavior, as well as PV-expressing interneurons and WFA-labeled PNNs in the PFC. Our findings suggest a sex-specific impact of early life adversity on adult locomotion and risk-assessment behavior, where females are more sensitive to adversity-induced deviations in performance. Our data also indicate a sex-specific additive effect of adversity on PNN structural integrity and PV cell count, suggesting that females may be at higher risk of long-term neural and extracellular consequences following multiple hits of adversity.

Behaviorally, our data indicate no change in the amount of time spent in the open area of the EZM, signifying a lack of effect of repeated developmental adversity on this measure of anxiety-like behavior. We

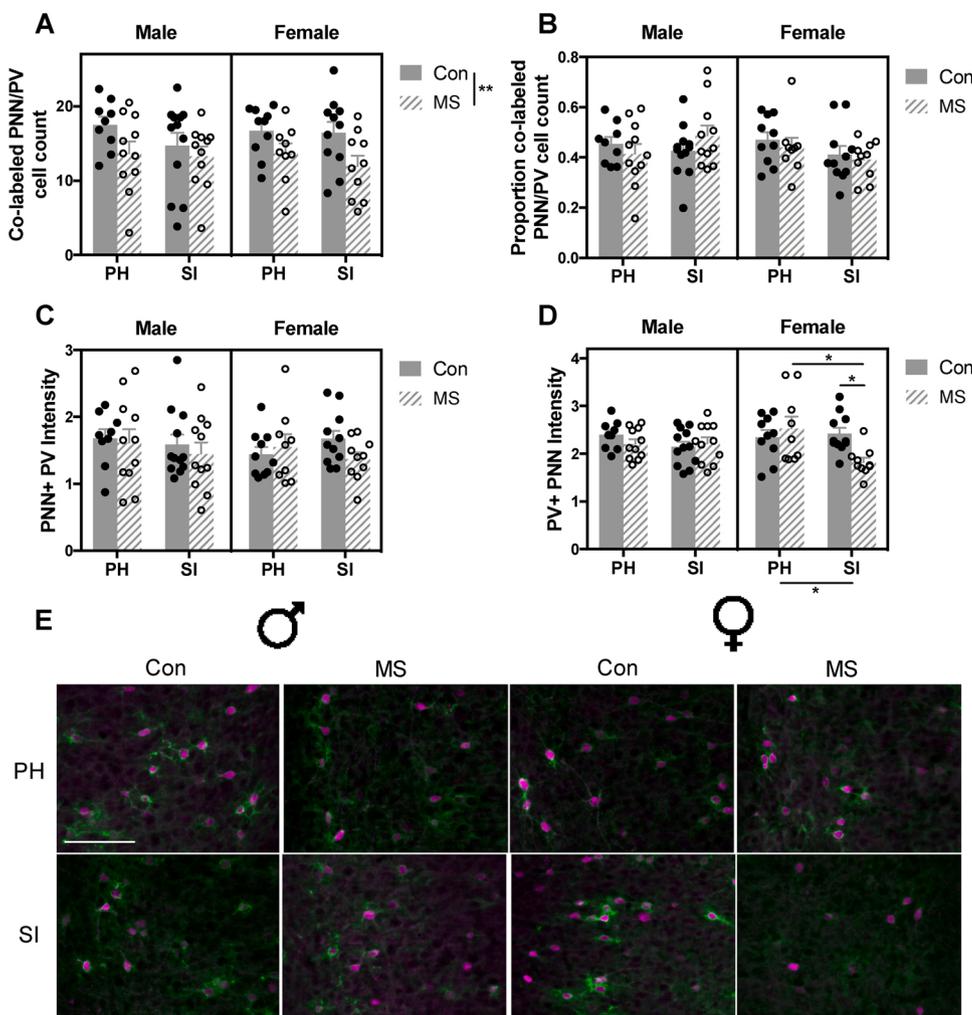


Fig. 7. Effects of maternal separation (MS) paired with social isolation (SI) on co-labeled parvalbumin (PV)-expressing neurons and perineuronal nets (PNNs) in the infralimbic (IL) prefrontal cortex. (A) The number of PNN + PV cells was decreased in the IL of both males and females. (B) The proportion and (C) intensity of PV cells surrounded by PNNs were not altered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) (D) An additive effect of MS and SI on the intensity of PNNs surrounding PV cells in the PL was observed only in females. (E) Representative photomicrographs of adult *Wisteria floribunda* agglutinin (WFA; green) + PNNs and PV cells (magenta) in the PL. Scale bar = 50 μm ; *: $p < 0.05$; $n = 9\text{--}13/\text{group}$

observed an increase in frequency to the open area in SI females, which may be driven by increased locomotion, displayed by crossings from one closed area of the EZM to the other. Contrary to our hypothesis, this finding may suggest an SI-induced decrease in anxiety-like behavior. In past work, adolescent SI (P28-70) has been found to have similar anxiolytic effects in female C57BL/6 J mice, as well as hypersociality (Riviera-Irizarry et al., 2020). While social behavior was not measured in the current study, it is possible that the observed hyperactivity may be explained by increased motivation for social contact. Notably, SI females also showed decreased risk-assessment behavior, suggested in the NIH Research Domain Criteria (National Institute of Mental Health., 2016) to reflect vigilance to potential threats. MS, however, had a contrasting effect on EZM performance, resulting in an overall decrease in frequency to open and crossings that was driven by females, suggesting that females may experience long term behavioral effects of neonatal adversity. Females exposed to MS paired with SI showed a decrease in locomotion and an increase in risk-assessment behavior compared to females exposed to SI alone. This may suggest that the SI-induced increase in exploratory behavior and reduced risk assessment is prevented by MS experience. We have previously shown that MS leads to increased anxiety-like behavior in adolescent males (Ganguly et al., 2015) and others have found that MS paired with early weaning resulted in increased anxiety-like behavior in adult males (Murthy et al., 2019). It is essential, however, to recognize the limitations of behavioral tasks – such as the EZM and Elevated Plus Maze – as indicators of anxiety-like behavior. Rodent-based anxiety-related tasks rely on observable behavior. In humans, however, Generalized Anxiety Disorder does not commonly manifest as distinct behavioral phenotypes, instead existing as a more internal, psychophysiological experience (Troisi, 1999). Importantly, meta-analyses have found a large amount of heterogeneity and discrepancy from studies assessing behavioral effects of early life adversity, especially between different measures of anxiety-like behavior (Bonapersona et al., 2019; Wang et al., 2020). Such conceptual and procedural concerns complicate generalization of rodent behavioral paradigms to human experience (Wall and Messier, 2001). Clinical literature points to aberrations in childhood and adolescent environment on gender disparities in later-life depression (Heim et al., 2009; Leach et al., 2008) and PTSD (McLaughlin et al., 2010). Findings regarding gender differences in anxiety following childhood adversity, however, are mixed (Gallo et al., 2018). While our findings may point to a role of early life adversity in female-specific behavioral development, future work in humans and animal models must disentangle the complex relationship between childhood maltreatment and mental health in males and females.

Few studies have investigated the effect of multiple hits of adversity on later-life PV interneurons and PNNs, and the current work is the first to assess these in the context of sex differences. Here, we demonstrate that neonatal MS paired with juvenile SI decreases the number of PV-expressing interneurons in the PFC and reduces the structural integrity of the PNNs that enwrap them, in females only. Previously, Castillo-Gómez et al. (2017) did not observe a potentiated decrease in PFC PV cell count, PNN number, or PV + PNN number following two hits of stress in male mice, corroborating our finding that compounding neurostructural deficits may occur specifically in females. The decrease in PV cell number seen in females exposed to both MS and SI did not occur in tandem with a decrease in PNN number, suggesting that the potentiated reduction in PNN intensity did not occur simply because there were fewer PV cells in the PFC. The lack of decrease in the proportion of PNN coverage, however, demonstrates that the overall PV decrease was not driven solely by the population of PV cells enwrapped by PNNs. Our data confirm prior evidence that both populations of PV cells (PNN-lacking and PNN-enwrapped) are vulnerable to the effects of stress (Cabungcal et al., 2013). Further, PV cells enwrapped by structurally aberrant PNNs may be at similar risk to those lacking any protection from PNNs. PNNs act as a physical and molecular barrier, with many extracellular components working in concert to maintain structural

integrity and protect the cells that they enwrap (Wang and Fawcett, 2012). We have previously found that MS alone delays PNN formation in the PFC with normal levels apparent by adulthood (Gildawie et al., 2020a), suggesting a transient effect of early life adversity on altered PNN expression. While altered PNN structural integrity in juvenility may have long term consequences on brain maturation, the current findings suggest that a second hit of juvenile adversity is required for prolonged PNN reduction in adulthood. Importantly, we have also found that PV cells in the PFC of juveniles exposed to neonatal MS undergo increased oxidative damage (Soares et al., 2020). As degradation of PNNs via chondroitinase ABC has been found to increase PV neuron vulnerability to oxidative stress (Cabungcal et al., 2013), this converging evidence suggests that a lack of PNNs following MS contributes to oxidative damage and consequential PV loss upon subsequent challenges.

PV neuron maturation during early development is essential for long term function. Reduction in adolescent PFC PV expression has been shown to decrease GABAergic transmission in adulthood, altering excitatory-inhibitory synaptic activity (Caballero et al., 2020). Indeed, PV reduction has long been implicated in mood disorders, such as schizophrenia (Beasley and Reynolds, 1997; Enwright et al., 2016). Importantly, the presence of PNNs has an important impact on synaptic input and physiology of PV positive cells (Riga et al., 2017). Typical PNN number and integrity is therefore critical for maintaining proper neural transmission throughout life. In postmortem human studies, individuals with schizophrenia (Mauney et al., 2013) and bipolar disorder (Alcaide et al., 2019) expressed fewer PNNs in the PFC, suggesting that PV and PNN disruption has important implications for the onset of neuropsychiatric disorders. These studies, however, were not sufficiently powered to assess differences between men and women. Our findings suggest that female PNNs and PV cells in the PFC are more susceptible to the effects of repeated adversity, which may underlie the increased risk of developing mood disorders for young girls following multiple instances of adversity during childhood. While changes in PV cell function were not assessed in the current work, a decrease in PFC PNN structural integrity following adversity during key developmental stages could have important consequences for cortical plasticity with implications for sex-specific response to stress.

When interpreting these findings, some considerations should be made. First, in designing the current study, we sought to quantify adversity-induced PNN/PV disruption and anxiety-like behavior; however, behavioral and neuroanatomical assessment was separated by 15 days, making interpretation of the brain-behavior relationship more complex. Since PNNs and PV cells are both highly sensitive to altered experience, even in adulthood (Riga et al., 2017), we chose to include a gap between behavioral testing and tissue collection to avoid potential confounds from the task. Secondly, while the EZM has been well-validated as a measure of anxiety-like behavior in rodents (Pähkla et al., 2000; Shepherd et al., 1994) and has been shown to have concurrent validity with other tests, such as the Open Field Test (Braun et al., 2011; Díaz-Morán et al., 2014), it was the only assessment of behavior in this study. It is important not to place undue weight on a single behavioral task. Future research, therefore, must continue to determine how anxiety-like behavior – and other PFC-mediated behaviors – interrelate and are affected by early life adversity. Next, we chose to use Pipsqueak for cell count and intensity analyses. Pipsqueak is a powerful tool that can provide faster and more reliable quantification than more manual methods (Slaker et al., 2016). We are, however, limited in our analysis, as Pipsqueak – in its current form – does not allow for count or intensity quantification for PNNs surrounding non-PV cells. Prior work from our lab has shown distinct differences in how MS affects the expression of PV cells with and without PNNs (Gildawie et al., 2020a). Given the important role of PNNs in PV expression and function, however, measuring the structural integrity of PNNs enwrapping PV cells versus other cell types could provide important information regarding cell-specific effects of early life adversity. Lastly, in adult mice previously exposed to MS, both males and females demonstrated

enhanced anxiety-like behavior; however, the effects in females were moderated by estrous phase (Romeo et al., 2003). While the current study was not powered to assess estrous phase, it is possible that female behavioral response to developmental adversity may be modulated by cycle. That said, female behavior was no more variable than that of males.

5. Conclusions

Early life is rife with sensitive periods during which aberrant experience can have lasting effects on plasticity and behavioral development (Bicks et al., 2020; Chocyk et al., 2010). Heightened neural malleability during early stages of development may – in part – stem from protracted formation of PNNs (Gildawie et al., 2020a; Mauney et al., 2013), and may also depend on sex and pubertal status (Drzewiecki et al., 2020). Here, we show a clear sex-specific effect of multiple hits of early life adversity during distinct windows of susceptibility, where females demonstrate a potentiated decrease in PV number and PNN structural integrity following neonatal MS and juvenile SI. Evidence suggests that reduced PNN number and structural integrity may result in a detrimental increase in plasticity (see review: Reichelt et al., 2019) and expose enwrapped PV-expressing interneurons to the effects of oxidative stress (Cabungcal et al., 2013). PNN degradation has been seen to disrupt GABAergic circuitry (Riga et al., 2017) and hamper synaptic transmission (Blosa et al., 2015), which has implications for behavioral dysfunction (Bicks et al., 2020). In the current study, we observed a sex-specific effect of adversity on hyperactivity and risk-assessment behavior; however, contrary to findings in the brain, the pattern of results was not additive in nature. This may suggest that adversity-induced PV and PNN disruption does not fully underlie alterations in anxiety-like behavior and that other mechanisms may be at play. Future work will investigate other PFC-mediated behaviors that may be regulated by alterations in neurostructural maturation, such as social behavior and working memory.

Recent work has elucidated potential mechanisms by which PNNs are degraded in response to altered experience and neuropsychiatric conditions. For example, microglia have been implicated in PNN structural development and degradation (Crapser et al., 2020a, b; Nguyen et al., 2020). Further, sex differences have been observed in the neuroimmune response to stress (Fonken et al., 2018; Gildawie et al., 2020b), suggesting a microglia-mediated mechanism may underlie differences between males and females in their behavioral and neural response to repeated adversity during sensitive periods of development. The evidence presented here has important implications for sex-specific neurostructural and behavioral effects of multiple hits of adversity. Moreover, the current work helps lay the foundation for mechanistic investigation of sex-specific changes in plasticity, which will further our understanding of neuropsychiatric sequelae and potential preventative therapies.

Data statement

The entirety of raw data from this study is available from the authors upon request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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