

## Does maternal separation accelerate maturation of perineuronal nets and parvalbumin-containing inhibitory interneurons in male and female rats?

Rick Richardson<sup>a,\*</sup>, Jeremy Bowers<sup>a</sup>, Bridget L. Callaghan<sup>b</sup>, Kathryn D. Baker<sup>a</sup>

<sup>a</sup> School of Psychology, UNSW Sydney, NSW, 2052, Australia

<sup>b</sup> Department of Psychology, University of California – Los Angeles, Los Angeles, CA, United States

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### ABSTRACT

Early life adversity impacts on a range of emotional, cognitive, and psychological processes. A recent theoretical model suggests that at least some of these effects are due to accelerated maturation of specific physiological systems and/or neural circuits. For example, maternal separation (MS), a model of early life adversity in rodents, accelerates maturation of memory systems, and here we examined its impact on maturation of perineuronal nets (PNNs) and parvalbumin (PV)-containing inhibitory interneurons. PNNs are specialized extracellular matrix structures suggested to be involved in stabilizing long-term memories and in the closure of a sensitive period in memory development. PV-containing inhibitory interneurons are the type of cell that PNNs preferentially surround, and are also thought to be involved in memory. In Experiment 1, with male rats, there was an increase in PNNs in both the amygdala and prefrontal cortex with age from infancy to juvenility. Contrary to prediction, MS had no impact on either PNN or PV expression. The same pattern was observed in female rats in Experiment 2. Taken together, these data show that the early maturation of memory in MS infants is not due to an accelerated maturation of PNNs or PV-containing cells in either the amygdala or prefrontal cortex.

### 1. Introduction

There is substantial evidence that early-life adversity has detrimental consequences for physical and mental health. As just a few examples, exposure to early-life adversity has been shown to increase the risk for metabolic syndrome, diabetes, cardiovascular disease, and Alzheimer's disease (Alastalo et al., 2009; Danese and McEwen, 2012; Thomas et al., 2008; see also Hoeijmakers et al., 2018; Nusslock and Miller, 2016). Children who experience adversity are also vulnerable to developing mental health disorders across the lifespan, including anxiety-, depressive-, and substance abuse-disorders (McLaughlin et al., 2010; McLaughlin et al., 2019; Teicher and Samson, 2013).

Many of the consequences observed in humans exposed to early-life adversity can be modelled in pre-clinical animal studies. There are a variety of procedures used to induce early-life adversity in such studies, with some involving pre-natal manipulations (e.g., stress during pregnancy) and others involving post-natal manipulations (e.g., limited bedding and maternal separation). Perhaps the two most frequently used post-natal procedures, at least in rodents, are the *maternal separation* and *limited bedding* models. In the latter model, the amount of bedding material provided to the dam (i.e., mother) is substantially

reduced, usually for a limited period of time shortly after parturition. In the former model, infants (i.e., pups) are separated from their mother for a period of time each day, and placed in an incubator. The exact parameters of this separation (i.e., length, days on which it occurs) varies across studies, but commonly the pups are separated for three hours a day on postnatal days 2–14 (e.g., Callaghan and Richardson, 2012; Gareau et al., 2007). Direct comparisons of the outcomes of these two models of early-life adversity have been rare (although see Demaestri, Pan, Critz, Ofray, Gallo, and Bath, 2020, for a recent exception), but there is substantial evidence from a large number of studies that exposure to either type of early life adversity has a variety of behavioral, physiological, and/or neural consequences (e.g., Francis et al., 2002; Kalinichev et al., 2002; Huot et al., 2004; Moriceau et al., 2009; Walker et al., 2017), including, for example, dysregulated hypothalamic-pituitary-adrenal stress reactions and heightened anxiety-related behaviors in adulthood. While both models have contributed to our understanding of the consequences of early-life adversity, in this study we focussed on the effects of maternal separation (MS).

We recently reported that exposure to early-life adversity, as modelled by MS, leads to an accelerated maturation of emotion

\* Corresponding author.

E-mail address: [r.richardson@unsw.edu.au](mailto:r.richardson@unsw.edu.au) (R. Richardson).

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regulation in rats. Specifically, a number of studies have shown that young animals forget more rapidly than do adults, a phenomenon referred to as *infantile amnesia* (Campbell and Campbell, 1962; Campbell and Spear, 1972; Josselyn and Frankland, 2012; Madsen and Kim, 2016). However, infant rats exposed to the MS procedure show more adult-like memory (Callaghan and Richardson, 2012; also see Peng et al., 2019). That is, unlike standard-reared infants that exhibit substantial forgetting after 7–14 days, infants exposed to MS exhibit essentially no forgetting over these intervals. This early termination of the infantile amnesia period is even passed on to subsequent generations that don't directly experience early-life adversity (Callaghan et al., 2016). In addition, previous work had shown that infant, in contrast to juvenile-aged, rats are relapse-resistant following extinction of an aversive association. That is, when tested in a different context, or given a pre-test reminder (e.g., an unsignalled presentation of the aversive stimulus used in training), juvenile rats (i.e., ~23 days of age) exhibit a return of an extinguished fear response, like adults; neither effect was observed in infant rats (~17 days of age; for review see Kim and Richardson, 2010). In contrast, infant rats exposed to MS exhibit both renewal and reinstatement following extinction (Callaghan and Richardson, 2011). Longer-lasting fear memories and increased relapse following extinction of a learned fear response earlier in development could both contribute to the development of an anxiety disorder often observed in those exposed to early-life adversity (Copeland et al., 2018).

Such findings led to the proposal of the *stress acceleration hypothesis* (e.g., Callaghan and Tottenham, 2016). From this perspective, early-life adversity accelerates the maturation of some physiological and cognitive systems/processes so that individuals can cope with potentially difficult future environments. Recent work suggests that the neural systems supporting long-lasting fear memories may be one such system exhibiting an acceleration. This has been demonstrated in studies examining the expression of phosphorylated mitogen-activated protein kinase (pMAPK), a marker of synaptic plasticity, in the medial prefrontal cortex (mPFC). Typically, adult and juvenile animals have increased expression of cells labelled with pMAPK in the prelimbic subregion of the mPFC when expressing a learned aversive association the day after training relative to untrained animals (e.g., Li et al., 2012). An elevation of pMAPK expression has also been detected in the infralimbic subregion of the mPFC of adult and juvenile rats following extinction training when they learn to inhibit a fear association (Kim et al., 2009). In contrast, standard-reared infant animals, unlike their older counterparts, do not exhibit an increase in pMAPK-labelled cells in the prelimbic region following the expression of a learned aversive association (Li et al., 2012), or in the infralimbic region following the extinction of such learning (Kim et al., 2009). These results were replicated in a study by Cowan et al. (2019), who also reported that infants exposed to early-life adversity (modelled by MS) exhibited a more mature pattern of neural function. That is, the MS infants exhibited an increased number of pMAPK-labelled cells in the medial prefrontal cortex following both the expression of, and the extinction of, a learned aversive association (within the prelimbic and infralimbic subregions, respectively). In other words, the MS animals responded more like juveniles when this activity-dependent neural measure was assessed. In the present study, we examined whether a similarly accelerated maturation at a structural level occurs. That is, we tested whether early-life adversity, modelled by MS, leads to accelerated maturation of structural neural components thought to be involved in long-term memory and/or relapse-prone extinction.

Perineuronal nets (PNNs) are dense extracellular matrix structures that preferentially surround mature GABAergic neurons. They have been shown to be critically involved in the closing of critical periods of sensory system development (e.g., Fawcett et al., 2019; Bavelier et al., 2010). That is, areas of the brain involved in processing of sensory information remain highly plastic until PNNs develop in sufficient numbers. Further, depletion of PNNs in sensory areas in adulthood leads to a re-opening of the critical period (i.e., the neurons in that area are

once again highly plastic; Pizzorusso et al., 2002). It has been suggested that infantile amnesia can be thought of as a critical period in the development of memory (Callaghan et al., 2014; Travaglia et al., 2016). Given that PNNs are likely involved in long-term memory storage in the mature animal (e.g., Fawcett et al., 2019; Tsien, 2013) perhaps accelerated maturation of PNNs in memory-related brain regions following early life adversity is involved in terminating the infantile amnesia period? In support of this suggestion, PNNs have been reported to protect long-term memories of fear and reward learning in adult rodents (Banerjee et al., 2017; Gogolla et al., 2009; Slaker et al., 2015) and contribute to relapse-related phenomena following extinction (Gogolla et al., 2009; Xue et al., 2014). For example, Gogolla et al. (2009) provided evidence that the transition from relapse-resistant extinction in infant mice to relapse-prone extinction in juvenile mice could be a consequence of an increased number of PNNs in the amygdala across these developmental stages. Specifically, Gogolla et al. (2009) reported an increase in the number of PNNs in the amygdala across these ages, and, further, that depletion of PNNs in the adult amygdala returned the animals to an infant-like state, in that they were relapse-resistant following extinction of learned fear. Given these findings, and those showing that MS infants, compared to standard-reared infants, exhibit longer-lasting fear memory as well as earlier emergence of a relapse-prone extinction profile, it has been suggested that accelerated maturation of PNNs might be a potential mechanism underlying these consequences of early life adversity (Callaghan et al., 2014). Nevertheless, as direct experimental evidence is lacking we tested this hypothesis here.

PNNs occur in multiple regions of the brain, and across regions they preferentially surround GABAergic interneurons, in particular those that contain parvalbumin (PV). PNNs function as a cation buffer which enhances the excitability of fast-spiking interneurons, such as PV interneurons (Balmer, 2016; Härtig et al., 1999). Given that GABAergic-containing interneurons have also been suggested to play a role in memory in adults (e.g., Donato et al., 2013; Shi et al., 2019), and the percentage of all PV neurons surrounded by PNNs predicts behavioral indices of the progression of critical periods (e.g., song maturity in birds; Balmer et al., 2009), we also measured PV-expressing cells and the percentage of these cells surrounded by PNNs. If the mechanism for the accelerated maturation of fear-related processes (i.e., long-lasting memory and relapse-prone extinction) observed in MS infants (i.e., Callaghan and Richardson, 2011, 2012) is an accelerated maturation of PNNs, then we would expect that MS infants, compared to standard-reared infants, would express more PNNs, an increase in the density of PV-expressing cells, and/or a change in the percentage of PV-expressing cells surrounded by PNNs.

We examined the effects of MS on PNNs and PV-labelled cells in two brain regions known to be involved in fear-related behaviors in rats and other rodents: the basolateral amygdala and the medial prefrontal cortex. We included a late-juvenile aged comparison group of standard-reared animals to serve as a developmental comparison and predicted that juveniles would express more PNNs than the standard-reared infants. If MS accelerates maturation of these structures, then the MS infants should exhibit a level of PNNs, PV cells, and/or percentage of PV-labelled cells surrounded by PNNs more similar to what is observed in juvenile standard-reared animals relative to what is observed in standard-reared infants.

## 2. General method

### 2.1. Subjects

Subjects were experimentally naïve Sprague-Dawley rats, sourced from a breeding colony maintained by the School of Psychology at UNSW Sydney. The adult male and female breeders were obtained from the Australian Resource Centre (Perth). The day of birth was designated as post-natal day (P) 0, and litters were culled to a standard size of eight,

and kept together, with the dam, until being used in an experiment. Male offspring were used in Experiment 1 while female offspring were used in Experiment 2. Although we did not predict any sex-specific effects MS on PNNs, male and female subjects were analyzed separately given that trajectories of some maturational processes in the brain regions of interest can slightly differ slightly between sexes (Hu et al., 2013) and may be impacted by MS (Holland et al., 2014). No more than one rat per litter was used in any experimental group in either experiment. Rats were maintained on a 12-h light/dark cycle (lights on at 0700).

Some animals were standard-reared (i.e., typical animal facility care involving weekly cage cleaning) while others were exposed to maternal separation. Animals in this latter condition were separated from their mothers for three hours per day, commencing between 0900–1200, on P 2–14 as in our past studies (e.g., Callaghan and Richardson, 2011, 2012). During this period of separation, the entire litter was removed from the home cage and placed together in an incubator, with three centimetres of sawdust bedding, maintained at approximately 27 °C.

There were three groups in both experiments: standard-reared 18-day old infants (SR18), maternally-separated 18-day old infants (MS18), and standard-reared 28-day old juveniles (SR28). All procedures were approved by the UNSW Animal Care and Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013).

## 2.2. Perfusions

Perfusion procedures were as in previous studies in our laboratory (e.g., Baker et al., 2017). Briefly, animals (either P18 or P28) were given an intraperitoneal injection of Pentobarbitone Sodium (108 mg/mL; 5 ml/kg). Once an effective plane of anaesthesia was achieved, the heart was exposed and the left ventricle was injected with a solution of heparin (5000U/mL) and 1 % sodium nitrite (2.5 ml/kg). Rats were then transcardially perfused with 50–100 ml of prewash (1 % sodium nitrite and heparin in 0.9 % saline) followed by 200–300 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4).

Brains were extracted and postfixed before being washed in 0.1 M phosphate buffered saline (pH 7.2; hereafter referred to as PBS) and then stored at 4 °C in 20 % sucrose in PBS for up to 48 h. A cryostat (CM1950, Leica Microsystems) was used to cut 40 µm coronal brain slices, which were grouped into four series of slices. These sections were stored at 4 °C in 0.1 % sodium azide in PBS.

## 2.3. Staining and mounting

PNNs in the basolateral amygdala and medial prefrontal cortex were detected using Wisteria floribunda agglutinin (WFA) fluorescence, a technique that has been validated in both animal and human imaging of PNNs in various brain regions (e.g., Baker et al., 2017; Carstens et al., 2016; Slaker et al., 2015). To label PNNs and PV cells, brain sections containing the prefrontal cortex and amygdala were washed three times, 10 min per wash, in PBS and then incubated for 2 h in a PBS solution containing 0.5 % Triton X-100, 10 % normal horse serum (NHS), and 5% normal goat serum (NGS). Sections were then incubated for 48 h at room temperature in Wisteria floribunda lectin (1:200; Vector Laboratories) along with a monoclonal mouse antibody against parvalbumin (1:2000; Sigma P3088) in a PBS blocking solution containing 0.5 % Triton-X 100, 0.1 % sodium azide, 1 % NGS, and 2 % NHS. After 48 h, sections were incubated in Alexa Fluor 594 goat anti-mouse IgG1 (γ1) (1:750; Life Technologies A-21125) in the PBS blocking solution. Sections were then washed three times in PBS, incubated for two minutes in DAPI, and then washed again. DAPI binds to adenine-thymine in DNA, therefore aiding in the identification of landmarks and cortical layers according to a brain atlas (Paxinos and Watson, 2009). Sections were then mounted onto gelatinized slides, air-dried, and coverslipped with PermaFluor mounting medium.

## 2.4. Imaging

Photomicrographs were acquired with Olympus Cell Sens software using a fluorescent microscope (Olympus BX53 equipped with an X-Cite 120 illuminator; 10x objective) and DP74 camera. Images were taken of the basolateral amygdala (between bregma –2.16 mm and –2.92 mm; see Fig. 1A for a diagram) and two sub-divisions of the medial prefrontal cortex – the prelimbic and infralimbic cortices (between bregma 3.72 mm and 3.00 mm) preferentially from the left hemisphere. As the area of the medial prefrontal cortex may differ across the rostral-caudal extent of the region as well as across animals, boundaries were created of 1 mm height × 1 mm width to define the prelimbic cortex (see Fig. 2A), while the infralimbic cortex was defined by boundaries of 0.7 mm height × 1 mm width (see Fig. 3A); where this area was smaller the area (mm<sup>2</sup>) of that region was manually traced using Cell Sens software. The area (mm<sup>2</sup>) of the basolateral amygdala for each section was also manually traced and computed. Separate fluorescent filters were used to identify WFA and/or PV stained cells in the images.

## 2.5. Density measurements

PNNs and PV cells were manually counted by an experimenter blind to the experimental condition of the animals. The single- and double-labelled cell counts were performed in Adobe Photoshop. The average density of PNNs (mm<sup>2</sup>) per slice was calculated from typically 3 slices (although the range was 1–6 slices) per region per animal.

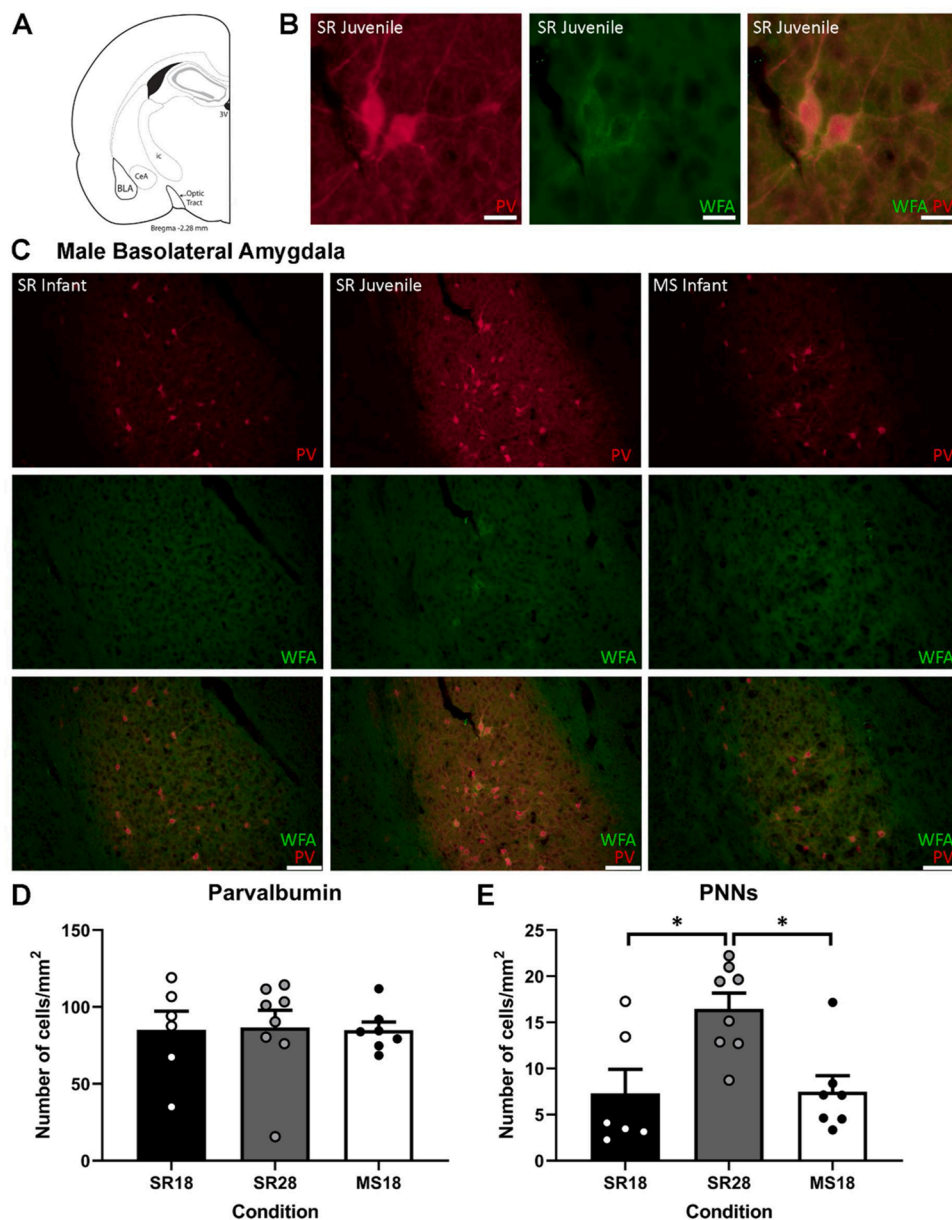
## 2.6. Exclusions

Sections with the prelimbic and infralimbic cortex from four subjects (1 male MS18, 1 male SR28, 1 female MS18, and 1 female SR18) were not counted because of tissue degradation, although, the basolateral amygdala from those subjects was intact.

## 2.7. Statistics

IBM SPSS Statistics 25 was used to conduct separate one-way Analyses of Variance (ANOVAs) for each region of interest to test for differences in density of PNNs, PV cells, and double-labelled cells across the three groups. Following this, pairwise comparisons were conducted with Tukey's honestly significant difference (HSD) tests, with  $\alpha$  controlled at 0.05. Data was checked for normality using Shapiro-Wilks test (see Table S1 and S3 for results). Although in some groups the data was not normally distributed (19 out of 108 tests), violations of normality were unlikely to affect the interpretation of our primary hypothesis. More specifically, the concern with such violations is that they can possibly inflate the Type I error rate, but given that no significant effect of rearing condition was detected in the analyses any such concern is not applicable here. Linear mixed model analyses were conducted using the GAMLj suite version 2.0.5 (Gallucci, 2019) for jamovi version 1.6.3 (The Jamovi Project, 2020). The mixed effects analyses were performed to primarily evaluate the relationship between early-life adversity and PV cells or PNNs across brain regions in males or females. Condition and region were entered as fixed effects and any statistically significant main effects of condition were followed up with post hoc comparisons with holm's corrections for multiple comparisons. Regions were not compared to each other since these comparisons were not of interest. Given that multiple density counts were obtained from each rat (i.e., from three brain regions), the measures were considered clustered by rat, and so a random effect intercept of rat was entered in the model to specify the variability between rats. This analysis had the advantage over repeated measures ANOVA in permitting different sample sizes across brain regions (i.e., the repeated factor).





**Fig. 1.** (A) A representative diagram of the basolateral amygdala (BLA; CeA = Central amygdala; ic = internal capsule; 3V = third ventricle). (B) Representative fluorescent photomicrographs of PV (left), WFA (middle) and combined immunoreactivity showing PNNs surrounding PV cells (right) in the basolateral amygdala of a standard-reared juvenile (SR28) male rat. Scale bars = 20  $\mu$ m. (C) Representative photomicrographs of PV (top row), WFA (middle row) and combined (bottom row) immunoreactivity in the basolateral amygdala of male rats. Columns are separated into different conditions: standard-reared infants (SR18, left), standard-reared juveniles (SR28, middle), and maternally-separated infants (MS18, right). Scale bars = 100  $\mu$ m. (D) Mean (+SEM) density of parvalbumin-labelled cells and (E) mean (+SEM) density of perineuronal nets, both with individual dot points. Group sizes: SR18 = 6, SR28 = 8, MS18 = 7. (\*) indicates significant differences ( $p < .05$ ).

### 3. Experiment 1 (males)

#### 3.1. Results

##### 3.1.1. Basolateral amygdala

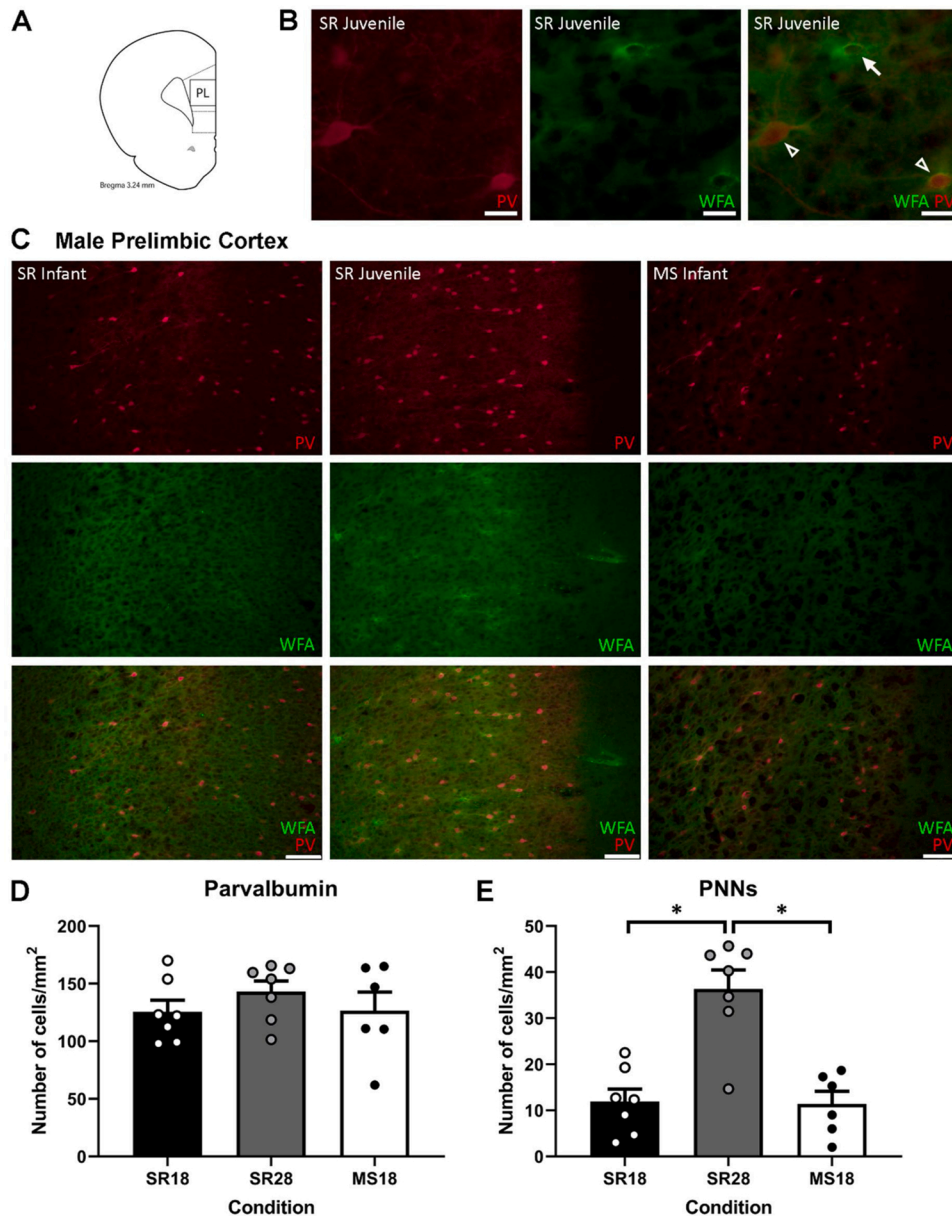
Representative photomicrographs of PNNs enwrapping PV cells in the basolateral amygdala are shown in Fig. 1B. Fig. 1C provides representative photomicrographs for each measure and for each group. The three groups did not differ on the density of PV-labelled cells in the basolateral amygdala,  $F(2, 18) = .01$ ,  $p = .99$  (Fig. 1D). That is, there was no effect of age or early-life adversity on this measure. However, the groups did differ in terms of the density of PNNs,  $F(2, 18) = 7.44$ ,  $p = .004$  (Fig. 1E). As predicted, standard-reared juveniles expressed more PNNs in this region in comparison to standard-reared infants ( $p = .012$ ). However, contrary to predictions, the density of PNNs in this region of the brain was similar between the standard-reared and MS infant groups ( $p = 1.0$ ), and the standard-reared juveniles expressed significantly more PNNs than the MS infants ( $p = .010$ ). As expected, PNNs preferentially surrounded PV-labelled cells (72–89%), but this did not differ across groups,  $F(2, 18) = .79$ ,  $p = .46$ . There was a significant group

difference in the percentage (7–14%) of PV-expressing cells surrounded by a PNN,  $F(2, 18) = 3.57$ ,  $p = .049$ , with the SR28 group appearing to have a higher percentage than either of the infant groups. However, none of the pairwise comparisons were significant according to a Tukey HSD test (SR18 v SR28,  $p = .082$ ; MS18 v SR28,  $p = .090$ ). A similar pattern was found for density of PV-labelled PNNs (see supplementary materials Table S2).

##### 3.1.2. Prelimbic cortex

Representative photomicrographs of PNNs enwrapping PV cells and non-PV expressing cells in the prelimbic cortex are shown in Fig. 2B. Representative photomicrographs for each measure and for each group are shown in Fig. 2C. The density of PV-labelled cells in the prelimbic cortex was equivalent across groups,  $F(2, 17) = .70$ ,  $p = 0.51$  (Fig. 2D). In terms of PNNs, the three groups differed, as was the case for the basolateral amygdala,  $F(2, 17) = 18.91$ ,  $p < .001$  (Fig. 2E). This group difference was due to the standard-reared juveniles having more PNNs than both the standard-reared and the MS infants ( $ps < .001$ ). The two infant groups did not differ from one another ( $p = .99$ ). A similar pattern was found for density of PV-labelled PNNs (Table S2). As expected,





**Fig. 2.** (A) A representative diagram of the prelimbic cortex. (B) Representative fluorescent photomicrographs of PV (left), WFA (middle), and combined immunoreactivity showing PNNs surrounding PV cells (right; white triangles) and a PNN surrounding a non-PV cell (right; white arrow) in the prelimbic cortex of a standard-reared juvenile (SR28) male rat. Scale bars = 20  $\mu$ m. (C) Representative photomicrographs of WFA (top row), PV (middle row) and combined (bottom row) immunoreactivity in the prelimbic cortex of male rats. Columns are separated into different conditions: standard-reared infants (SR18, left), standard-reared juveniles (SR28, middle) and maternally-separated infants (MS18, right). Scale bars = 100  $\mu$ m. (D) Mean ( $\pm$ SEM) density of parvalbumin-labelled cells and (E) mean ( $\pm$ SEM) density of perineuronal nets, both with individual dot points. Group sizes: SR18 = 7, SR28 = 7, MS18 = 6. (\*) indicates significant differences ( $p < .05$ ).

PNNs preferentially surrounded PV-labelled cells (81–92 %), but this did not differ across groups,  $F(2, 17) = 1.33$ ,  $p = .29$ . There was a group difference in the percentage of PV-expressing cells surrounded by a PNN,  $F(2, 17) = 19.26$ ,  $p < .001$ , with both of the infant groups expressing lower percentages (7.5 % and 7.8 %) than the standard-reared juveniles (23.58 %,  $ps < .001$ ).

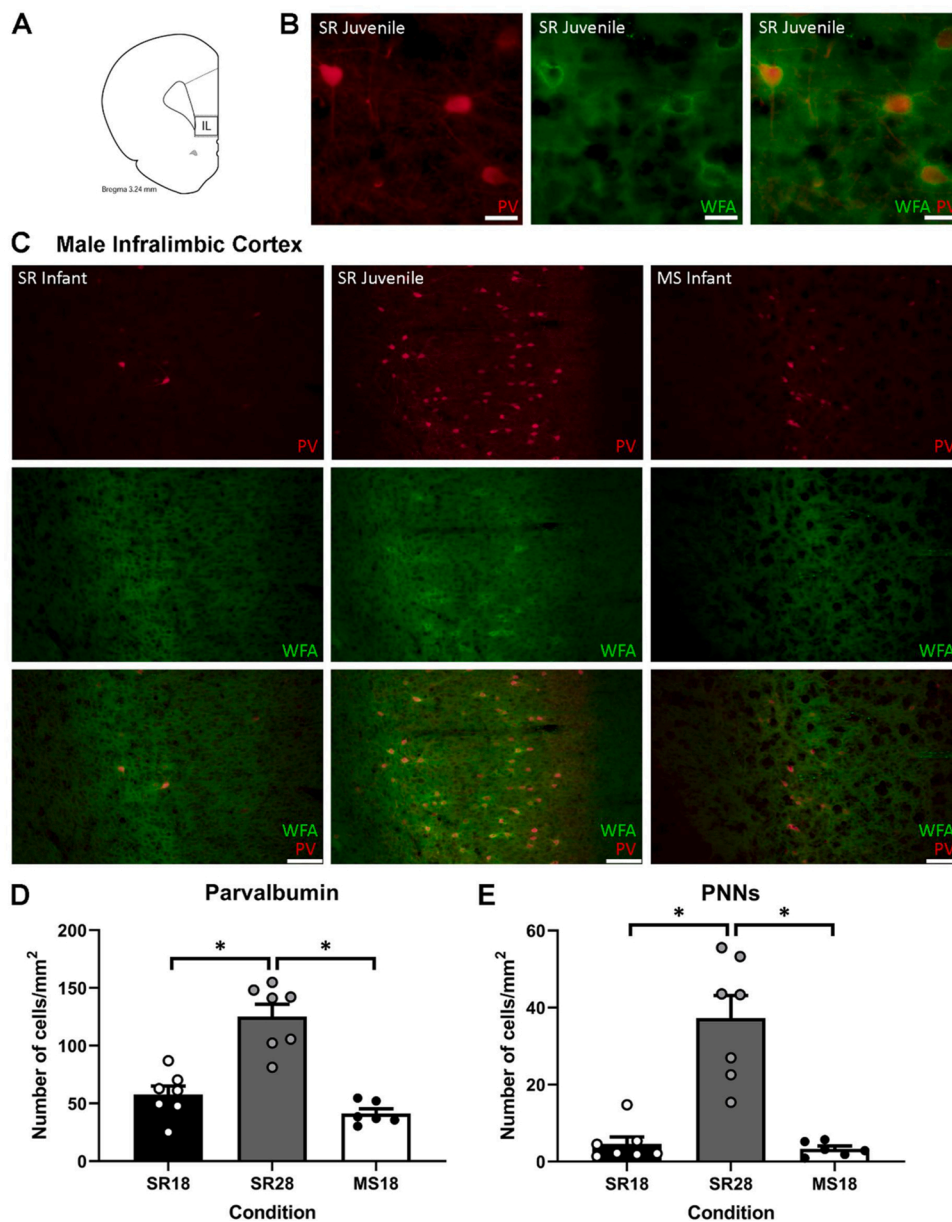
### 3.1.3. Infralimbic cortex

Fig. 3B shows representative photomicrographs of PNNs surrounding PV cells in the infralimbic cortex. Representative photomicrographs for each measure and for each group are shown in Fig. 3C. The three groups differed in the density of PV-labelled neurons,  $F(2, 17) = 29.77$ ,  $p < .001$  (Fig. 3D). Post hoc tests showed that this difference was due to standard-reared juveniles having more PV-labelled cells than both the standard-reared and the MS infants ( $ps < .001$ ); the two infant groups did not differ ( $p = .36$ ). As with the basolateral amygdala and the prelimbic cortex, the three groups differed in the density of PNNs in the infralimbic cortex,  $F(2, 17) = 26.44$ ,  $p < .001$  (Fig. 3E). Again, this difference was due to the standard-reared juveniles expressing more PNNs than either of the two infant groups ( $ps < .001$ ), which did not

differ from one another ( $p = .96$ ). The density of PV-labelled PNNs varied across groups in a similar way (Table S2). As expected PNNs preferentially surrounded PV-labelled cells (54–87 %), but unlike in the basolateral amygdala and the prelimbic cortex, this differed across groups,  $F(2, 17) = 7.38$ ,  $p = .005$ , with the two infant groups (SR18 = 54 % and MS18 = 69 %) different from the standard-reared juveniles (87 %,  $ps < .004$ ); the two infant groups did not differ ( $p = .22$ ). The percentage of PV-expressing cells surrounded by a PNN also increased with age,  $F(2, 17) = 18.19$ ,  $p < .001$ , with the two infant groups (6.01 % and 6.39 %) having a smaller percentage than the standard-reared juveniles (25.1 %,  $ps < .001$ ) and not differing from each other ( $p = .99$ ).

### 3.1.4. Across regions

We used linear mixed models to examine the relationship between PV and PNNs variables with respect to condition (SR18, SR28, MS18) and region. We were primarily interested in evaluating whether early-life adversity affected PV cells or PNNs when analysing across brain regions. As cells were counted in three regions from each animal, rat was included in each model as a random effect. As fixed effects we entered condition, region, and their interaction into each model. The models



**Fig. 3.** (A) A representative diagram of the infralimbic cortex. (B) Representative fluorescent photomicrographs of PV (left), WFA (middle), and combined immunoreactivity showing PNNs surrounding PV cells (right) in the infralimbic cortex of a standard-reared juvenile (SR28) male rat. Scale bars = 20  $\mu$ m. (C) Representative photomicrographs of PV (top row), WFA (middle row) and combined (bottom row) immunoreactivity in the infralimbic cortex of male rats. Columns are separated into different conditions: standard-reared infants (SR18, left), standard-reared juveniles (SR28, middle) and maternally-separated infants (MS18, right). Scale bars = 100 $\mu$ m. (D) Mean (+SEM) density of parvalbumin-labelled cells and (E) mean (+SEM) density of perineuronal nets, both with individual dot points. Group sizes: SR18 = 7, SR28 = 7, MS18 = 6. (\*) indicates significant differences ( $p < .05$ ).

were of PV density, PNN density, PNN PV-labelled density, non-PV-labelled PNNs density, percentage of PNNs surrounding PV cells, and the percentage of PV cells with a PNN. The full outcomes of each model are included in the supplementary materials whereas Table 1 shows the outcomes of the fixed effects and the post hoc comparisons with holm’s corrections for the condition effects. As shown in Table 1, statistically significant effects for condition were noted for every marker averaged across the basolateral amygdala, prelimbic, and infralimbic cortex in males. Post hoc comparisons revealed that early-life adversity did not alter density of PV-labelled cells, PNNs, PV-labelled PNNs, or non-PV-labelled PNNs. Early-life adversity also did not affect the percentage of co-localization of PNNs and PV when compared to SR18 animals. The only measure where MS18 animals did not differ significantly from the older standard-reared juveniles was the percentage of PNNs labelled with PV. On every measure a developmental effect was confirmed in standard-reared males.

#### 4. Experiment 2

Our past work on the effects of MS on fear memory and fear

extinction involved only male rats (Callaghan and Richardson, 2011, 2012), which is one reason why males were used in Experiment 1. However, females are more likely to experience an affective disorder than are males (McLean et al., 2011). Given that early-life adversity is a leading risk factor for such disorders perhaps the impact of MS on brain development will be more robustly expressed in females. We tested this hypothesis in Experiment 2, which had the same design and procedures as the first experiment.

#### 4.1. Results

##### 4.1.1. Basolateral amygdala

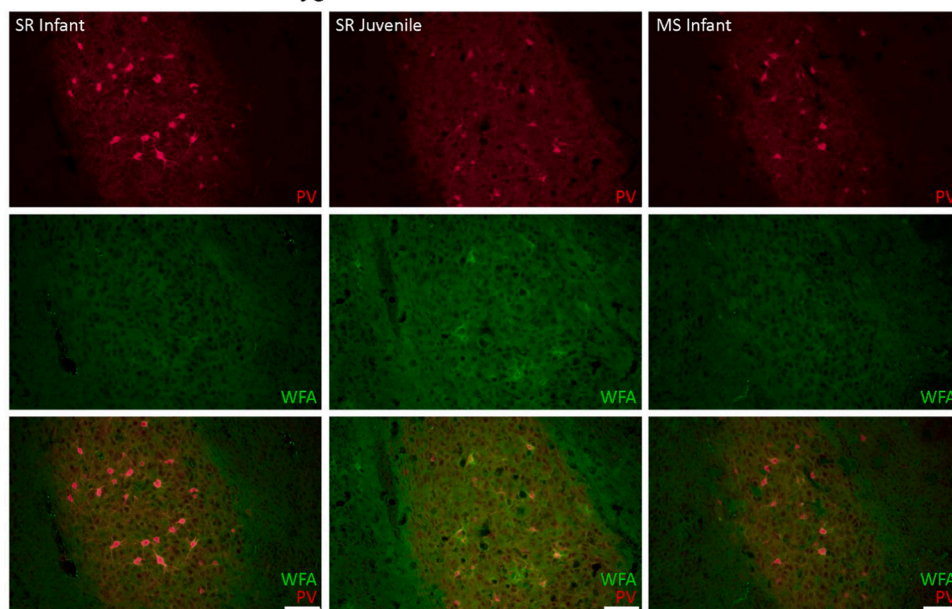
Representative photomicrographs for each measure and for each group are shown in Fig. 4A. The three groups didn’t differ on the density of PV-containing cells in the basolateral amygdala,  $F(2, 19) = 1.40, p = .27$  (Fig. 4B); however, the density of PNNs significantly differed between groups,  $F(2, 19) = 6.18, p = .009$  (Fig. 4C). Post hoc tests showed that this was due to the standard-reared juveniles having more PNNs than the standard-reared infants ( $p = .007$ ), as expected. The MS infant females were not different from either group on this measure (smallest

**Table 1**  
Linear Mixed Model Fixed Effects for Males.

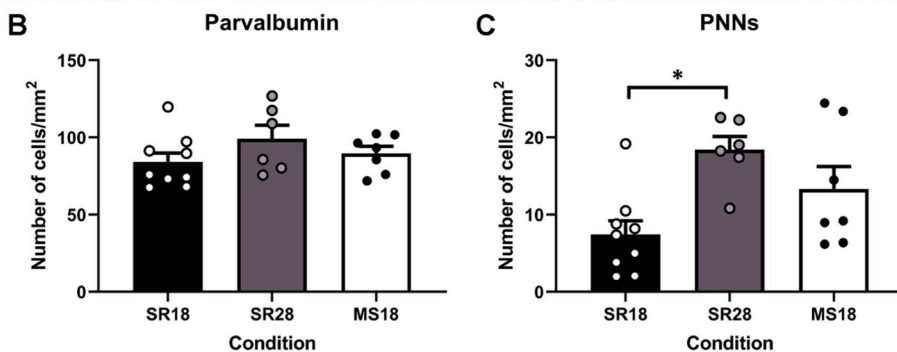
Measure	Effect	F	df	p	Post hoc comparisons with Holm's correction p		
					SR18 v SR28	MS18 v SR28	SR18 v MS18
PV	Condition	7.51	2, 19.1	.004	.014	.007	.60
	Region	33.06	2, 35.5	<.001			
	Condition x Region	6.66	4, 35.5	<.001			
PNNs	Condition	28.5	2, 19.1	<.001	<.001	<.001	.80
	Region	11.7	2, 34.6	<.001			
	Condition x Region	10.3	4, 34.6	<.001			
PV + PNNs	Condition	24.3	2, 19.3	<.001	<.001	<.001	.80
	Region	16.2	2, 34.4	<.001			
	Condition x Region	15.5	4, 34.5	<.001			
Non-PV PNNs	Condition	5.45	2, 52	.007	.032	.032	.93
	Region	0.05	2, 52	.95			
	Condition x Region	1.21	4, 52	.32			
% PNNs around PV cells	Condition	3.87	2, 17.5	.041	.048	.58	.12
	Region	4.17	2, 34.7	.024			
	Condition x Region	1.70	4, 34.7	.17			
% PV + with PNNs	Condition	15.11	2, 18.6	<.001	<.001	<.001	.83
	Region	2.72	2, 33.5	.081			
	Condition x Region	8.36	4, 33.5	<.001			

Note. PNNs = perineuronal nets, PV = parvalbumin, df = degrees of freedom using satterthwaite method, SR18 = standard-reared 18-day old infants, MS18 = maternally-separated 18-day old infants, SR28 = standard-reared 28-day old juveniles.

**A Female Basolateral Amygdala**



**Fig. 4.** (A) Representative photomicrographs of PV (top row), WFA (middle row) and combined (bottom row) immunoreactivity in the basolateral amygdala of female rats. Columns are separated into different conditions: standard-reared infants (SR18, left), standard-reared juveniles (SR28, middle) and maternally-separated infants (MS18, right). Scale bars = 100um. (B) Mean (+SEM) density of parvalbumin-labelled cells and (C) mean (+SEM) density of perineuronal nets, both with individual dot points. Group sizes: SR18 = 9, SR28 = 6, MS18 = 7. (\*) indicates significant differences ( $p < .05$ ).



$p = .15$ ). A similar pattern was found for density of PV-labelled PNNs (Table S4). As expected, PNNs preferentially surrounded PV-labelled cells (76–83 %), but this did not differ across groups,  $F(2, 19) = 0.24$ ,  $p = .79$ . There was trend for a group difference for percentage of PV-

expressing cells with a PNN,  $F(2, 19) = 3.46$ ,  $p = .052$ . Pairwise comparisons showed that the standard-reared infants (7.68 %) were different from the standard-reared juveniles (15.98 %,  $p = .047$ ) while the MS infants were intermediate between the two (12.49 %, smallest



$p = .29$ ).

#### 4.1.2. Prelimbic cortex

Representative photomicrographs for each measure and for each group are shown in Fig. 5A. The groups were equivalent in the density of PV-containing cells,  $F(2, 17) = 3.48, p = .054$  (Fig. 5B). The groups did, however, differ in the mean density of PNNs,  $F(2, 17) = 6.67, p = .007$  (Fig. 5C). The difference in the density of PNNs was due to the SR28 juveniles expressing more than the SR18 infants ( $p = .006$ ); the MS18 female infants did not differ significantly from the SR28 juveniles ( $p = .051$ ) nor the SR18 infants ( $p = .68$ ). However, analysis of density of PNNs around PV-labelled cells did suggest that MS18 females had fewer PNNs around PV-labelled cells than SR28 females (group effect:  $F(2, 17) = 6.71, p = .007$ ; MS18 vs SR28:  $p = .048$ ; see also Table S4). As expected, PNNs preferentially surrounded PV-labelled cells (84–89%), but this did not differ across groups,  $F(2, 17) = 0.31, p = .74$ . There was a group difference in the percentage of PV-expressing cells surrounded by a PNN,  $F(2, 17) = 6.16, p = .010$ . The standard-reared infants had a smaller percentage (6.6%) than the standard-reared juveniles (17.8%,  $p = .008$ ) whilst the MS infants were intermediate between the two (9.5%, MS18 vs SR28:  $p = .07$ ; MS18 vs SR18  $p = .65$ ).

#### 4.1.3. Infralimbic cortex

Representative photomicrographs for each measure and for each group are shown in Fig. 6A. The groups differed in both the density of PV cells,  $F(2, 18) = 16.1, p < .001$  (Fig. 6B), and PNNs,  $F(2, 18) = 11.35, p = .001$  (Fig. 6C). Post hoc analysis showed that the group difference

was due to the SR28 juveniles expressing more PV-containing cells and more PNNs than either of the infant groups (all  $ps \leq .006$ ), which did not differ (smallest  $p = .78$ ). Likewise, density of PNNs around PV-labelled cells had a similar pattern across groups (Table S4). Once again, PNNs were found to preferentially surround PV-labelled cells (66–90%), and the groups did not differ,  $F(2, 18) = 2.82, p = .086$ . The percentage of PV-expressing cells with a PNN differed across groups,  $F(2, 18) = 7.66, p = .004$ . Both the standard-reared infant group (8.8%) and the MS infant group (7.1%) had a lower percentage than the standard-reared juveniles (17.51%, smallest  $p = .025$ ).

#### 4.1.4. Across regions

As for the analyses across regions in males, we used linear mixed models to evaluate the relationship between PV and PNNs variables with respect to condition (SR18, SR28, MS18) and region with rat as a random effect. The full outcomes of each model are included in the supplementary materials. Table 2 shows the outcomes of the fixed effects and the post hoc comparisons with holm's corrections for the condition effects. As in males, there was no statistical evidence that MS18 animals differed from SR18 animals on density of PV-labelled cells, PNNs, PV-labelled PNNs, non-PV-labelled PNNs, percentage of PNNs around PV-labelled cells, nor percentage of PV cells with a PNN when these measures were examined across the basolateral amygdala, prelimbic, and infralimbic cortex in females. While developmental effects were observed in standard-reared animals for some measures, the MS18 animals did not show any evidence of accelerated maturation.

### A Female Prelimbic Cortex

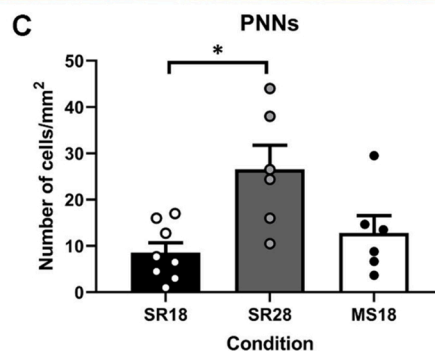
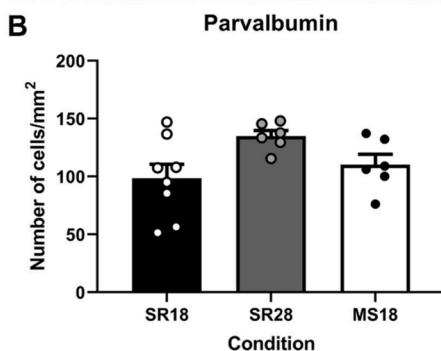
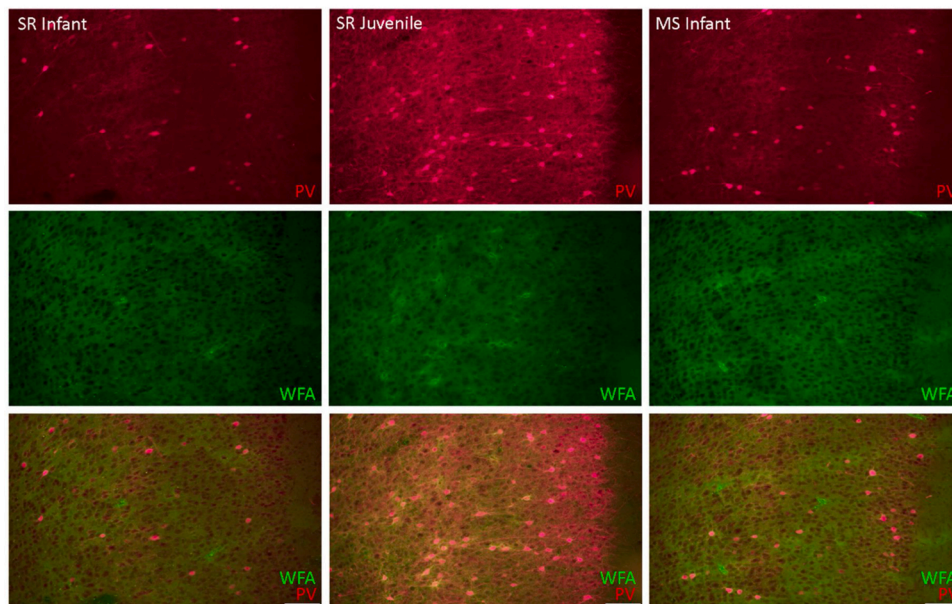
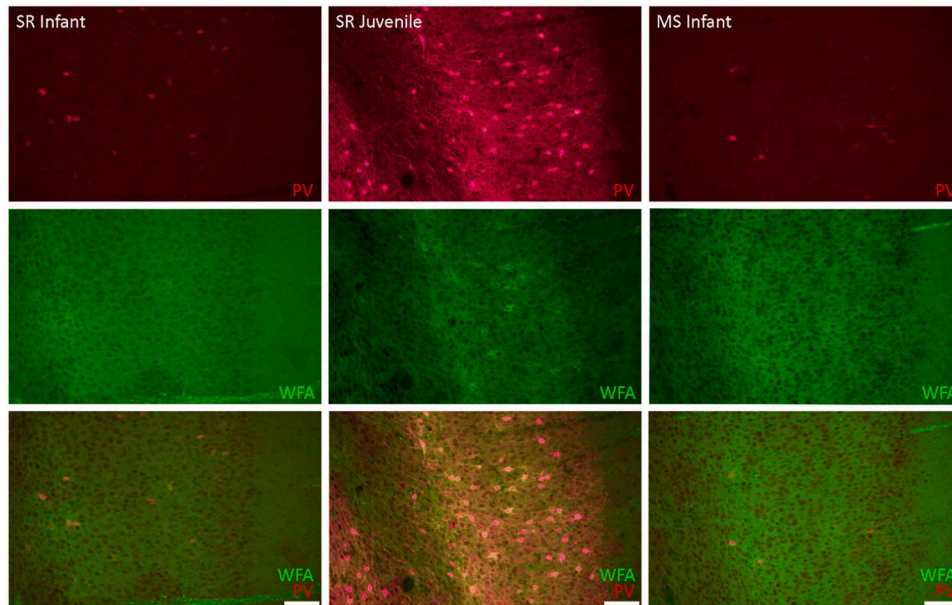
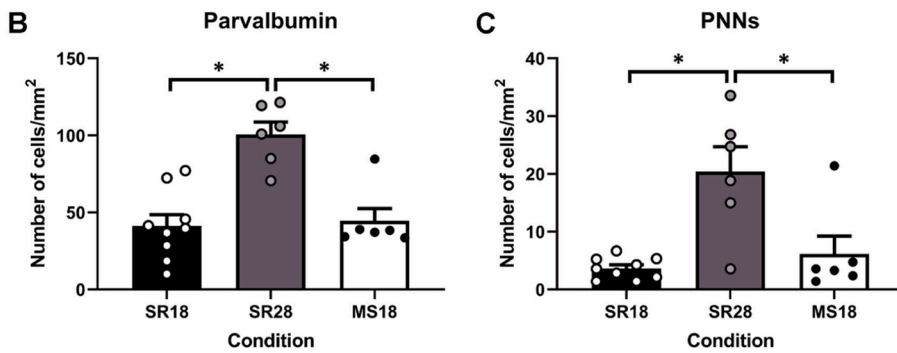


Fig. 5. (A) Representative photomicrographs of PV (top row), WFA (middle row) and combined (bottom row) immunoreactivity in the prelimbic cortex of female rats. Columns are separated into different conditions: standard-reared infants (SR18, left), standard-reared juveniles (SR28, middle) and maternally-separated infants (MS18, right). Scale bars = 100um. (B) Mean (+SEM) density of parvalbumin-labelled cells and (C) mean (+SEM) density of perineuronal nets, both with individual dot points. Group sizes: SR18 = 8, SR28 = 6, MS18 = 6. (\*) indicates significant differences ( $p < .05$ ).

**A Female Infralimbic Cortex**



**Fig. 6.** (A) Representative photomicrographs of PV (top row), WFA (middle row) and combined (bottom row) immunoreactivity in the infralimbic cortex of female rats. Columns are separated into different conditions: standard-reared infants (SR18, left), standard-reared juveniles (SR28, middle) and maternally-separated infants (MS18, right). Scale bars = 100um. (B) Mean (+SEM) density of parvalbumin-labelled cells and (C) mean (+SEM) density of perineuronal nets, both with individual dot points. Group sizes: SR18 = 9, SR28 = 6, MS18 = 6. (\*) indicates significant differences ( $p < .05$ ).



**Table 2**  
Linear Mixed Model Fixed Effects for Females.

Measure	Effect	F	df	p	Post hoc comparisons with Holm's correction p		
					SR18 v SR28	MS18 v SR28	SR18 v MS18
PV	Condition	9.35	2, 19	.001	.002	.009	.45
	Region	52.91	2, 35.9	< .001			
	Condition x Region	4.59	4, 35.9	.004			
PNNs	Condition	11.07	2, 19	< .001	< .001	.008	.24
	Region	6.72	2, 35.7	.003			
	Condition x Region	2.07	4, 35.7	.11			
PV + PNNs	Condition	10.40	2, 18.8	< .001	< .001	.008	.32
	Region	7.09	2, 35.5	.003			
	Condition x Region	3.04	4, 35.5	.030			
Non-PV PNNs	Condition	2.88	2, 19.2	.081			
	Region	1.82	2, 36.7	.18			
	Condition x Region	0.78	4, 36.7	.54			
% PNNs around PV cells	Condition	2.33	2, 54	.11			
	Region	2.30	2, 54	.11			
	Condition x Region	0.76	4, 54	.56			
% PV + with PNNs	Condition	8.21	2, 18.3	.003	.002	.027	.27
	Region	0.48	2, 35.2	.62			
	Condition x Region	1.04	4, 35.2	.40			

Note. PNNs = perineuronal nets, PV = parvalbumin, df = degrees of freedom using satterthwaite method, SR18 = standard-reared 18-day old infants, MS18 = maternally-separated 18-day old infants, SR28 = standard-reared 28-day old juveniles.

**5. Discussion**

In past work we reported that infant rats exposed to early-life adversity (modelled by the maternal separation procedure) exhibit an

earlier maturation of long-lasting memory and relapse-prone extinction (i.e., the adult phenotype rather than the infant phenotype of infantile amnesia and relapse-resistant extinction; Callaghan and Richardson, 2011, 2012; also see Peng et al., 2019). Here we examined a specific

prediction of the stress acceleration hypothesis (Callaghan and Tottenham, 2016) in regards to these findings by testing whether infants exposed to maternal separation exhibit an accelerated maturation of two neural structural elements thought to be involved in long-term memory and relapse-prone extinction: PNNs and PV-containing neurons. We assessed this prediction in two areas of the brain thought to be involved in memory and extinction: the basolateral amygdala and the prefrontal cortex (both the prelimbic and the infralimbic sub-regions). Further, we tested this prediction in both males (Experiment 1) and females (Experiment 2). As can be seen in Table 3, the results consistently failed to support our prediction that MS leads to accelerated maturation of PNNs and/or PV-expressing cells. Nor did we observe any effects of MS on the percentage of PV cells surrounded by PNNs.

Our primary hypothesis concerned PNNs, which have been shown to be involved in long-term memory in adult rodents (e.g., Fawcett et al., 2019; Shi et al., 2019). While we observed a striking effect of age on the density of PNNs, in both standard-reared males and females in the basolateral amygdala, prelimbic cortex, and infralimbic cortex, we failed to observe a single instance where the MS18 group differed from the SR18 group, contrary to our predictions. A different approach to testing for accelerated maturation would be to compare the MS infants to the SR juveniles. In those comparisons, the MS18 females failed to differ from the SR28 females as to the density of PNNs in both the basolateral amygdala and the prelimbic cortex, offering some support for accelerated maturation of PNNs, at least in the females. However, the subsequent cross-region analyses (see Table 2) detected a significant difference between these groups, indicating that MS did not lead to an accelerated maturation of PNNs across the regions examined. Inspection of the respective figures shows that this discrepancy in the statistical outcomes is likely due to the small sample size for the prelimbic cortex, as the two infant groups are very similar, and a single animal in the MS18 group having a high density of PNNs; see Fig. 5C). In contrast, the density of PNNs in the female basolateral amygdala does appear to be intermediate between that of the infant and juvenile standard-reared groups (Fig. 4C). That is, one might take this as some evidence of accelerated maturation of PNNs in the basolateral amygdala, at least in females, following early-life adversity. However, on the whole the

evidence presented here clearly does not provide support for early life adversity, at least as modelled by MS, accelerating the maturation of PNNs in either males or females, in the basolateral amygdala, infralimbic, or prelimbic cortex.

By examining the normative developmental trajectory of PNNs in the basolateral amygdala and two sub-regions of the prefrontal cortex from infancy to the juvenile period, these results extend our recent work on the maturation of PNNs in these regions (Baker et al., 2017). In that study we showed, in male rats, that there was a notable increase in the number of PNNs in the PL and IL from the juvenile stage to adolescence and from adolescence to adulthood in the basolateral amygdala. Here, we show that in the standard-reared animal, both males and females, there is an increase in PNN density from infancy to the juvenile period as well, replicating previous reports of this maturation in the basolateral amygdala in both male and female rodents (Umemori et al., 2015) or males only (Gogolla et al., 2009). In contrast, there is a dearth of literature on the maturation of PNNs in the medial prefrontal cortex earlier than the juvenile period and our findings that PNNs increase in both the prelimbic and infralimbic cortex from infancy to juvenility is novel.

We failed to find any evidence in support of our secondary and tertiary hypotheses that early-life adversity would increase the percentage of PV-expressing cells with a PNN in the MS infants relative to the standard-reared infants (see Table 3) and/or alter the density of PV cells. In both the prelimbic and infralimbic cortex, the standard-reared infants had a lower percentage of PV-expressing cells with a PNN than the standard-reared juveniles, and the MS infants were similar to their age-matched standard-reared counterparts in both prefrontal regions, regardless of sex. Generally, and as confirmed by the cross-region analyses, MS infants also had smaller percentages of PV-expressing cells with a PNN; only in the female prelimbic region did this comparison not reach statistical significance ( $p = .07$ ). The higher percentages of PV cells with PNNs in juveniles than infants in the prefrontal cortex suggests these inhibitory cells are maturing, yet, MS is not affecting this process. A clear developmental effect was also evident in the density of PV cells increasing in the infralimbic cortex from infancy to the late juvenile period. This was observed in both females and males. Importantly, there was, once again, no evidence of any effect of early-life adversity on this

**Table 3**  
Summary of the results.

Experiment 1 - Males		PV+	PNNs	PV-labelled PNNs	% PNNs around PV cells	% PV+ with PNNs
Basolateral Amygdala	age	no	yes	no	no	no
	rearing	no	no	no	no	no
Prelimbic Cortex	age	no	yes	yes	no	yes
	rearing	no	no	no	no	no
Infralimbic Cortex	age	yes	yes	yes	yes	yes
	rearing	no	no	no	no	no
Across regions	age	yes	yes	yes	yes	yes
	rearing	no	no	no	no	no
Experiment 2 - Females		PV+	PNNs	PV-labelled PNNs	% PNNs around PV cells	% PV+ with PNNs
Basolateral Amygdala	age	no	yes	yes	no	maybe
	rearing	no	no	no	no	no
Prelimbic Cortex	age	no	yes	yes	no	yes
	rearing	no	no	no	no	no
Infralimbic Cortex	age	yes	yes	yes	no	yes
	rearing	no	no	no	no	no
Across regions	age	yes	yes	yes	no	yes
	rearing	no	no	no	no	no

Note. An “age” effect means that the SR28 group was statistically greater than the SR18 group, and a “rearing” effect means that the MS18 group was statistically greater than the SR18 group (i.e., exhibited accelerated maturation).



measure. Given that the number of PV cells in the prelimbic and infralimbic areas in standard-reared juveniles is comparable to that seen in standard-reared adults (Baker et al., 2017), our results suggest that the emergence of adult-like numbers of PV cells, and hence mature inhibitory function to support cognitive function, may be more delayed in the infralimbic than the prelimbic region.

We consistently observed that PNNs preferentially surrounded PV cells, across regions and sex, and there was no indication that early-life adversity shifted this preference. It may have been difficult to detect an accelerated maturation due to early-life adversity on this measure because there was a developmental increase in the predominance of PNNs being around PV cells only in the infralimbic cortex of males. In the basolateral amygdala and prelimbic cortex, in both sexes, the percentage of PNNs around PV was similar across age and rearing conditions, despite the density of PNNs increasing in each region with maturation from infancy to juvenility. When interpreted with the measures of density of PNNs co-labelled for PV or not, such findings suggest that PNNs are predominantly forming around cells high in PV protein expression (and hence detected by immunoreactivity) but more subtly around cells low in PV (which may not be detected by immunoreactivity), or which do not express PV. Nonetheless, an increase in PNNs around cells low in PV (or which do not express PV) was only detected to a statistically significant level in the male, but not female, infralimbic cortex. Overall, a shift in percentage of PV-labelled PNNs is perhaps not the most sensitive measure of PNN maturation in the basolateral amygdala or prefrontal cortex from infancy to the juvenile period.

Taken together, these results indicate that the observed effects of MS on memory maturation and the faster transition to relapse-prone extinction (i.e., Callaghan and Richardson, 2011, 2012) are likely not mediated by either of these structural elements. Although not designed to test the accelerated maturation hypothesis of early-life adversity the results of a recent study by Santiago et al. (2018) also fail to provide support for this hypothesis. In that study rats were exposed to a different form of early-life adversity than was employed here – the “limited bedding” model. Santiago et al. predicted that exposure to adversity early in life would decrease both PNNs and PV-expressing cells in the basolateral amygdala, thereby reducing inhibition of the amygdala which would lead to increases in threat-related responding in the infant. When comparing across infancy (i.e., 12, 15, and 18 days old) exposure to early-life adversity decreased, rather than increased, rate of maturation of PV-expressing cells (in terms of intensity) and of PNNs (in terms of density) in the basolateral amygdala. We did not observe a decrease in the density of either PNNs or PV-expressing cells in the basolateral amygdala of our MS infants (we did not measure intensity). The results of Santiago et al. clearly do not support the prediction made by the accelerated maturation hypothesis and rather suggest the possibility of some delay in the maturation in the basolateral amygdala, at least with the limited bedding model of early life adversity.

Early-life adversity may have effects on PNNs or PV + cells at different ages than tested in our study. For example, in a recent study Gildawie et al. (2020) reported a complex pattern of region- and sex-specific effects of early-life adversity (modelled by MS) on both PNNs and PV + cells in the basolateral amygdala and the medial prefrontal cortex (i.e., the prelimbic and the infralimbic regions) of rats. That study was not specifically designed to assess the acceleration maturation hypothesis and involved different age groups than the current study (i.e., rats at 20, 40, and 70 days of age were assessed). A large, complex set of results were reported by Gildawie et al., but in terms of PNN density, they reported a decrease following MS in the 20 day-old animals, in both males and females, in the prelimbic region (with no differences being seen at that age in the infralimbic region and the basolateral amygdala). We did not observe such a decrease in the prelimbic region, but did employ a different MS procedure (i.e., 3 h per day from P2-P14 while Gildawie et al. did 4 h per day from P2-P20), and also tested a slightly younger age group (i.e., P18 rather than P20).

In another recent study, again, not explicitly designed to assess the

stress acceleration hypothesis, Guadagno et al. (2020) reported a number of effects of early life adversity. Of most direct relevance to the current study, Guadagno and colleagues reported that early life adversity increased the density of PV-labelled interneurons with a PNN, an effect that we did not observe. However, the two studies differ on a number of characteristics. For example, Guadagno et al. used the limited bedding model of early-life adversity while we used the maternal separation model. In addition, Guadagno et al. found this effect only in the right basolateral amygdala, whereas our study was not designed to explore hemispheric differences (i.e., we preferentially counted cells from the left hemisphere). Also, Guadagno et al. tested rats that were P22–28, and collapsed them into a single group (i.e., the study was focused on assessing potential effects of early life adversity rather than testing the stress acceleration hypothesis). In any case, future research will have to determine the reasons for the differences in the results between these various studies, but it is clear that none of these studies, including ours, provide support for the conclusion that early-life adversity leads to an accelerated maturation of PNNs in the basolateral amygdala or the medial prefrontal cortex, at least with the specific measures assessed.

In this study we focused our analyses on the density of PNNs and PV cells and did not evaluate the complexity of PNN structure nor the intensity of staining of PNNs and PV. Perineuronal nets in the rat cortex have been qualitatively described as transitioning from faint, diffuse (or “fuzzy”) structures around the cell body in the first and second postnatal weeks (i.e., by P14) to net-like structures clearly ensheathing the cell body, axon initial segments as well as proximal parts of dendrites by juvenility (i.e., by P21), before extending even further around the distal parts of dendrites in adolescence (i.e., by P35) to resemble adult-like structures (Köppe et al., 1997). Our observations of PNNs in the basolateral amygdala, prelimbic, and infralimbic cortices from SR18 infants to SR28 juveniles is in agreement with those previous reports; PNNs primarily appeared as fuzzy perisomatic sheaths in infants, regardless of rearing condition, but ensheathed more of the proximal dendrites in juveniles (see examples in Figs. 1B, 2 B, and 3B). However, detailed analysis of PNN complexity would best be done with images obtained with a confocal microscope. Such analyses may reveal subtle, yet important, structural changes in PNNs which may impact their function as regulators of plasticity.

In terms of PV-labelled interneurons, our results very clearly show that MS had no impact, at least in terms of a density measure. However, there is some evidence from studies of the hippocampus that low-PV and high-PV expression states of PV-circuits are associated with different plasticity levels in adult male mice after experiences such as learning and environmental enrichment, and that adults have a higher proportion of PV neurons in high-PV expression states than infants (Donato et al., 2013). Based on such findings, it could be the case that if early-life adversity did accelerate maturation of the cells, maternally-separated infants would have an increased ratio of high to low PV expression relative to standard-reared infants. Yet, MS has the opposite effect on the intensity of PNNs and PV-expression in the adult mouse ventral hippocampus (Murthy et al., 2019), suggesting that PV-expression states may not be associated with long-term behavioral consequences of MS.

Although the results reported here do not provide support for our predictions, or the stress acceleration hypothesis, as noted in the Introduction we have recently reported results with a functional measure (i.e., pMAPK expression following either fear expression or fear extinction) that supports this hypothesis (also see Callaghan et al., 2019). In addition, a precocial maturation of adolescent basolateral amygdala to infralimbic cortex anatomical connectivity has been detected in rats exposed to early-life adversity, with females affected earlier than males (Honeycutt et al., 2020), providing evidence for adversity-driven effects on corticolimbic circuitry later in development. Given that accelerated maturation of basolateral amygdala to infralimbic cortex connectivity was correlated with anxiety-like behavior, and preceded disruptions in functional resting-state connectivity in late adolescence in females,

precocious maturation may come at the expense of an overall disruption in the stability of the functional basolateral amygdala-prefrontal cortex network as it develops (Honeycutt et al., 2020). The antecedents of such effects remain unknown. Determining the neural effects, both structural and functional, of early-life adversity will clearly be important in providing further insights into how these experiences impact on mental and physical health as well as insights into how to either treat or prevent their negative consequences.

### CRedit authorship contribution statement

**Rick Richardson:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing. **Jeremy Bowers:** Methodology, Data curation, Formal analysis. **Bridget L. Callaghan:** Conceptualization, Writing - review & editing. **Kathryn D. Baker:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing - review & editing.

### 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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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.dcn.2020.100905>.

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