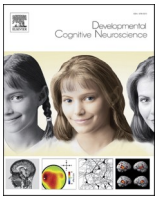
Developmental Cognitive Neuroscience 41 (2020) 100737

Contents lists available at ScienceDirect

Developmental Cognitive Neuroscience

journal homepage: www.elsevier.com/locate/dcn



Variation in early life maternal care predicts later long range frontal cortex synapse development in mice

A. Wren Thomas [a], Kristen Delevich [b,c], Irene Chang [b], Linda Wilbrecht [b,c,*]

[a] Helen Wills Neuroscience Graduate Program, University of California, Berkeley, CA, 94720, USA
[b] Department of Psychology, University of California, Berkeley, CA, 94720, USA
[c] Helen Wills Neuroscience Institute, University of California, Berkeley, CA, 94720 USA

ARTICLE INFO

Keywords:
Adolescence
Frontal cortex
Amygdala
Stress
Synapse
Pruning

ABSTRACT

Empirical and theoretical work suggests that early postnatal experience may inform later developing synaptic connectivity to adapt the brain to its environment. We hypothesized that early maternal experience may program the development of synaptic density on long range frontal cortex projections. To test this idea, we used maternal separation (MS) to generate environmental variability and examined how MS affected 1) maternal care and 2) synapse density on virally-labeled long range axons of offspring reared in MS or control conditions. We found that MS and variation in maternal care predicted bouton density on dorsal frontal cortex axons that terminated in the basolateral amygdala (BLA) and dorsomedial striatum (DMS) with more, fragmented care associated with higher density. The effects of maternal care on these distinct axonal projections of the frontal cortex were manifest at different ages. Maternal care measures were correlated with frontal cortex → BLA bouton density at mid-adolescence postnatal (P) day 35 and frontal cortex → DMS bouton density in adulthood (P85). Meanwhile, we found no evidence that MS or maternal care affected bouton density on ascending orbitofrontal cortex (OFC) or BLA axons that terminated in the dorsal frontal cortices. Our data show that variation in early experience can alter development in a circuit-specific and age-dependent manner that may be relevant to understanding the effects of early life adversity.

1. Introduction

Adolescence is transitional period between childhood and adulthood that is marked by striking changes in neural circuits and behavior. The frontal cortices develop at this time and are considered the last regions of the brain to mature. Within the frontal cortices, different circuits show different developmental trajectories (Delevich et al., 2019). While dendritic spine density and overall excitatory synapse density decreases, a subset of frontal connections exhibit growth in axonal length and gain in density of synapses during adolescence (humans and non human primates:Huttenlocher and Dabholkar, 1997; Petanjek et al., 2011; Anderson et al., 1995; Bourgeois et al., 1994; mice: Johnson et al., 2016a, 2016b; Johnson et al., 2016a, 2016b; multiple species: Delevich et al., 2019). In a striking example, it has been observed in rats and mice that axonal projections from the frontal cortices (along the medial wall) to the basolateral amygdala (BLA) and from the BLA to the frontal cortices grow dramatically. This growth includes increases in axonal innervation and gain of new synapses during the adolescent to adult transition (Arruda-Carvalho et al., 2017; Cunningham et al., 2002; Johnson et al., 2016a, 2016b; Landers and Sullivan, 2012; but see Cressman et al., 2010). Studies in humans also find that frontal-BLA connectivity is remodeled during late childhood and adolescence (Gee et al., 2013). Others show frontal cortico-striatal connectivity is also strengthened at this time (Larsen et al., 2017; van den Bos et al., 2012). Dopaminergic axons also increasingly innervate the frontal cortices into young adulthood in rats and mice (Benes et al., 2000; Cunningham et al., 2002; Hoops et al., 2018; Reynolds et al., 2018), and inhibitory synapses onto subtypes of frontal cortical neurons are also remodeled in rats and mice (Piekarski et al., 2017a, 2017b; Tseng and O'Donnell, 2006; Vandenberg et al., 2015).

It is currently unclear why these specific circuits develop late relative to others in the brain. Theoretical work on the evolution of plasticity suggests developmental plasticity, particularly plasticity that accompanies an extended process of maturation and/or physical differentiation, may emerge to support adaptation to an individual's specific environment (Nettle and Bateson, 2015; Hostinar and Gunnar, 2013;

* Corresponding author at: Department of Psychology, University of California, Berkeley, CA, 94720, USA.
E-mail address: wilbrecht@berkeley.edu (L. Wilbrecht).

https://doi.org/10.1016/j.dcn.2019.100737
Received 25 April 2019; Received in revised form 3 November 2019; Accepted 18 November 2019
Available online 20 November 2019

Panchanathan and Frankenhuis, 2016; Mabry and Stamps, 2008; Frankenhuis and Walasek, in press). In this adaptive theoretical framework, cues sampled from the environment may be used to inform development during a sensitive window. Changes may occur immediately, directly in response to the cue experience, but also may extend to longer term qualitative and/or quantitative changes in developmental program trajectory (Nettle and Bateson, 2015; Panchanathan and Frankenhuis, 2016; Frankenhuis and Walasek, in press). For example, it has been proposed that individuals that experience cues that indicate scarcity or uncertainty in the environment may speed the trajectory of development to enhance genetic fitness (Belsky et al., 2010; Rickard et al., 2014; Hostinar and Gunnar, 2013; Bath et al., 2016).

Whether we adopt an adaptive theoretical framework or interpret experience dependent changes in response to harsh environments as pathological, it is important to understand what factors in early life affect short and long term brain development. Early life adversity is associated with increased risk for the development of a number of physical and mental health conditions including depression and anxiety disorders, substance abuse (Felitti et al., 1998; McLaughlin et al., 2012) and may also drive differences in executive function (Lovic and Fleming, 2004; Buss et al., 2010; Sheridan and McLaughlin, 2014). Particular inspiration for our study comes from work showing that early institutional care in children (followed by adoption) alters the development of connectivity between the frontal cortices and the amygdala (Gee et al., 2013), which is a circuit central to affective processes, addiction as well as flexible learning and updating.

Here we tested how an early life maternal separation (MS) paradigm (Parfitt et al., 2004; Romeo et al., 2003; Thomas et al., 2016) impacts the trajectory of development of synapse density on long range axons that emerge from the soma of neurons in the dorsal frontal cortex and project to the BLA and dorsomedial striatum (DMS) in mice. We also investigated ascending axons that emerge from soma in the BLA and orbitofrontal cortex (OFC) and target layer 2/3 of the dorsal frontal cortex in mice.

We first justify the choice of MS as a model of early life adversity and then justify the choice of circuit focus. MS is a paradigm that has been used for decades to model early life adversity by interrupting continuous access to nourishment and care provided by the dam to the pup (Francis et al., 1999; Hofer, 1994). Separation from the dam for some parts of the day is likely to occur in the wild, and may vary depending on richness of the foraging environment. It has been pointed out that dam behavior in response to environmental variables or treatments like MS may also serve as an informative signal about the type of environment which offspring will soon enter (Baram et al., 2012). A number of studies have shown that MS in rodents leads to both immediate and long-term effects on the hypothalamic-pituitary-adrenal axis and anxiety-like behavior (Huot et al., 2002; Ladd et al., 2004; Nishi et al., 2013; Plotsky and Meaney, 1993; Wigger and Neumann, 1999). The MS experiments we present here follow up from a previous behavioral study from our lab, in which we found that mice that experienced MS during development were less flexible than controls in a reversal learning task when tested in adolescence but not in adulthood (Thomas et al., 2016). Mice that experienced MS as pups also consumed more alcohol in adulthood than controls (Thomas et al., 2016). To remain consistent with our previous work we chose to use an MS paradigm from P1-10 in the current study and to focus on male mice. To connect the current data to other early life adversity work on high and low care rodent dams (Caldji et al., 1998; Liu et al., 1997) and the effects of limited nesting material (Rice et al., 2008), we also added quantification of maternal care to our experiments. This allowed us to test if variation in maternal care, as a continuous variable, sculpted later anatomical measures from developing circuits. This work should therefore help to interpret and translate work with mice and the MS paradigm to other rodent models of early life adversity.

We choose to study frontal cortex projections to the BLA and DMS due to their relevance to mental health and addictive behavior, and their importance for learning and flexible updating (Haber and Behrens, 2014) (relevant to our previous behavioral study Thomas et al., 2016). The frontal cortex and its amygdala and striatum projections are considered core nodes in the heuristic 'Triadic model' of adolescent brain development (Ernst, 2014) and these hubs have been highlighted as sensitive to early life stress (Fareri and Tottenham, 2016). Early life and chronic adult stress are known to affect spine density within the BLA and frontal cortices in animal models (Vyas et al., 2002, 2006; Bock et al., 2005; Chocyk et al., 2013; Koe et al., 2016; Monroy et al., 2010; Muhammad et al., 2012; Ng et al., 2018; Pascual and Zamora-León, 2007). Also, dendritic spines in the frontal cortex region in which we seed our virus injection have been shown to be gained and lost with fear learning and extinction, respectively (Lai et al., 2012). Here we move beyond dendritic spines to observe how the structural connectivity between these regions, manifest by synaptic boutons on long range axons, are affected by MS. These data can inform human MRI based connectivity studies with high resolution information about synaptic structural connectivity and inspire future behavioral, electrophysiological and/or pharmacological interventions.

2. Methods

2.1. Animals

Male C57Bl/6 *Mus musculus* (lines originally obtained from Charles River) were used for this study. Dams and sires were housed in pairs throughout the breeding and rearing period. At postnatal (P) day 21, experimental mice were weaned and group housed in same-sex cages, 2–5 per cage. All cages were kept on a 12/12 reverse light dark cycle (lights off at 10 AM). All animals received nesting material and paper huts in their home cage. All procedures were approved by the UC Berkeley Animal Care and Use Committee.

The number of mice used in each experiment are reported in results as the N = number of mice. Note that multiple axon segments were quantified for each mouse and are noted as n = number of axon segments. The average linear distance of axon sampled in each mouse for the descending projections from the frontal cortices was $M = 459.05$ microns, SD = 23.14 per mouse and did not differ significantly between time points or groups. The average linear distance sampled in each mouse for the ascending projections from the BLA and OFC was $M = 593.81$, SD = 145.81.

The number of litters sampled by projection type, treatment and age group was the following: Frontal → BLA (Adolescent: MS = 3 litters, Control = 4 litters; Adult: MS = 6 litters, Control = 3 litters), Frontal → DMS (Juvenile: MS = 3 litters, Control = 4 litters; Adult MS = 4 litters, Control = 4 litters), BLA → Frontal (MS = 8 litters, Control = 7 litters) and OFC → Frontal (MS = 8 litters and Control = 6 litters).

2.2. Maternal separation (MS)

From P1 to 10, pups from the MS group were removed daily from their home cage for 3 h from 11:30 AM to 2:30 PM. During the 3 h separation, pups were kept in a clean cage placed on an electric heating pad. MS pups were separated from each other by dividers and thus could hear and smell each other, but not touch during the separation. The control group stayed in the home cage with the dam and the litter was not handled.

To better understand our MS manipulation and enhance potential for comparison to high low care rat studies and an alternate mouse model of early life adversity that provides dams with limited nesting material (LNM) (Rice et al., 2008), we also quantified maternal care in MS and control dams. It has been shown that the LNM manipulation leads to greater fragmentation of care, indicated by an increase in the number of sorties, instances when the dams leave the nest (Rice et al., 2008).

2.3. Maternal care quantification

Maternal care monitoring 1 (P1-P10): Maternal behavior was monitored in both control (n = 18) and MS (n = 15) cages every other day from P1-P10, for a total of 5 monitoring days. Observations were sampled from three periods of the day during the subjective dark phase (11 AM, 2:30 PM and 5 PM) under infrared light. Within these times, cages were monitored for 30 min, in which dam behavior was scored every other minute (15 min. total of observation per session). The amount of time the dam spent on the nest and the number of sorties (instances the dam left the nest) were scored rounding to full seconds. Nests were built up into dome-like structures, which have been described in detail in other studies assessing the C57Bl/6 strain (Millstein and Holmes, 2007; Rice et al., 2008). These domes typically occluded behavior in the nest, thus more detailed maternal behaviors such as arched-back nursing and licking and grooming could not be scored without disruption.

Maternal care monitoring 2 (P16-P20): Maternal and pup behavior was also monitored just before weaning from P16-P20 in both control (n = 16) and MS (n = 14) cages in order to understand whether maternal care was affected beyond the MS manipulation period. Additionally, mounts of the pups by the dam have been observed near weaning age and is thought to drive weaning (Franks et al., 2015). We hypothesized that MS litters would experience more mounts by the dam. P16-20 observations were made on the same schedule and litters as those monitored as P1-10, except the number of litters monitored during this period differed from the earlier monitoring period due to errors in data collection. Cages were observed at 11 AM, 2:30 PM and 5 PM over 3 days. Here we marked each observation minute as simply positive or negative for dam sortie, pup sortie, all pups in nest and instances of dam mounting pups. The maximum possible score for each item (in P16-20 monitoring) was therefore 15 min x 9 sessions = 135.

2.4. Viral injections

Stereotaxic viral injections were performed under isoflurane anesthesia. In the first cohort of mice, both juvenile (P21) and adult (P67-75) mice were injected with a Nanoject II injector (Drummond Scientific Company, Broomall, PA) to deliver 50 nl of AAV2/1-CAG-eGFP (UNC Vector Core) to the left frontal cortex, (juvenile: AP + 2.6 mm, ML + 1.0 mm, DV 0.5 mm relative to bregma; adult: AP + 2.7 mm, ML + 1.0 mm, DV 0.5 mm relative to bregma). In the second cohort, juvenile (P21) mice were injected with 50 nl of AAV2/1-CAG-eGFP or AAV2/1-CAG-Tdtomato to both the left lateral OFC (AP + 2.6 mm, ML + 1.65 mm, DV 2.1 mm) and bilateral BLA (AP -1.0 mm, ML +/- 3.2, DV 4.25; reporter virus was counterbalanced between brain regions). Before surgery, mice were given an analgesic (10 mg/kg of meloxicam). Dosing was repeated twice post-surgery at 24 h intervals and mice were monitored for health and weight gain.

Nomenclature for mouse frontal and prefrontal cortex varies greatly across studies and species (Carlén, 2017; Laubach et al., 2018). Here, we use frontal cortex to describe the agranular dorsal and rostral frontal regions collectively. The seed region for descending axons in cohort 1 is called frontal association area (FrA) in the Paxinos and Franklin, 2012. Our sampling of afferent axons that target the frontal cortices in cohort 2 extended in the rostral and medial direction including secondary motor (M2) and cingulate regions (Cg) regions.

2.5. Histology

Mice were transcardially perfused at a mid-adolescent time point or in adulthood with 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Perfusion age was P35 for all data in Fig. 2 and P28 for data in Fig. 4. Perfusion age was P81-89 for data in Fig. 3. Brains were extracted and post-fixed in 4% paraformaldehyde overnight and then placed in PB. Coronal sections (200 μm) were cut on a vibratome and mounted on slides with Fluoromont-G (Southern Biotech). Sections that included the injection sites were stained with DAPI and checked for accuracy.

2.6. Microscopy

To image descending frontal axons, we used an Ultima IV laser scanning microscope (Bruker, Middleton, WI) and a 40 × 0.8 NA water immersion objective (Olympus, Center Valley, PA). A Mai Tai HP laser (Spectra physics, Santa Clara, CA) was tuned to 910 nm in order to excite GFP. We imaged axon segments (~40 microns in length) and obtained image stacks with a 1 μm z-step. To image ascending BLA and OFC axons that overlap in the L2/3 region of the dorsal frontal cortex (sampling regions labeled with GFP and td-tomato in the same mouse), we used a Zeiss LSM 710 laser scanning confocal microscope using 40 × 0.8 NA oil immersion objective. Axon length sampling was comparable across MS and control groups (Supplementary Table 1).

2.7. Image processing and analysis

Images were scored as median-filtered 3-dimensional z stacks. All images were analyzed blind to experimental group. Axonal boutons were scored based on established criteria from a consortium of imaging labs (Holtmaat et al., 2009) using custom Matlab software (Mathworks). Briefly, axonal boutons were scored if the intensity was more than 3 times as bright as the adjacent axon shaft, a conservative criterion established using imaging and electron microscopy (Holtmaat et al., 2009). Bouton density was calculated as the number of boutons, divided by the length of analyzed axon. We also looked at bouton volume, motivated by work that shows a strong relationship between bouton size and synaptic strength (Cheetham et al., 2014; Murthy et al., 2001). We used bouton brightness as a proxy for bouton volume, based on established criteria (Holtmaat et al., 2009). We measured the average intensity of the adjacent axon backbone and the bouton of interest and a nearby region of the background. Background subtracted bouton intensity was then divided by the background subtracted backbone intensity to get a background subtracted normalized intensity for the bouton of interest.

2.8. Statistics

All statistical comparisons were performed using GraphPad Prism 7 (GraphPad, San Diego, CA) and R (version1.1.463; R Foundation for Statistical Computing, Vienna, AT). Maternal care quantification data were tested for normality using D'Agostino and Shapiro-Wilk normality tests. Data that were normally distributed were analyzed using unpaired student's t-tests for group comparisons. Pearson's correlations were used to test the linear relationship between variables. Data that were not normally distributed were analyzed using the Mann-Whitney *U* test for group comparisons and non-parametric Pearson's correlations for linear relationship between variables. All normally distributed data are presented as means ± SEM. Data that were not normally distributed are presented as medians ± IQR.

Because axon bouton measures included segments imaged within and between subjects, data were analyzed by fitting a linear mixed model using residual maximum likelihood in the lme4 package (version 1.1–19) (Bates et al., 2015). The categorical variable treatment (MS or control) was included as the fixed effect and subject as the random effect, and data were analyzed separately by age and brain region. We inspected residual plots to determine whether they deviated from homoscedasticity or normality, and if they did, density data were square root transformed (DMS bouton density data in Fig. 2J-L and Fig. 4J-L). In the case of BLA bouton brightness square root transformation did not result in normally distributed residuals, so these data were transformed using the powerTransform function in the car package (version 3.0-2) (Fox et al., 2011).

The fit of different linear mixed models was compared by AIC score,

and p-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. P values for these analyses were determined using the car package (version 3.0-2). The r.squaredGLMM function of the MuMIn package (version 1.42.1) was used to calculate the conditional coefficient of determination explained by the model including both fixed and random effects (r_c). In Supplementary Fig. 1, post hoc pair-wise comparisons of mixed effect models were performed using the emmeans package (version 1.4). Statistical significance was $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3. Results

3.1. MS alters early maternal care

To measure how P1-10 MS impacts maternal care, we quantified the behavior of dams from both control and MS cages from P1-P10 (Fig. 1a). We recorded the amount of time dams spent over the nest and the number of sorties (instances when the dam left the nest) during each monitoring session. We found that dams experiencing the MS manipulation spent significantly more time over the nest compared to control dams during observations from P1-P10 [Sum of all observation sessions: Control: $M = 8865 \pm 346.4$, n = 18; MS: $M = 10{,}128 \pm 143.5$, n = 15; t = 3.139 df = 31, $P = 0.0037$] (Fig. 1b). Dams experiencing the MS manipulation made significantly more sorties than control dams [Sum of all observation sessions: Control: $M = 16.89 \pm 1.193$, n = 18; MS: $M = 22.93 \pm 2.345$, n = 15; t = 2.414 df = 31, $P = 0.0219$] (Fig. 1c). There was no difference between treatment groups in offspring weight at weaning (P21) [Control: $M = 10.92 \pm 0.2954$, n = 18; MS: $M = 10.6 \pm 0.3242$, n = 19, t = 0.7396 df = 35, $P = 0.4645$] (Fig. 1d).

We also measured maternal care just before weaning from P16-20. Maternal care measures during this period did not differ between control and MS mice [Dam out of nest: Control: $M = 59.88 \pm 5.491$, n = 16; MS: $M = 56.93 \pm 6.328$, n = 14, t = 0.3535 df = 28, $P = 0.7264$; Pups out of nest: Control: $M = 64.94 \pm 5.915$, n = 16; MS: $M = 60.5 \pm 5.073$, n = 14; t = 0.561 df = 28, $P = 0.5793$; All in nest: Control: $M = 69.63 \pm 6.353$, n = 16; MS: $M = 77.36 \pm 4.008$, n = 14; t = 0.9956 df = 28, $P = 0.3280$; Mounting behavior: Control: $Mdn = 0$ (0–2.5), n = 16; MS $Mdn = 0$ (0–2.25), n = 14, U = 106, $P = 0.7847$].

These observations indicate that MS (P1-10) impacts maternal care during early life (P1-10), but not dam/pup behavior that we observed in the later pre-weaning period (P16-20). During P1-10, MS altered both the quantity and quality of early care. We speculate that the greater amount of time MS dams spend over nest is a compensatory response, making up for missed maternal care bouts. Increases in maternal care after reunion with pups have been noted in other studies as well (Millstein and Holmes, 2007). The increase in sorties suggests MS has parallels to the limited nesting material model of adversity (Fig. 1e) (Rice et al., 2008).

3.2. At an adolescent time point, variation in maternal care shows a significant relationship with synapse density on frontal afferents that target the BLA

We next used a viral strategy to label frontal cortex axons with GFP in the MS and control group offspring (Fig. 2a,b) and examined innervation of two target regions, the BLA (Fig. 2c,d) and the dorsomedial striatum (DMS) (Fig. 2h,i). When comparing linear bouton density, we found that Frontal → BLA axons exhibit higher bouton density in MS mice compared to control mice at P35 [Control: $M = 0.1197 \pm 0.0121$ (n = 59

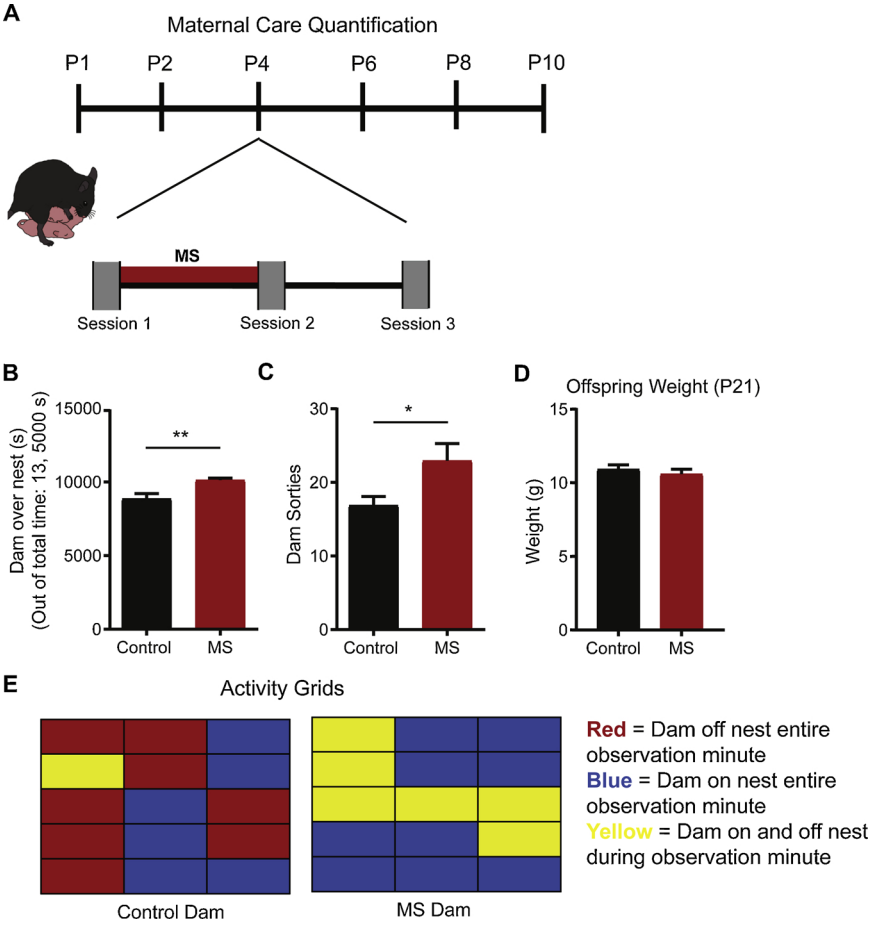


Fig. 1. MS dams displayed more time over nest and more sorties compared to control dams. A, Timeline of maternal care quantification and maternal separation. Cages were monitored for 5 days during the P1-P10 period. Each day of monitoring, cages were monitored 3 times a day (sessions shown in grey bars). MS offspring were separated from the homecage for 3 h (red bar). B, MS dams spent more time over the nest compared to control dams [Control: M = 8865 ± 346.4, n = 18; MS: M = 10,128 ± 143.5, n = 15; t = 3.139 df = 31, P = 0.0037]. **C**, MS dams displayed significantly more sorties compared to control dams [Control: M = 16.89 ± 1.193, n = 18; MS: M = 22.93 ± 2.345, n = 15; t = 2.414 df = 31, P = 0.0219]. **D**, MS and Control offspring did not differ in weight at P21 [Control: M = 10.92 ± 0.2954, n = 18; MS: M = 10.6 ± 0.3242, n = 19, t = 0.7396 df = 35, P = 0.4645]. E, Activity grids representing two example dams. Red squares = dam off nest entire observation minute, blue squares = dam on nest entire observation minute, yellow = mixed minute (dam on and off nest during observation minute). Bars represent mean ± SEM. *P < 0.05; **P < 0.01.

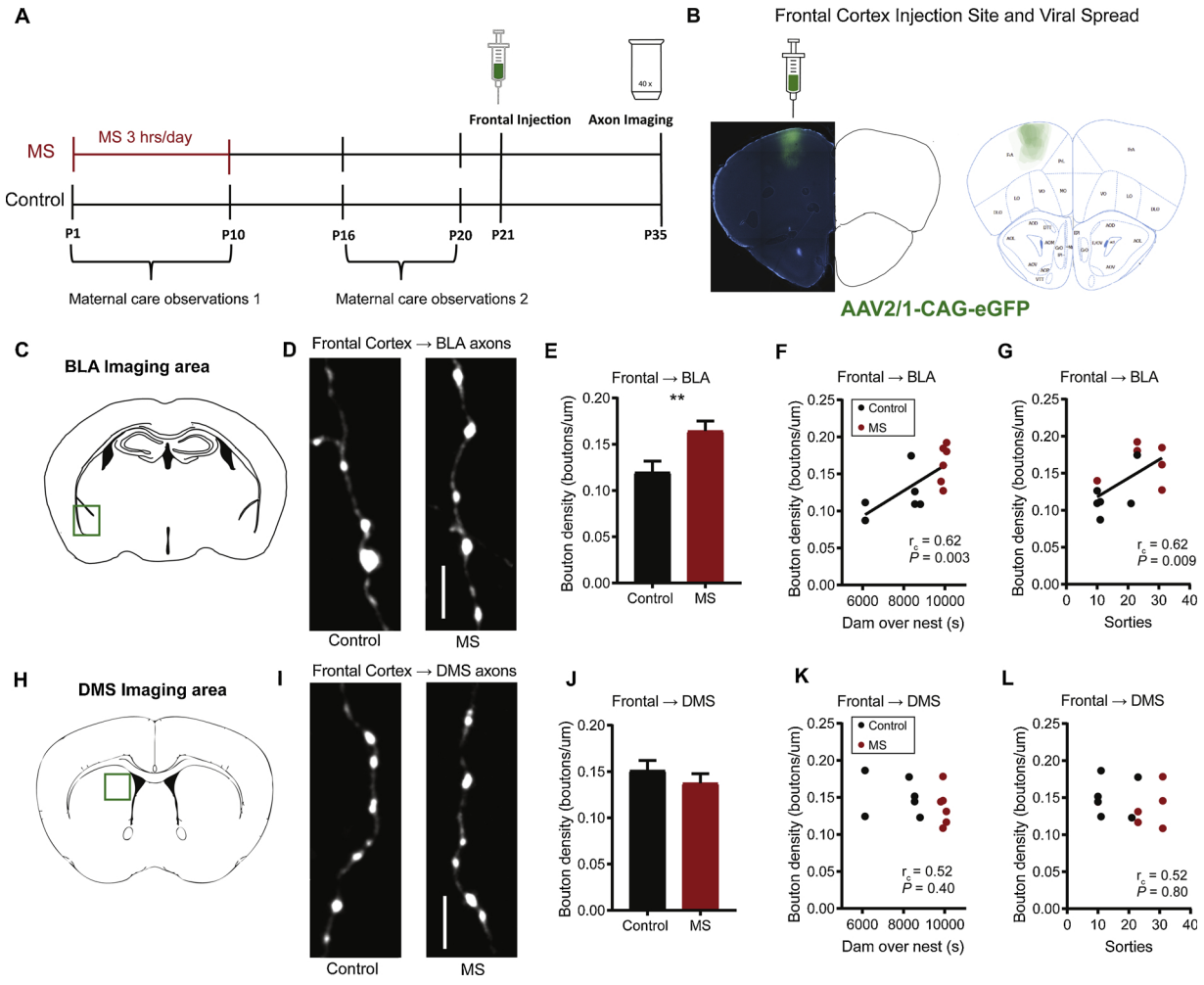


Fig. 2. Maternal care affects Frontal Cortex→BLA bouton development at P35.
A, Experimental Timeline. B, Frontal cortex injection site single example (left) and overlay of all injection histology (right). C, Schematic of Frontal → BLA imaging area. D, Frontal Cortex →BLA example axons (scale bar = 5 microns). E, MS mice had higher Frontal Cortex →BLA bouton density compared to controls at P35 [Control: $M = 0.1197 \pm 0.0121$ (n = 6) versus MS: $M = 0.1643 \pm 0.01069$ (n = 6) bouton density, $P = 0.0079$]. F, The amount of time dams spent over the nest in early life correlated with Frontal Cortex →BLA bouton density in adolescent offspring (rc = 0.6172, $P = 0.0059$), with higher time over nest associated with higher bouton density. G, Early life dam sorties were correlated with Frontal Cortex →BLA bouton density in adolescent offspring (rc = 0.6212, $P = 0.012$), with more sorties associated with higher bouton density. H, Schematic of Frontal Cortex→DMS imaging area I, Frontal Cortex →DMS example axons (scale bar = 5 microns). J, MS mice did not differ in Frontal Cortex →DMS bouton density compared to controls at P35 [Control: $M = 0.1512 \pm 0.01082$ (n = 6) versus MS: $M = 0.1374 \pm 0.01016$ (n = 6) bouton density; $P = 0.459$]. K, The amount of time dams spent over the nest did not correlate with Frontal Cortex→DMS bouton density in the adolescent mice (rc = 0.517, $P = 0.367$). L, The number of dam sorties did not correlate with Frontal Cortex →DMS bouton density in adolescent offspring (rc = 0.519, $P = 0.778$). Bars represent mean ± SEM. $^{**}P < 0.01$.

axon segments, N = 6 mice) versus MS: $M = 0.1643 \pm 0.01069$ (n = 61 axon segments, N = 6 mice) bouton density; $\chi^2(1) = 7.07$, $P = 0.0079$] (Fig. 2e). Furthermore, Frontal → BLA bouton density correlated with early maternal care measures. There was a positive correlation between Frontal → BLA bouton density and P1-10 time over nest ($\chi^2(1) = 7.58$, $P = 0.0059$; $r_c = 0.6172$) (Fig. 2f) and dam sorties ($\chi^2(1) = 6.25$, $P = 0.0124$; $r_c = 0.6212$) (Fig. 2g).

In contrast to Frontal → BLA axons, the density of boutons on Frontal → DMS axons (labeled by the same viral injection) did not differ between controls and MS mice in adolescence [Control: $M = 0.1512 \pm 0.01082$ (n = 60 axon segments; N = 6 mice) versus MS: $M = 0.1374 \pm 0.01016$ (n = 61 axon segments, N = 6 mice) bouton density; $\chi^2(1) = 0.55$, $P = 0.459$] (Fig. 2j). Also, Frontal → DMS axonal bouton density did not show any relationship with amount of time dam spent over nest ($\chi^2(1) = 0.81$, $P = 0.367$; $r_c = 0.517$) (Fig. 2k) or with dam sorties ($\chi^2(1) = 0.08$, $P = 0.778$; $r_c = 0.519$) (Fig. 2l).

3.3. Bouton volume on Frontal → BLA afferents correlates with a measure of maternal care

To further explore the nature of the differences in bouton density in the frontal cortex projection to the BLA in adolescence, we performed additional analyses of bouton volume using normalized and background subtracted fluorescent intensity as a proxy measure for bouton volume (Holtmaat et al., 2009). We did not find a significant effect of MS treatment on bouton brightness ($\chi^2(1) = 2.57$, $P = 0.109$), but 'time over nest' showed a significant relationship with bouton brightness on Frontal → BLA afferents ($\chi^2(1) = 5.0$, $P = 0.024$), with more time spent over the nest associated with dimmer (and therefore smaller) boutons (data not shown). Together with our previous findings on bouton density, these data show that time over nest P1-10 is associated with higher density, smaller volume boutons on Frontal → BLA axons in the offspring during adolescence.

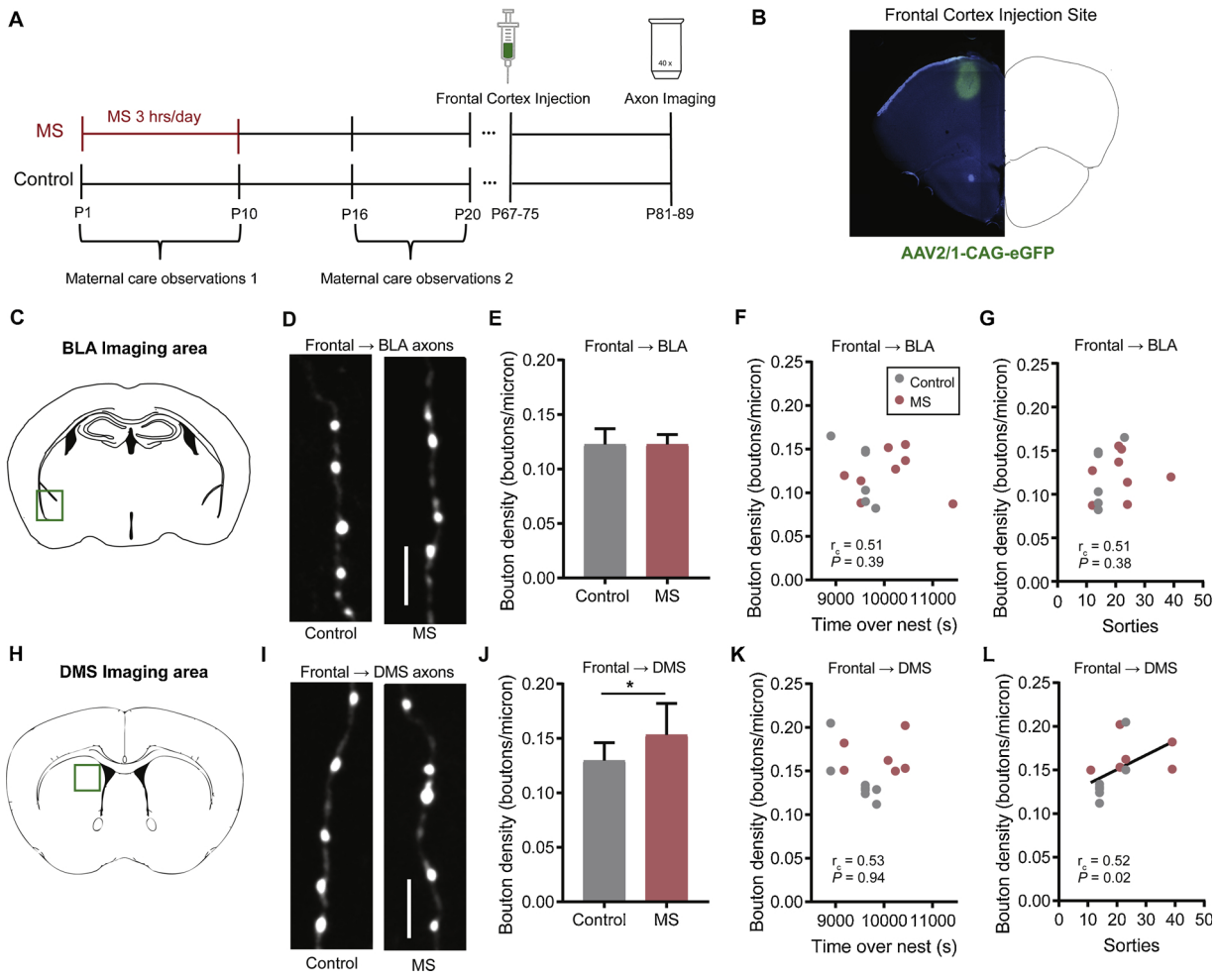


Fig. 3. Maternal care affects Frontal → DMS bouton density in adulthood.
A, Experimental Timeline. B, Injection Site image of Frontal Cortex. C, Schematic of BLA imaging area. D, Representative Frontal → BLA axons (scale bar = 5 microns). E, Adult MS mice did not differ in Frontal → BLA bouton density compared to adult controls. [Control: M = 0.1225 ± 0.01433 (n = 6) versus MS: M = 0.1225 ± 0.009137 (n = 8) bouton density; P = 0.345]. Bars represent mean ± SEM. F, The amount of time dams spent over the nest in early life did not correlate with Frontal → BLA bouton density in adult offspring (rc = 0.5124, P = 0.3899). G, The number of sorties in early life did not correlate with Frontal → BLA bouton density in adult offspring (rc = 0.5124, P = 0.3835). H, Schematic of DMS imaging area. I, Representative Frontal → DMS axons (scale bar = 5 microns). J, Adult MS mice had higher Frontal → DMS bouton density compared to adult controls [Control: Mdn = 0.1296 (n = 8) versus MS: Mdn = 0.1532 (n = 7), P = 0.016]. Bars represent median ± interquartile range. K, The amount of time dams spent over the nest in early life did not correlate with Frontal → DMS bouton density in adult offspring (rc = 0.53, P = 0.9388). L, The number of dam sorties in early life did correlate with Frontal → DMS bouton density in adulthood (rc = 0.5217, P = 0.0236). *P < 0.05.

3.4. At an adult time point, variation in maternal behavior shows a significant relationship with synapse density on frontal cortex afferents that target the striatum

We next labeled frontal cortex neurons with GFP in MS and control offspring in a separate cohort and measured axonal bouton density in the BLA and DMS in adulthood (Fig. 3a,b). We found that MS and control mice now showed comparable Frontal → BLA bouton density [Control: $M = 0.1225 \pm 0.01433$ (n = 57 axon segments, N = 6 mice) versus MS: $M = 0.1225 \pm 0.009137$ (n = 80 axon segments, N = 8 mice) bouton density; $\chi^2(1) = 0.05$, $P = 0.82$] (Fig. 3d,e), potentially due to a decrease in frontal cortex boutons in the BLA in MS mice (Supplementary Fig. 1). Additionally, the amount of time the dam spent over the nest and sorties P1-10 no longer showed a relationship with Frontal → BLA bouton density measured in adult offspring (time over nest: $\chi^2(1) = 0.84$, $P = 0.36$; $r_c = 0.5124$; sorties: $\chi^2(1) = 0.86$, $P = 0.35$; $r_c = 0.5124$) (Fig. 3f, g).

However, at this adult time point, MS offspring now had higher Frontal → DMS bouton density when compared to controls [Control: *Mdn* = 0.1296 (n = axon segments, N = 8 mice) versus MS: *Mdn* = 0.1532 (n = 69 axon segments, N = 7 mice) bouton density; $\chi^2(1) = 5.55$, $P = 0.018$] (Fig. 3i,j), potentially due to new bouton gains and/or maintenance of density levels in the MS group (Supplementary Fig. 1). The number of dam sorties in early life showed a positive correlation with Frontal → DMS bouton density in adulthood ($\chi^2(1) = 4.99$, $P = 0.025$; $r_c = 0.5217$,) (Fig. 3l), but the amount of time dams spent over the nest showed no significant relationship ($\chi^2(1) = 0.007$, $P = 0.93$; $r_c = 0.53$) (Fig. 3k).

3.5. BLA axons that target the frontal cortex were not sensitive to MS and changes in maternal care at an adolescent time point

In a final experiment, we examined if ascending BLA axons that target the dorsal frontal cortex were impacted by MS treatment. We chose an adolescent time point rather than an adult time point because MS impacted descending frontal to BLA axonal bouton density significantly in adolescence (Fig. 2). We found that BLA axons that targeted the dorsal frontal cortex did not differ in bouton density between control

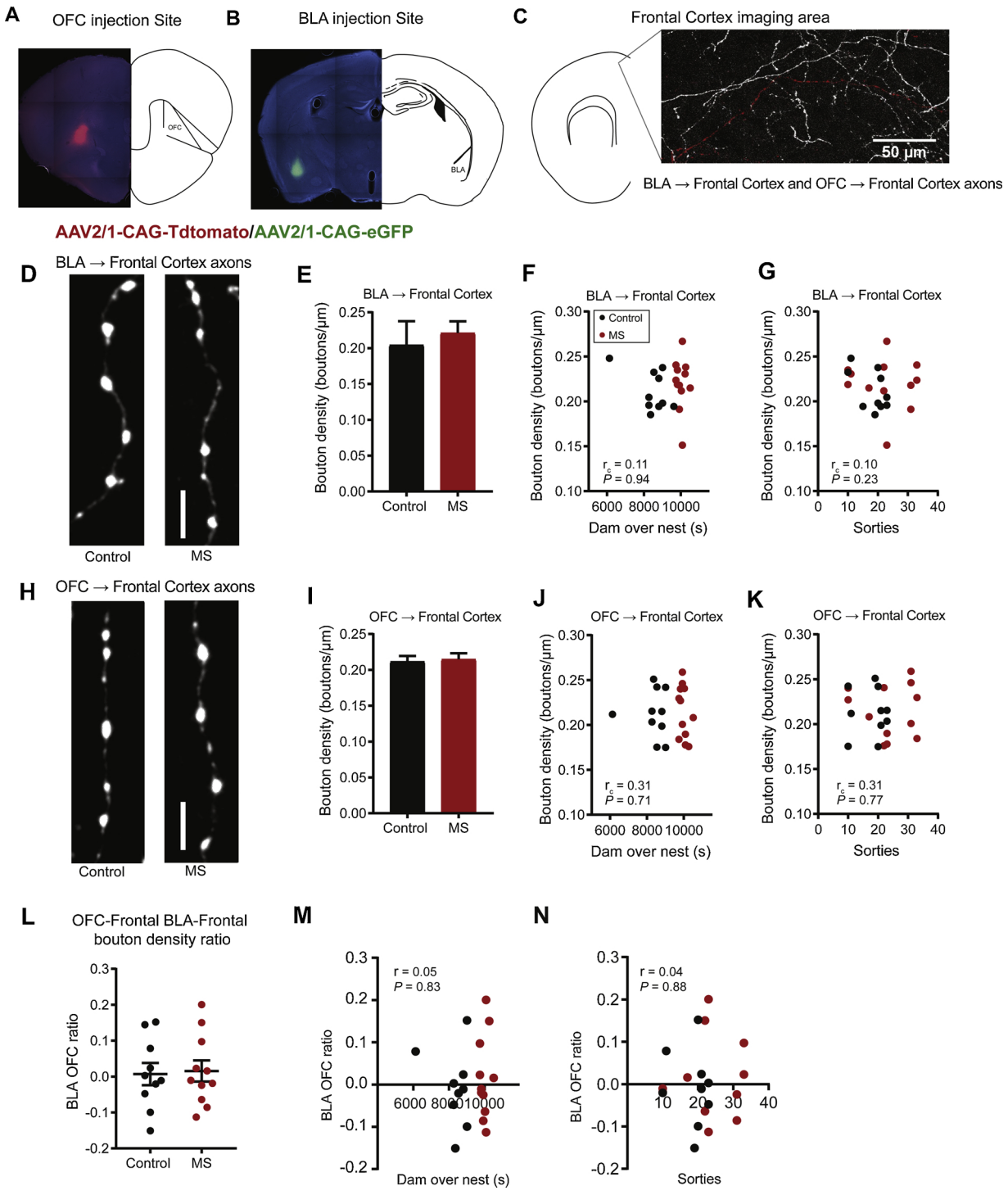


Fig. 4. BLA→ Frontal Cortex and OFC→ Frontal Cortex axons are not sensitive to variations in maternal care. A, Example OFC injection. B, Example BLA injection. C, Image of both axons converging in dorsal medial frontal cortex. D, BLA→ Frontal Cortex example axons (scale bar = 5 microns). E, MS mice did not differ from controls in BLA→ Frontal Cortex bouton density [Control: Mdn = 0.2044 (n = 11) versus MS: Mdn = 0.2211 (n = 12) bouton density; P = 0.2023]. Bars represent median ± interquartile range. F, There was no relationship between BLA→ Frontal Cortex bouton density and dam time over nest (rc = 0.1076, P = 0.9418). G, There was no relationship between bouton density and dam sorties (rc = 0.1003, P = 0.2331). H, OFC→ Frontal Cortex example axons (scale bar = 5 microns). I, MS mice did not differ from controls in OFC→ Frontal Cortex bouton density [Control: M = 0.2117 ± 0.007673 (n = 11) versus MS: M = 0.2149 ± 0.008394 (n = 12) bouton density; P = 0.5228]. Bars represent mean ± SEM. J, There was no relationship between OFC→ Frontal Cortex bouton density and dam time over nest (rc = 0.3104, P = 0.7104). K, There was no relationship between OFC→ Frontal Cortex bouton density and dam sorties (rc = 0.3129, P = 0.7737). L, BLA and OFC bouton density ratio (BLA density-OFC density/BLA density + OFC density) did not differ between controls and MS mice [Control: M = 0.007103 ± 0.03086, n = 10; MS: M = 0.01546 ± 0.02947, n = 11, t = 0.1958 df = 19, P = 0.8469]. Bars represent mean ± SEM. M, There was no relationship between the ratio and dam time over nest (r = 0.051, P = 0.8309). N, There was no relationship between the ratio and dam sorties (r = 0.03589, P = 0.8806).

and MS mice [Control: Mdn = 0.2044 (n = 70 axon segments, N = 11 mice) versus MS: Mdn = 0.2211, (n = 80 axon segments, N = 12 mice) boutons, $\chi^2(1) = 1.64$, $P = 0.20$] (Fig. 4e). Additionally, there was no relationship between BLA → dorsal frontal cortex bouton density and dam time over nest ($\chi^2(1) = 0.004$, $P = 0.95$; $r_c = 0.1076$) (Fig. 4f), or dam sorties ($\chi^2(1) = 1.44$, $P = 0.23$; $r_c = 0.1003$) (Fig. 4g). For comparison we also examined axonal bouton density on OFC axons that also target overlapping ROIs. Potentially, BLA axons could out-compete these overlapping OFC axons in competition for synaptic 'territory.' There was no significant difference in OFC → Frontal bouton density between control and MS mice [Control: M = 0.2117 ± 0.007673 (n = 66 axon segments, N = 11 mice) versus MS: M = 0.2149 ± 0.008394, (n = 76 axon segments, N = 12 mice); $\chi^2(1) = 0.45$, $P = 0.50$] (Fig. 4i). There was no relationship between OFC → Frontal bouton density and time over nest ($\chi^2(1) = 0.45$, $P = 0.69$; $r_c = 0.3104$) (Fig. 4j), or dam sorties ($\chi^2(1) = 0.09$, $P = 0.76$; $r_c = 0.3129$) (Fig. 4k). We also calculated the ratio between OFC and BLA bouton density (BLA density – OFC density/BLA density + OFC density) and found no difference in BLA: OFC ratio between MS and controls [Control: $M = 0.007103 \pm 0.03086$, N = 10 mice; MS: $M = 0.01546 \pm 0.02947$, N = 11 mice, $t = 0.1958$ $df = 19$, $P = 0.8469$] (Fig. 4l) and no relationship between the ratio and time over nest ($r = 0.051$, $P = 0.8309$) (Fig. 4m) or sorties ($r = 0.03589$, $P = 0.8806$) (Fig. 4n). These negative data show that while MS treatment impacts descending frontal inputs to the BLA in adolescence (shown in Fig. 2) parallel effects are not observed on ascending BLA afferents at this time.

4. Discussion

Prior to our study, it was clear from the literature that MS and other forms of early life disruption can impact dendritic spine density and behavior. Our MS data add a new level of detail to how environmental disruption during early life can alter maternal care and the trajectory of brain development in long range axonal projections. Additionally, our data provide new empirical evidence for adaptive developmental plasticity models in which protracted neural circuit development is informed by conditions present in the postnatal environment (Nettle and Bateson, 2015; Panchanathan and Frankenhuis, 2016).

Examining maternal behavior, we find that MS (applied as a 3 h paradigm from P1-10) can produce paradoxical changes in maternal care during the ten day treatment period, without grossly affecting maternal behavior during P16-20. Moreover, we find an increase in dam sorties during P1-10 in dams experiencing separation from their pups. These data suggest that MS can "fragment" dam care in a manner similar to an alternate model of early life adversity in which mouse dams are provided with limited amounts of nesting material (Rice et al., 2008) (although changes in amount of care are not parallel). These observations should be valuable to those seeking to connect, compare and contrast the diverse literature on models of early life adversity.

Next, by quantifying boutons that emerge from the same frontal cortex region to innervate two different targets, we find MS treatment has complex effects on the trajectory of development of these pathways in offspring and does not support the popular hypothesis that early adversity drives earlier maturation of the brain in a general fashion. When analyzed across age groups, there was a significant interaction between age and MS treatment on frontal → BLA bouton density such that bouton density was significantly higher in P35 MS mice compared to P35 controls and P85 MS mice (Supplementary Fig. 1A). Meanwhile, Frontal → BLA bouton density did not differ between P35 and P85 control mice. This suggests that MS caused transient changes in Frontal → BLA bouton density, not earlier attainment of adult-like levels. In addition, there was a significant interaction between age and MS treatment on bouton density on Frontal → DMS axons: bouton density was significantly higher in MS mice compared to controls at P85 but not P35 timepoints, but we did not observe a significant age effect when comparing within MS or control groups between P35 and P85 (Supplementary Fig. 1B).

4.1. Data may inform studies of human brain development

Our findings in mice support parallels to research in human subjects who experienced early life institutional care. Previous brain imaging work in children who experienced early life institutional care followed by adoption, found abnormal functional connectivity between prefrontal cortex and BLA while viewing fearful faces, compared to age-matched controls (Gee et al., 2013). Our data, are not from a fully homologous prefrontal cortical region but together with these human data suggest that frontal cortical projections to the amygdala are sensitive to care experience. Additionally, our data suggest that differences in development of descending connectivity from the frontal cortex into the BLA, rather than ascending connectivity, may be responsible for these findings.

4.2. What is the function of the change in frontal cortex to BLA and frontal cortex to DMS synapses?

Rodent and human studies have identified the frontal cortex to amygdala projections (each area broadly defined) as playing a key role in fear expression and extinction as well as emotion regulation (Arruda-Carvalho and Clem, 2015; Cho et al., 2013; Corcoran and Quirk, 2007; Lai et al., 2012; Likhtik et al., 2005). A separate literature has identified a role for frontal cortex to amygdala connections in appetitive associative learning and flexible goal directed behavior (Costa et al., 2016; Haber and Behrens, 2014; Stuber et al., 2011). Dysfunction of frontal cortex to amygdala connectivity has been implicated in various psychiatric disorders (Maren et al., 2013). The ventromedial frontal cortex is typically thought to exert top-down dampening control over BLA in order to regulate emotional behavior (Sotres-Bayon and Quirk, 2010), but more dorsal frontal projections could also invigorate associative learning and affect flexible decision-making (see Lai et al., 2012 and below on Ito et al., 2015).

Here, we have focused on dorsal frontal cortex projections and see an upregulation of boutons on BLA targeting axons at an adolescent timepoint. How this change in bouton density impacts the function of this circuit is unclear and could be involved in pathological processes, adaptation to the environment, or resilience. One study found that rats that experienced MS had higher dendritic spine density in the BLA in adulthood, as well as increased anxiety-like behavior (Koe et al., 2016). While we did not measure spine density in the BLA, possibly, MS can modify presynaptic and postsynaptic structures in a coordinated manner to increase the number of synaptic connections between frontal cortex and the BLA (in our data this would be at a specific time point in development). Another study in mice found that observing a distressed conspecific generated 'silent synapses', glutamatergic synapses lacking functional AMPAR, specifically in a dorsal frontal cortex -BLA projection. This same stress model also augmented passive avoidance learning (Ito et al., 2015). This enhanced learning may potentially be due to these newly generated glutamatergic synapses, which likely strengthen circuit transmission between the dorsal frontal -BLA pathway (Hanse et al., 2013). It is plausible that the boutons on Frontal → BLA axons in MS mice at P35 generate similar changes. Further research is needed to test whether these additional boutons form functional or silent synapses, and whether they are behaviorally relevant.

In a previous behavioral study of MS in mice, we found that P26 mice that experienced MS show inflexibility in an appetitive odor-based reversal task (Thomas et al., 2016), that is known to depend on the integrity of the dorsal medial regions of the frontal cortex (medial M2, Cg and FrA)(Johnson and Wilbrecht, 2011), the BLA (Loucks, 2014), as well as OFC (Bissonette et al., 2008; McAlonan and Brown, 2003). Based on the coincident timing of the MS effect in these circuits at this age, we speculate that changes in the Frontal → BLA pathway may play a role in sculpting performance in this flexible decision-making task. It is also

notable, that in our past study, inflexibility in MS mice did not continue into adulthood and in the current study these axonal effects were also absent in adulthood.

In our previous behavioral study, we also found adult offspring that experienced MS consumed more alcohol in an intermittent access ethanol paradigm compared to controls (Thomas et al., 2016). In the current study the difference we see in Frontal → DMS bouton density is specifically seen in adult MS mice. Previous studies have found a role for the DMS in alcohol consumption behavior in mice (Cheng et al., 2017), and implicated frontal cortex to DMS inputs in alcohol seeking and consumption behavior in mice (Ma et al., 2018; Warnault et al., 2016).

There is evidence from human studies that greater amygdala reactivity may serve as a mechanism for both risk and resilience in maltreated children. Studies indicate that childhood maltreatment is associated with more severe and chronic cases of depression (Wiersma et al., 2009) as well as poor treatment outcomes (Nanni et al., 2012). However, amygdala activity in individuals who have experienced early life adversity may also be protective. Goldstein-Piekarski et al. (2016) found that depressed people with high levels of early life adversity who show higher amygdala reactivity to happy faces are more likely to experience remission from depression following antidepressant exposure, whereas the opposite is true for depressed individuals with low adversity experience.

In sum, it is difficult to say in laboratory mice whether the changes we see after MS and changes in maternal care are "good" or "bad" without the context of the natural environment. However, our data are consistent with life history models that predict that brain development should be sculpted by the statistics of the early environment. Often in summaries of brain development, a canonical developmental trajectory is portrayed in which synapses are "overproduced" in an experience-independent manner and then are selectively pruned by an experience (Rakic et al., 1986). Here, we see a different pattern in which the order is flipped such that early experience may be a critical variable in informing and sculpting later developing connectivity (Baram et al., 2012; Panchanathan and Frankenhuis, 2016; see also experience-expectant plasticity in Greenough et al., 1987).

4.3. What is the mechanism underlying the changes in frontal cortex long range synapses?

The potential mechanisms that might underlie the changes in BLA and DMS bouton density observed here will require further study. One possible candidate mechanism is corticotropin releasing factor (CRF). Mice lacking the CRF1 receptor are resistant to the cognitive effects of early life stress (Wang et al., 2011a) and adult chronic stress (Wang et al., 2011b). CRF gene expression has also been shown to be sensitive to early life maternal care as adult rats that experienced handling (which leads to increased maternal care upon reunion with the pups) showed reduced CRF expression in hypothalamic neurons (Plotsky and Meaney, 1993; Liu et al., 1997). How this might come into play in frontal cortex → BLA axons or frontal cortex → DMS axons is unclear, but it is known that CRF can act on the amygdala (Gallagher et al., 2008; Regev et al., 2012; Roozendaal et al., 2002) and CRF is released within layer 5 of the PFC (Yan et al., 1998), the same layer that the cell bodies that project to the BLA and DMS reside. Based on these data, we speculate that CRF may act on cortical projection neurons and/or their targets with temporal and regional or cell type specificity.

5. Limitations of our study

Although we did not see differences in bouton densities in adolescence in the projection from the frontal cortex to the DMS or projections from the BLA and OFC to the dorsal frontal cortices (superficial layers), our sampling was not exhaustive. Alternate time points and alternate projections may have revealed MS effects or correlation with variation in maternal care. Importantly, we cannot say whether MS induces changes immediately following the MS manipulation, or rather sculpts later development. However, we do find delayed effects of the MS manipulation on frontal axons that target the DMS, as these axons do not differ between MS and control mice in adolescence, but do so in adulthood. Additionally, these data were also collected postmortem, and cannot reveal changes in the dynamic turnover of synaptic structures which can be highly responsive to experience during development and adulthood (Johnson et al., 2016a, 2016b,; Lai et al., 2012). Finally, while we speculate about multiple mechanisms, investigations of these mechanisms were beyond the scope of the study.

6. Conclusion

Adolescent development is a time of flux for the prefrontal (and larger frontal cortex) and a period of vulnerability for mental health (Paus et al., 2008; Silberg et al., 1999). Here we provide evidence that variables in the early life environment (i.e. caregiver variability) may modify long range frontal circuit development in a projection specific and age dependent manner. We speculate that these changes may support developmental differences in learning in mice that experienced differences in maternal care (Thomas et al., 2016), and potentially serve to adapt the brain to environments with different statistics. These data are consistent with imaging studies of children who experienced early life institutional care (Gee et al., 2013), and at a more granular level point to which circuits and neurons are likely to be affected. In future, our knowledge of human brain development and health should be enriched by further rodent studies with high resolution circuit and cell-type specificity. Additionally, future studies should compare how stress at a different developmental time point, such as during adolescence, impacts the development of these same neural circuits under study. Our data suggests that while circuits may be sensitive to early life disruption, the temporal relationship between experience and experience dependent structural plasticity may be complex and not fit into simple models.

Studies that take adaptive developmental plasticity models into account may yield new insights about the most effective timing for interventions and treatments. We envision studies with increasing temporal and circuit specificity will enhance our capacity to design and deliver more effective interventions to promote positive development (Crone and Dahl, 2012; Dahl et al., 2018).

Funding sources

This project was supported in part by NIHR01MH087542 (L.W.), a National Science Foundation Graduate Research Fellowship (A.W.T) under Grant No. (DGE 1106400), a UC Berkeley Elizabeth Roboz Einstein Fellowship and by the National Institutes of Health S10 program under award number 1S10RR026866-01. The contents solely the responsibility of the authors and do not necessarily represent the official view of the NIH or NSF.

Declaration of Competing Interest

The authors declare that there are not conflicts of interest.

Acknowledgements

The authors thank Benjamin Tang, Corinna Wong, Michelle Matvey, Jenna Martin, Amy Kim, Carolyn Johnson, Josiah Boivin, David Piekarski, Lung-Hao Tai, and Wan Chen Lin for assistance with experiments, analysis and discussion; Silvia Bunge, Ron Dahl, Willem Frankenhuis, Daniela Kaufer, and Judy Stamps for discussions.

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.2019.100737.

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