Effects of early life adversity and adolescent basolateral amygdala inhibition on corticolimbic connectivity and anxiety behaviors

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Abstract

Early development of postnatal corticolimbic circuitry is vulnerable to challenges from the early environment. Prior work has shown hyper-innervation of glutamatergic basolateral amygdala (BLA) projections to the prefrontal cortex (PFC) in the adolescent timeperiod following rearing in an adverse early life environment, but how these ELA-induced connectivity changes alter the startle circuitry required to execute a startle response and whether the BLA-PFC pathway is responsible for previously reported blunted startle response remains unknown. We directly tested whether BLA activity during a discrete adolescent period altered later anxiety behaviors, and if behavioral changes were driven by altered PFC innervation. To test these questions, we exposed rat pups to an ELA model of maternal separation on postnatal days (P) 2-21. Rats then underwent an injection of an inhibitory DREADD in the BLA in order to silence the excitatory projections during the adolescent time period of P33-39. Finally, rats were tested in an acoustic startle paradigm in late adolescence to assess the effects of reduced BLA-PFC connectivity on the ELA-induced blunted startle response. Density, intensity, and volume of axonal boutons in the BLA projecting PFC neurons were then assessed to elucidate how BLA inhibition during adolescence affects innervation later in life. These results explore how altered early life environments may affect the development of anxiety-related circuitry and anxiety responses later in life, and how rescue of this maladaptive response may be sex and developmentally specific.

1. Introduction

Early postnatal life is a dynamic period of brain development that involves a coordinated symphony of circuit maturation. As many regions within the brain continue to develop and mature postnatally, external stimuli impact this development and regulate the degree of connectivity to other brain areas. Since subcortical limbic regions undergo substantial synapse formation and pruning during early development while higher-order areas such as the prefrontal cortex (PFC) mature during adolescence (Huttenlocher, 1984, 1990, 1997; Konrad et al., 2013; Drzewiecki et al., 2016), early postnatal life and adolescence are two sensitive periods when limbic and prefrontal regions form functional connections in response to external stimuli and neuronal activity. Thus, activity within limbic regions over the course of development can affect the strength and quantity of efferent connections to cortical areas.

The infant amygdala undergoes substantial structural and functional changes in the postnatal period (Gilmore et al., 2012; Payne et al. 2009; Gee et al., 2013; Swartz et al., 2014; Silvers et al., 2016; Decety et al., 2012; Vink et al., 2014), receiving environmental input via the sensory thalamus and cortices (Janak & Tye, 2015). Seminal lesion studies performed in rodents, non-human primates, and humans reported that amygdala ablation in adulthood results in decreased recognition of and response to fearful stimuli (Brown & Schäfer 1888; Klüver & Bucy, 1937; Blanchard & Blanchard, 1972; LeDoux et al., 1990; Adolphs et al., 1994; Anderson et al., 2001). The basolateral amygdala (BLA) in particular has unique integration with aversion- and reward-related circuitry and serves as the primary input nucleus of the amygdala, communicating sensory, executive, and memory-related bidirectionally connections with the prefrontal cortex (PFC) (Janak & Tye, 2015). These connections allow the BLA to

function as an integration point of emotional processing, receiving sensory and affect-valanced stimuli and then funneling the relevant information to multiple higher order areas (Cunningham et al., 2002). Monosynpatic BLA connectivity with the PFC (Bacon et al., 1996; Krettek and Price, 1977; McDonald, 1996; Verwer et al., 1996) is known to increase with development throughout adolescence in healthy rats, and reportedly facilitate the integration of emotional information with cognitive appraisals of the environment (Cunningham et al., 2002). Challenges to the early amygdala that are capable of altering local activity may therefore induce activity changes within the cortical efferent projections, which may lead to altered behaviors once the circuit is recruited for adult-like demands.

Adolescence is a dynamic period where synapses undergo significant pruning in some regions, and strengthening in others. Human studies show that white and gray matter cortical volume decreased drastically during the early adolescent period, (Huttenlocher 1979; Huttenlocher & de Courten, 1987; Benes et al., 1987) resulting in adult levels of synaptogenesis by the time of sexual maturity in humans (Glantz et al., 2007) and monkeys (Zecevic et al., 1989). These reported frontal cortex changes are thought to involve glutamatergic afferents and local remodeling of inhibitory-excitatory synaptic balance (Caballero et al., 2016; 2021), and coincide with the onset of working memory (Satterthwaite et al., 2013) and heightened emotional regulation (Gee et al., 2013; Swartz el al., 2014; Honeycutt et al., 2020). While significant synaptic pruning occurs in the frontal cortex during adolescence, the amygdala exhibits an increase in gray matter during childhood. The BLA in particular reaches its adult volume in human girls by the age of 4 and in boys by the age of 18 (Brenhouse & Anderson, 2011).

Importantly, neuronal activity during sensitive periods directly impacts pre- and post-synaptic plasticity (Guadagno et al., 2021). The present study therefore investigated the impacts of early life environmental inputs and adolescent BLA activity on the development of BLA-PFC circuitry and corresponding behaviors.

Postnatal brain circuitry has evolved to “expect” appropriate quantity and quality of caregiving stimuli in order to develop typically (Zeanah et al., 2011). Disruptions to typical development can be detrimental to the postnatal brain and can exert cascading effects on subsequent maturation (Knudsen, 2004; Hensch, 2004; Tseng et al., 2009; Tottenham & Gabrad-Durnam 2017). One such form of disruption includes early life adversity (ELA) (Lupien, 2009). ELA produces several affective consequences that emerge in adolescence, and continue through adulthood (Kessler et al., 2001; Davey et al, 2008; Anderson & Teicher 2008). These consequences include psychiatric disorders in humans such as anxiety and major depressive disorder (MDD) (Smyke et al., 2007; McEwan, 2008; Maccari et al., 2014; Callaghan & Tottenham, 2016; Hane & Fox, 2016; Krugers et al., 2017). Consequences of ELA are often sex and age dependent, with females experiencing anxiolytic symptoms earlier than males (Rudolph & Flynn (2007; Schuch et al., 2014; Weeks et al., 2014), likely due to sex-dependent trajectories of circuit development.

The maternal separation (MS) model of ELA in rodents induces corticolimbic and anxiety-related changes that resemble those reported in ELA-exposed humans. Particularly, MS induces hyperinnervation of the PFC from glutamatergic BLA projections. These effects were seen in adolescent rodents as early as postnatal day (p)40 in male rats, and p30 in females. Early maturation of corticolimbic connectivity

can exert deleterious consequences because PFC immaturity is needed in juvenility to learn safety signals (Yang et al., 2012). Therefore, here we hypothesized that preventing the consequences of accelerated BLA-to-PFC excitation in ELA-exposed adolescents could prevent later-life behavioral dysfunction.

Increased connectivity following MS is predictive of increased anxiety-like behavior (Honeycutt et al., 2020), however convergent and divergent mechanisms underlying connectivity and behavior remain unknown. Because anxiety is a common adverse outcome following ELA, studying discrete anxiety-related behaviors can provide insight into the mechanistic underpinnings following adverse rearing. Anxiety is a multi-dimensional disorder, which can present as aversion or heightened responsivity to potential threats (Leonardo & Hen, 2017). Aversion can be studied via a variety of behavioral assays, including the open field test (OFT) and the elevated plus maze (EPM), which assess rodent's aversiveness to enter bright open spaces where they are more prone to predator attacks. In contrast, threat anticipation and hyperarousal are domains of anxiety that exhibit heightened responsivity (Edmonton & Blackford., 2013; Silvers et al., 2016) and are explicitly regulated by the BLA (Terburg et al., 2018). The acoustic startle reflex (ASR) is a defensive behavior that provides a measurement of hyperarousal in rodents and humans, making ASR a valuable translational assessment of anxiety-provoked responsivity. ELA exposure enhances ASR (citations) in both rodents and humans, however the developmental influence of BLA activity on ASR is unknown.

Due to the fluctuating balance between BLA and PFC control during adolescence, it is crucial to investigate the role heightened BLA activity plays in driving

altered PFC activity and innervation after ELA. We hypothesized that ELA leads to pre- and post-synaptic changes to BLA-PFC connectivity, and that preventing heightened presynaptic BLA activity during mid adolescence would interrupt hyperinnervation and result in reduced anxiety behaviors.

We first determined whether MS exposure led to facilitation of BLA-evoked activity within the PFC during adolescence. To determine the role that altered corticolimbic circuitry plays in anxiety and the role of both pre and post synaptic inputs of this circuit, the BLA-PFC pathway was manipulated during development via an inhibitory chemogenetic injection into the BLA to induce repeated inhibition during adolescence. Histology was performed to determine if BLA inhibition in adolescence was capable of preventing presynaptic changes via glutamatergic PFC hyperinnervation from the BLA. Rats were also tested in multiple assays designed to measure anxiety-like behaviors in early and late adolescence to determine if acute and sub-chronic inhibition of the BLA is sufficient to prevent anxiety behaviors.

Methods

All experimental procedures were conducted in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (NIH) with approval from the Institutional Animal Care and Use Committee at Northeastern University and the University of Illinois Chicago.

2.1 Subjects and Maternal Separation (MS)

All rats were housed under standard laboratory conditions in a 12h light/dark cycle (lights on at 0700h), in a temperature and humidity-controlled room with access to food and water ad libitum. All experimental subjects were bred in house. Male and female rats were co-housed, and pregnancy was assessed daily. Males were removed once pregnancy was confirmed. Dams gave birth on p0. Litters were called on p1 to 12 pups (6 male) when possible, toe clipped on p5 for animal identification, and weaned on p21. Whole litters were randomly assigned to control (CON) or maternal separation (MS) rearing conditions. Pups in control litters were left undisturbed except for the husbandry procedures described above and regular weight recording and handling twice per week. Pups in the MS condition were separated from their dams and littermates in individual cups with home cage pine shavings within a circulating water bath thermo-regulated at 37C from P2-P10. At P11-20, when body temperature can be self-regulated, pups were separated into individual mouse cages. All separations lasted for a total of 4 hours each day beginning at 0900h and pups were kept in a separate room from the dam. Upon completions pups were reunited with their dams and littermates and left undisturbed until the next day. On p21, pups were weaned from the dam into standard rat cages with an age, sex, and condition matched partner.

2.2 Local Field Potential (LFP) recordings of prefrontal response to basolateral amygdala (BLA) stimulation

Mid-adolesent (p30-45) rats were anesthetized with 8% chloral hydrate (400 mg/kg, i.p.), placed in a stereotaxic apparatus (ASI Instruments, MI), and maintained at 37–38

°C using a Physitemp TCAT-2LV Controller (Physitemp Instruments, NJ), as previously described (Thomases et al., 2013; Cass et al., 2013). All recordings were conducted in the prelimbic region using a concentric bipolar electrode (SNE-100 × 50 mm; Rhodes Medical Instruments, CA) while stimulating the BLA (concentric bipolar electrode NE-100 × 50 mm; Rhodes Medical Instruments, CA) through a computer-controlled pulse generator (Master-8 AMPI, Jerusalem, Israel). Changes in the input-output curve of PFC responses were estimated by comparing the amplitude of BLA-evoked LFP at different stimulating intensities (0, 0.25, 0.5, 0.75, and 1.0 mA) using single (300µS duration) square pulses delivered every 15s in both control and MS rats.

2.3 Viral Injections and Chemogenetic Inhibition

In a separate cohort of male and female rats, on p26 subjects underwent a stereotaxic surgical injection of the inhibitory DREADD AAV-CAMKIIa-HM4DGi-mCherry (Addgene, Watertown, MA) to the BLA (A/P: -1.9mm, L/M: ±4.5mm, D/V: -6.75mm). Rats were anesthetized with 5% isoflurane mixed with oxygen, placed in a stereotaxic apparatus (Kopf, Tujunga, CA) and maintained at 37-38°C using a heating pad. Constant level of isoflurane (2–3%) was delivered during the surgical procedure and the level of anesthesia was monitored closely by breathing rate and the pedal reflex. After exposing the skull, two small (1 mm) holes were drilled above the M/L and A/P coordinates of the BLA. A Hamilton syringe was slowly lowered to the D/V coordinates and 200 nL of virus was injected at a rate of 50 nL/min via a Harvard Apparatus Pump 11 Elite Microinfusion pump for a total of 4 minutes. The Hamilton was left at the surgical site for 1 additional minute, for a total of 5 minutes/hemisphere, before slowly being withdrawn. The surgical

incision was closed with sterile sutures and treated with triple antibiotic ointment. Rats were placed back into the home cage following the return of movement and reflexes. Rats were allowed to recover for 1 week before behavioral testing.

2.4 Functional Validation of DREADD in the BLA

All brain slicing procedures were conducted as previously described (Flores-Barrera et al., 2017; 2020). Specifically, coronal brain slices (350µm-thick) containing the BLA were obtained within 6-7 days post-DREADD delivery to validate the functional expression of the hM4D-Gi receptor on amygdalar pyramidal neurons. All recordings were conducted at 33–35 °C in artificial cerebrospinal fluid (aCSF) containing (in mM): 122.5 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 20 glucose, 1.0 ascorbic acid (pH 7.40–7.43, 295–305 mOsm) under constant oxygenation with 95 % O_2–5 % CO_2. The patch electrode was pulled from borosilicate glass (8-10 MΩ) and filled with a potassium-based internal solution containing 0.125 % Neurobiotin and (in mM): 115 K-gluconate, 10 HEPES, 20 KCl, 2.0 $MgCl_2$, 2.0 Mg-ATP, 2.0 Na_2-ATP, 0.3 Na_2-GTP (pH 7.23–7.28, 280–282 mOsm). After 5 min of obtaining the whole-cell configuration, a set of somatic current pulses was applied in current-clamp mode to measure changes in neuronal excitability (input resistance, rheobase, latency to the first spike) prior and after bath application of the DREADD ligand CNO (10µM, 10 min).

2.5 DREADD Mediated Inhibition

Beginning on p33 and continuing until p39, rats were weighed and given an intraperitoneal injection of the DREADD agonist clozapine-N-oxide dihydrochloride

(CNO, 2mg/kg, Bio-Techne Tocris, Minneapolis, MN) or vehicle saline (SAL). CNO was prepared fresh prior to the start of injections and was dissolved in sterile saline.

2.6 Behavioral Assays

Rats were brought into the behavioral suite and allowed to habituate for 15 minutes. For early adolescent testing (p33-34), CNO or SAL injections were conducted at 15-minute intervals. 30 minutes after the first injection, rats were placed into the behavioral apparatus. Video monitoring of behavior was done via overhead CCTV camera (Panasonic WV-CP500, Secaucus, NJ), and behavioral scoring was done via Noldus Ethovision 9.0 Behavioral Tracking software.

2.6.1 Elevated Plus Maze

Elevated Plus Maze testing occurred on p33 and p34. The apparatus was a melamine maze elevated 100 cm above the floor with four radiating arms (50cm x 10cm) around a center square (10cm x 10cm), with 40cm-high walls surrounding two opposing arms, leaving the other two arms open. Behavior was recorded and videos were scored for time spent and number of entries in the open arms of the maze. The arena was cleaned with 50% ethanol solution between each animal.

2.6.2 Open Field Test

Open Field testing occurred on p33 and p34. The apparatus was a 100 cm x 100 cm black acrylic apparatus. Behavior was recorded and videos were scored for time and

number of entries spent in the center zone of the arena are measured. The arena was cleaned with 50% ethanol solution between each animal.

2.6.3 Acoustic Startle Response

Rats underwent the ASR test on p40 and p54-55, coinciding with the end of BLA inhibition and late adolescence, respectively. Rats were brought into the testing room and allowed to habituate for 15 minutes. On p40 and 54, were placed into a sound attenuated startle chamber (MedAssociates Inc, St. Albens, VT) and played a series of 30 105dB white noise bursts for an assessment of baseline startle. In late adolescence, rats underwent a second ASR test 24 hrs following baseline assessment, during which ASR was tested following exposure to 5 minutes of a 22 kHz rat ultrasonic vocalization (USV) recording; 22 kHz USV is a call type used by rodents to signal threat in the environment and has been shown previously to elicit activity in the BLA (Demaestri et al., 2019).

2.7 Histology

Following all behavioral testing, rats were deeply anesthetized and transcardially perfused with ice cold saline and 4% paraformaldehyde (PFA) solutions. Brains were extracted and preserved in 4% PFA for 24 hours in individual scintillation vials, followed by cryoprotection in 30% sucrose solution until brains reached optimal density. Brains were then cryosectioned on a Leica 6800 cryostat in 100um sections to isolate the PFC and BLA regions within 1 week of cryoprotection. Slices were placed in well plates with PBS until mounting and staining. Sections were collected in 6 well increments (600um).

2.8 Microscopy and Analysis

2.8.1 Viral Expression

BLA slices were mounted on Fisherbrand ColorFrost Plus slides. In order to quantify the amount of DREADD expression within and outside of the BLA, slices were imaged on a Zeiss Imager M.2 microscope for bolus verification. Slices with dual hemispheric expression in the BLA underwent volumetric analysis via the Cavalieri probe within StereoInvestigator. Sections containing DREADD expression were analyzed at 2.5X magnification to determine the extent of bolus infection within and spread outside of the BLA. Average BLA volume was previously measured in a subset of animals, and the volume of DREADD expression was quantified as a percent of the total BLA volume. Animals with a bolus that filled >60% of the BLA were excluded from analysis.

2.8.2 Axonal Innervation

The corresponding PFC of all included animals underwent immunohistochemsitry staining (IHC) to identify mCherry expression. Sections containing the infra- and pre-limbic regions were prepared for immunohistochemistry as detailed in Falcy et al. 2020. Sections were stained with a rabbit anti mCherry primary antibody (1:2500, Abcam ab167453) and a Alexafluor 488 Goat anti Rabbit secondary antibody (1:1000, Jackson ImmunoResearch Laboratories). Three 100um slices of the PFC were imaged on a LM800 Zeiss confocal microscope. Four Z-stacks were taken in three representative PFC slices, totaling 12 z-stacks per animal. Z-Stacks were taken in .5um steps between image planes with a 63X/ 1.4 oil objective with a pinhole size of 1 Airy unit. Images were

acquired with a voxel size of 0.09 um x 0.09 um x 0.5. Image stacks were 3-D reconstructed using Arivis Vision4D software (Carl Zeiss) and analyzed for bouton detection via the BlobFinder pipeline. Settings were determined using representative stacks from all groups and optimized for bouton detection with minimal noise. Z-stacks were analyzed for axonal bouton count, volume, and intensity.

2.9 Statistical Analysis

GraphPad Prism 10.0 Software was used for all data analyses and graphical representation. The sample size was determined from power analysis from prior experiments. Sex x rearing x treatment 3-way ANOVAs were used to compare performance in behavioral tests and innervation measures. 2-way ANOVAs with factors of age and rearing were conducted with Post-hoc Tukey's multiple comparison's tests when appropriate. p-values and effect sizes are reported. Cohen's d effect size was determined by calculating the mean difference between groups, then dividing the result by the pooled SD. For simplicity, statistical details are only provided for significant comparisons.

3. Results

3.1 BLA inhibition during the early adolescent time period is capable of altering BLA-PFC connectivity in male rats

Rats were sacrificed following behavioral testing (p56-60) and BLA innervation to the PFC was quantified via axonal bouton count, volume, and fluorescence intensity. Two-way ANOVAs revealed a main effect of rearing on male PL count ($F_{1,25}$ = 4.661; p=0.0407; partial η = 0.1571; Figure 3E), and intensity ($F_{1,25}$ = 36.09; p<0.0001; partial η

= 0.5908; Figure 3F). Analysis also revealed a main effect of rearing on male IL count ($F_{1,25}$ = 5.098; p=0.0329; partial η = 0.1694; Figure 3G) and intensity ($F_{1,25}$ = 29.53; p<0.0001; partial η = 0.5416; Figure 3H)). No effects of treatment were found on bouton volume in either region. No significant interaction or effects of rearing were seen across all measures: count, volume, and fluorescent intensity in either region. In females, there were no significant interactions or effects of rearing or treatment on either PL or IL count, volume, or intensity (Figure 3I-L).

3.2 Exposure to MS and mid adolescent BLA inhibition alters behavior in a sex dependent manner.

Here, we first assessed the effect of BLA inhibition on anxiety like behavior during the early adolescent timeperiod. Rats with AAV9-CaMKIIa-HM4DGi-mCherry viral expression in the BLA were given an intraperitoneal (i.p) injection of CNO (2 mg/kg) 30 minutes before OFT and EPM testing on p33 and 34. Animals were assessed for time and entries into the open arms (EPM) and into the center of the arena (OFT) (Figure 3A). Each sex was analyzed separately, with 2-way ANOVAs assessing effects of rearing condition and treatment (CNO or saline).

For males tested in the EPM, there was a main effect of treatment on open arm duration ($F_{1,37}$ = 5.681; p=0.0224; partial η = 0.133; Figure 4B), but no effect of rearing, or rearing x treatment interactions. No effects of rearing or treatment were found on number of entries to the open arms, or latency to enter the open arm of the EPM (Figure 4C). However, a rearing x treatment interaction was found in male distance moved during the EPM ($F_{1,37}$ = 58.481; p=0.0061; partial η = 0.186; Figure 4A), with MS CNO

treated animals traveling more. No effects of treatment or rearing were seen in females tested in the EPM across all measures: distance moved, open arm duration, number of entries to the open arm, or latency to enter the open arm of the EPM (Figure 4D-F).

In the OFT, for males, there was a main effect of treatment on center frequency ($F_{1,33}$ = 4.735; p=0.0368; partial η = 0.1255; Figure 4I) and center duration ($F_{1,33}$ = 7.722; p=0.0089; partial η = 0.1896; Figure 4H). No effects were seen in distance moved or latency to enter the center(Figure 4G). Additionally, no interactions or effects of rearing were seen across all measures: Distance moved, center frequency, center duration, or latency to enter. In females, a main effect of rearing was found for center frequency ($F_{1,39}$ = 4.544; p=0.0394; partial η = 0.1044; Figure 4L), but not center duration (Figure 4K). No effects were seen in distance moved, or the latency to enter the center (Figure 4J). Additionally, no interactions or effects of treatment were seen across all measures: Distance moved, center frequency, center duration, or latency to enter (Figure 4J-L).

3.3 Exposure to MS and early adolescent BLA inhibition does not alter threat responsivity in male or female animals in late adolescence

On p54 (late adolescence), rats were tested for baseline startle levels to 30 white noise bursts. No effects of either rearing or treatment were seen on latency to startle, peak time, peak value, duration, total, or average startle in male or female animals (Figure 5A-B).

On p55, rats were placed in the same chambers and tested for changes to their baseline startle. Rats were first presented with a 5-minute recording of a 22kHz USV

recording which was immediately followed by the same 30 white noise bursts as on prior days. Under these conditions, rearing and treatment had no effect on latency to startle, peak time, peak value, duration, total, or average startle in male or female animals (Figure 5C-D).

4. Discussion

The current study identifies dissociable pre- and post-synaptic contributions to sex and age specific effects of ELA on corticolimbic connectivity and behavior. We sought to investigate if an adverse rearing environment and interrupted BLA activity during in adolescence were differentially capable of altering BLA-PFC connectivity, and if these changes in connectivity drove changes to anxiety-like behavioral responses in early and late adolescence. We observed that MS rearing yielded postsynaptic facilitation of PFC responsivity to BLA activation by mid-adolescence. We hypothesized that presynaptic inhibition of the BLA during this period would prevent MS-driven hyperinnervation from the BLA to the PFC. As we previously observed (Honeycutt et al., 2020), MS resulted in hyperinnervation of the PFC by glutamatergic BLA neurons, however by late adolescence hyperinnervation was only apparent males. However, despite increases to both pre- and post-synaptic measures of connectivity, there were no changes to anxiety related behaviors in late adolescence as measured by the acoustic startle reflex (ASR). MS exposure led to decreased thigmotactic behavior in mid-adolescent females, which was unexpected. However, the assays used in this experiment to test for anxiety have also been used as measures of risk-seeking (Walf & Frye, 2013; Palm et al., 2014). ELA is known to increase risk-seeking and impulsivity (Rincón-Cortés, 2023). It is therefore possible that MS induced increased risk-seeking in

adolescent females that would not be seen if testing occurred in adulthood. Local BLA inhibition did not impact female behavior, but resulted in increased thigmotaxis in male animals. Taken together, these data suggest that MS induces divergent effects in early adolescence and late adolescence and that interrupting early adolescent BLA activity impacted adolescent behavior in males but was not sufficient to reverse any MS-related long-term consequences. Hyper-innervation of the PFC was not prevented by interrupting BLA activity, suggesting that sustained BLA activity during early adolescence is not necessary for long term development of pre-synaptic PFC inputs following MS.

This project intended to discern if interruption of mid-adolescent BLA activity disrupted our previously observed hyperinnervation of BLA-PFC projecting neurons, which began in early adolescence in females and mid-adolescence in males. Our results show that bouton count is increased in both the prelimbic (PL) and infralimbic (IL) regions of the PFC in male rats at P55. However, this increase was not attenuated by mid- adolescent BLA inhibition, suggesting that sustained activity of the BLA does not regulate the development of presynaptic connectivity with the PFC. Additionally, no increases in innervation were seen in females, indicating that by late adolescence, any previously observed increases in BLA-PFC connectivity may have pruned back to control levels. Both males and females exhibit significant pruning within the PFC between adolescence and adulthood, however females display greater losses than males (Koss et al., 2014) It is unknown at what age male and female PFC pruning spikes, which allows for the possibility that inhibition of BLA activity from p33-39 had differential effects on PFC innervation, and that innervation in female controls reached

adult levels by p55 when histology was performed (Juraska, 2024). Additionally, previous work from our group showed increases in female BLA-PFC innervation that were already apparent by p28, therefore if BLA activity does provoke innervation to the PFC in females, inhibition from p33-39 may have been too late to affect any MS-evoked changes. Thus, it will be important to repeat this study with an earlier intervention time point as well as earlier ages of assessment to capture the effects of juvenile BLA inhibition on adolescent innervation.

The impact of ELA on BLA-PFC connectivity was found to have both presynaptic and postsynaptic components which begin as early as adolescence. However, inhibiting presynaptic activity was not sufficient to reverse hyperinnervation. It is therefore possible that postsynaptic plasticity within the PFC precedes and drives these connectivity changes. For example, MS results in heightened expression of GluN2A-containing NMDA receptors within the PL (Wieck et al., 2013). While the PFC displays protracted development comprised of late-maturing connectivity with regions such as the BLA (Honeycutt et al., 2020; Cunningham et al., 2002), the postnatal environment is capable of directly impacting PFC activity and receptivity (Stern et al., 2024; Ayala-Rodríguez and García-Colunga, 2023), and the nature of glutamate receptivity can influence development of inputs (Kelsch et al., 2014). Future studies will focus on the postsynaptic contributions of aberrant corticolimbic development after ELA.

Another intention of the current study was to investigate how persistent BLA inhibition affected anxiety like behavior in late adolescence (P54-55), and if the observed changes to corticolimbic innervation drove any alterations to anxiety, as measured by arousal and threat perception. The ASR is highly evolutionarily conserved

and has been shown in both humans and rodents to be a reliable metric of anxiety (Zheng & Schmid, 2023; Granata et al., 2022; Davis et al., 1982). This reflex relies on quick relay of information via the primary acoustic startle pathway, including the auditory nerve, the ventral cochlear nucleus, the dorsal nucleus of the lateral lemniscus, the caudal pontine reticular nucleus, spinal interneurons, and spinal motor neurons (Davis et al., 1993). However, the amygdala has been posited as “the command center” responsible for the enhancement of ASR, with the BLA in particular heavily involved in the expression of anxiety-potentiated startle (Koch & Schnitzler 1997; Davis et al., 1993), and is poised to respond to threats from the environment.

The main mechanism via which rats signal threat is ultrasonic vocalization (USV) communication, in which a 22 kHZ USV represents anxiety or fear (Burgdorf et al., 2008; Wöhr et al., 2016), making the 22kHZ USV an ethologically relevant cue to evoke awareness of a distal threat. Previous work from our lab has shown enhanced BLA activity following presentation of a 22 kHz USV (Demaestri et al., 2019), indicating the role of the BLA in the processing of this fearful stimuli. We therefore presented rats with a five-minute 22kHz USV playback in order to measure changes in baseline startle following a threat cue.

Due to the involvement of the BLA in threat potentiated ASR (Vásquez-Roque et al., 2012), as well as the BLA-PFC pathway’s influence on ELA induced and general anxiety (Shin and Liberzon, 2010; Burghy et al., 2012; Godsil et al., 2013; Gee and Casey, 2015; Yan et al., 2017, Guadagno et al., 2021) we rationalized that BLA-PFC connectivity may be mediating the ASR and that this response may be heightened following USV presentation. Previous work from our group has shown that MS-exposed

animals exhibited reduced changes to baseline startle magnitude following the presentation of a 22kHz USV, which indicates a USV-induced blunted startle response (Granata et al., 2022). We therefore sought to investigate if hyperactivity of th BLA and the subsequent increase in BLA-PFC connectivity was driving this blunted startle response, and if inhibition of the BLA would rescue behavior to control startle levels. However, our results showed no effects of early adolescent BLA inhibition on the ASR at baseline or following USV exposure. Additionally, we were unable to replicate our previous findings of blunted USV-potentiated ASR following MS exposure (Granata et al., 2022), which is likely due to a difference in startle paradigms. These data indicate that MS-attributable increases to PFC connectivity from the BLA are not associated with, and therefore do not mediate baseline ASR or USV mediated ASR. This finding is interesting because it indicates that the developmental stage in which BLA activity is capable of altering later life anxiety behavior as it relates to distal threat is not during the early adolescent time period. These findings should be considered with the following limitations. Surgery and chronic injections, which are stressful experiences in and of themselves, were conducted during juvenility and early adolescence. This may be considered a second hit of stress later in life following MS exposure and may therefore reflect a culmination of a two-hit model of stress exposure. Secondary hits of stress following ELA rearing have been shown to alter typical stress responses (Kuhlman 2023).

In addition to examining the developmental trajectories of reduced BLA-PFC connectivity, this study also sought to examine the acute effects of local BLA activity manipulation. We tested rats on p33 and 34, the first two days of BLA inhibition, using

the open field test (OFT) and elevated plus maze (EPM) assays. These tests were chosen to illuminate the effects on BLA inhibition on anxiety behavior before any lasting changes to PFC connectivity were induces via persistent BLA inhibition. Our results show that BLA inhibition decreased exploratory behaviors in males, such that rats exposed to BLA inhibition exhibited decreased center time and entries in the OFT and decreased open arm duration in the EPM. Female rats exposed to MS rearing exhibited center frequency in the OFT. Previous work from our group indicates that adolescent female rats (p38) exposed to MS exhibit increased anxiety in the elevated zero maze assay, a comparable test to the EPM. This is contrary to our findings showing decreased anxiety in MS females. In addition to anxiety like behaviors, ELA has also been shown to induce increases in impulsivity (Kim et al., 2018; Duckworth 2013; Hostinar et al., 2012; Lovallo, 2013; Orshri, 2018; Lovic et al., 2011), novelty-seeking, and risk-taking behaviors (Roman et al., 2006; Marmendal et al., 2006, Lundberg et al., 2017). It is possible therefore that the anxiogenic behaviors seen in females during the OFT assay may be attributable to increases in risk-seeking behaviors. Further work will tease apart the discrete mechanisms of adolescent risk-seeking following MS, and how BLA activity drives these behaviors. Our previous work also showed no changes in male anxiety like behaviors at this age (Honeycutt et al., 2020), which is consistent with our findings here showing no changes in anxiety behavior in MS exposed males.

The current findings provide evidence of sex- and developmental- dependent effects of early life adversity on pre- and post-synaptic mechanisms of innervation that do not mediate changes in anxiety behaviors. Inhibition of the BLA during a discrete developmental time point is capable of altering the behavioral repertoire of male animals

in early adolescence. MS rearing is capable of inducing increased axonal innervation and LFP in the PFC in male animals. MS exposure decreased anxiety in early adolescent females, however no changes to anxiety or pre-synaptic PFC axonal innervation were seen in late adolescence. These results indicate disparate sex specific pre and post-synaptic outcomes following adverse early rearing environments.

Figure Legends

Figure 1. (**A**) Relative to the control group (Ctrl), BLA hyper- innervation in the prelimbic PFC appears by P40 in male rats that underwent maternal separation (MS) ($p<0.05$, main group effect) (**B**) Yet, the prelimbic PFC response to BLA inputs is already enhanced by P30 as shown by local field potential (LFP) recordings ($p<0.05$, main group effect). (**C**) In females, the BLA hyper-innervation is already apparent by P30 following MS ($p<0.05$, main group effect). (**D**) BLA-evoked LFP in the prelimbic PFC is also enhanced in MS females by P30 ($p<0.05$, main group effect). N=5-6/group.

Figure 2. (**A**) DREADD validation timeline. (**B**) CNO application decreased RMP (**C**) Increased Rheobase.

Figure 3. (A) Timeline of DREADD experiment. (B) Representative DREADD Injections in the BLA (C-D) BLA-PFC terminals in the PFC. (E-F) Male PL bouton count and intensity. (G-H) Male IL bouton count and intensity. (I-J) Female PL bouton count and intensity. (K-L) Female IL bouton count and intensity.

Figure 4. EPM results for male **(A)** Distance moved **(B)** Open arm duration **(C)** Open arm frequency and female **(D)** Distance moved **(E)** Open arm duration **(F)** Open arm frequency. OFT results for male **(G)** Distance Moved **(H)** Center Duration **(I)** Center frequency and female **(J)** Distance Moved **(K)** Center Duration **(L)** Center frequency.

Figure 5. Acoustic startle results (**A**) Male Baseline peak startle value (**B**) Male Percent change from baseline following USV presentation (**C**) Female Baseline peak startle value (**D**) Female Percent change from baseline following USV presentation.

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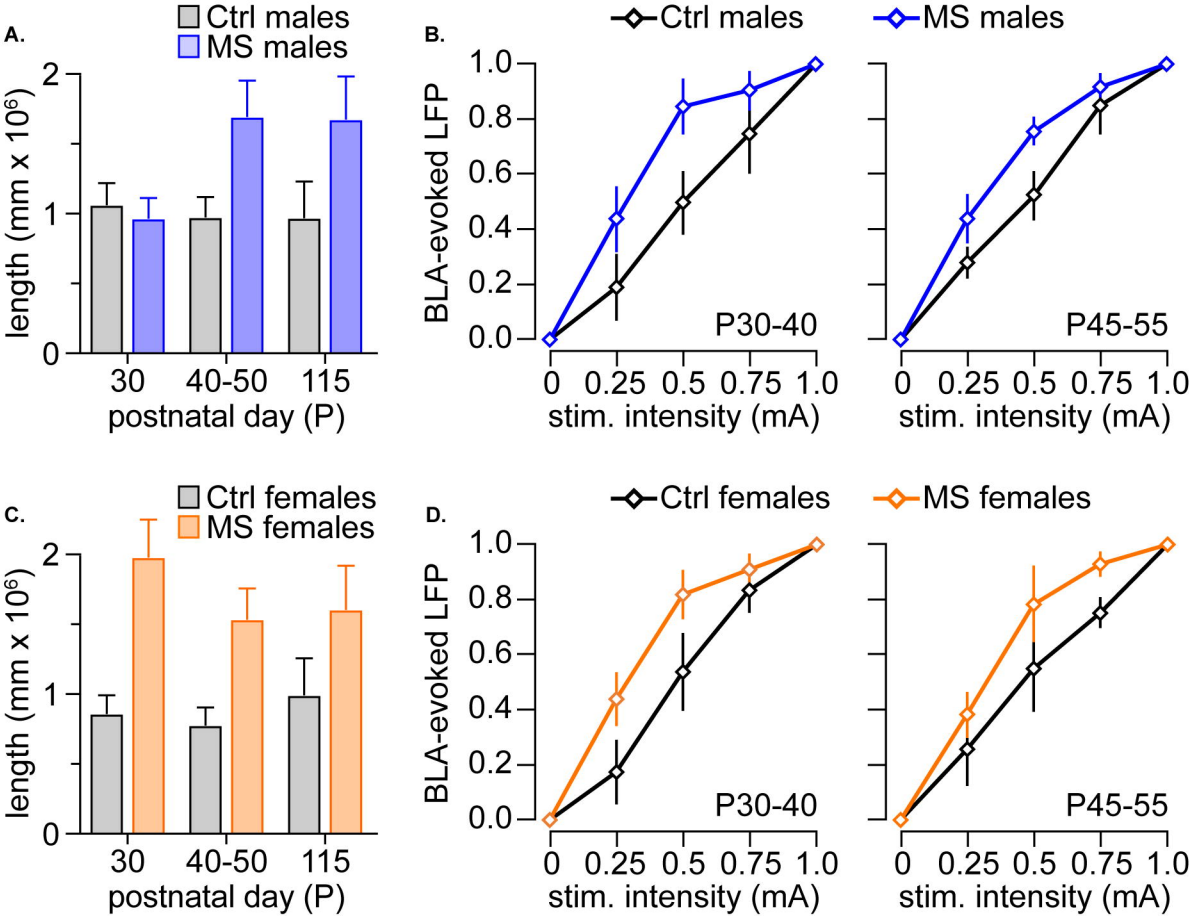
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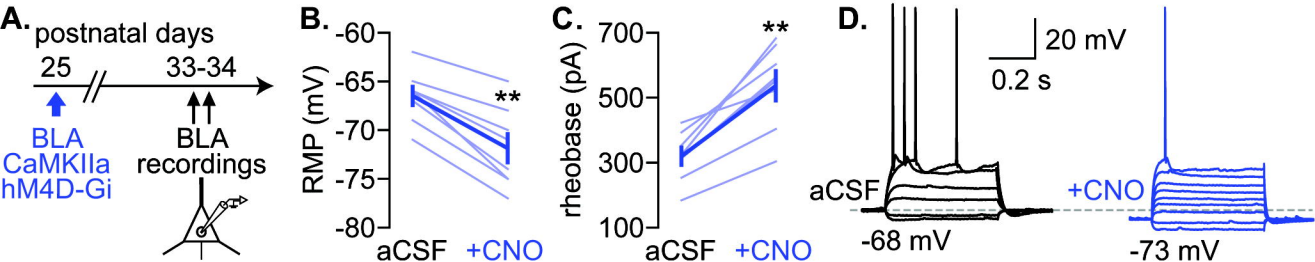
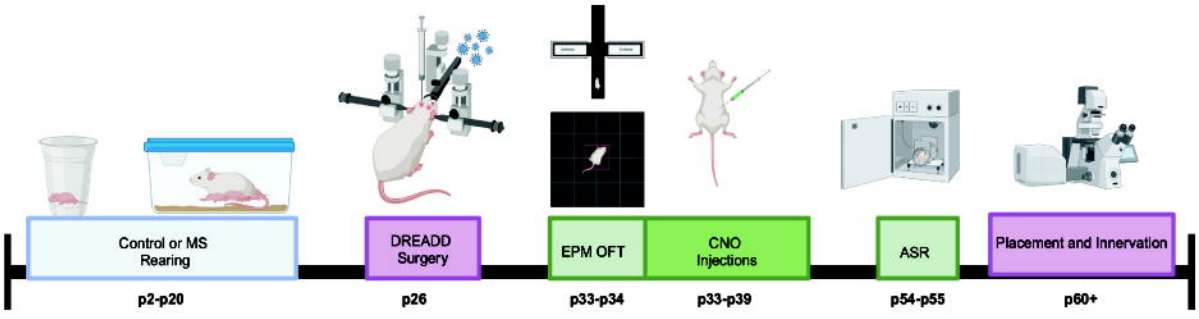
Ctrl males
MS males
length (mm x 10^6)
2
1
0
30
40-50
115
postnatal day (P)
B.
Ctrl males
MS males
BLA-evoked LFP
1.0
0.8
0.6
0.4
0.2
0.0
P30-40
P45-55
0
0.25
0.5
0.75
1.0
stim. intensity (mA)
C.
Ctrl females
MS females
length (mm x 10^6)
2
1
0
30
40-50
115
postnatal day (P)
D.
Ctrl females
MS females
BLA-evoked LFP
1.0
0.8
0.6
0.4
0.2
0.0
P30-40
P45-55
0
0.25
0.5
0.75
1.0
stim. intensity (mA)


A.
postnatal days
25
33-34
BLA
CaMKIIa
hM4D-Gi
BLA
recordings
B.
RMP (mV)
-60
-65
-70
-75
-80
**
aCSF
+CNO
C.
rheobase (pA)
700
500
300
100
**
aCSF
+CNO
D.
20 mV
0.2 s
aCSF
-68 mV
+CNO
-73 mV

A.



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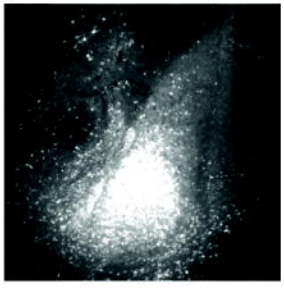
Representative BLA DREADD Injection



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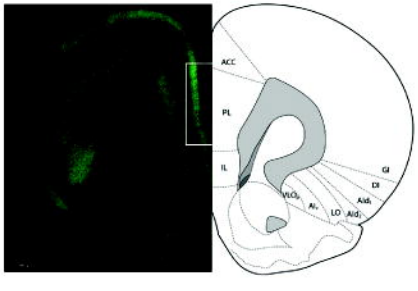
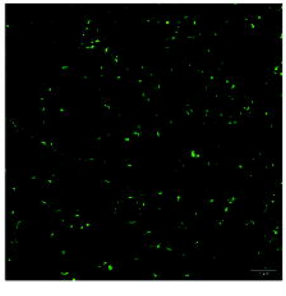
Representative BLA Terminals in PFC



D.

Representtive BLA-PFC Boutons



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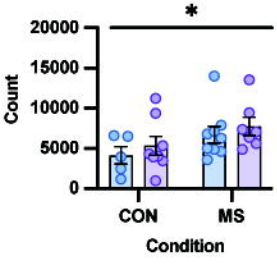

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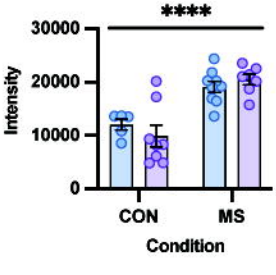

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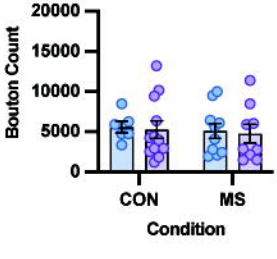

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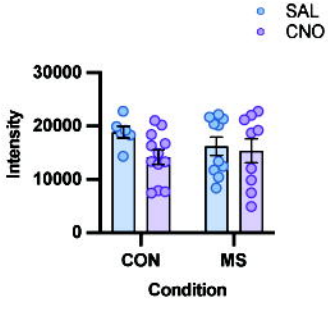

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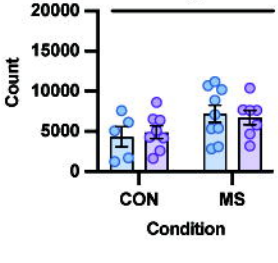

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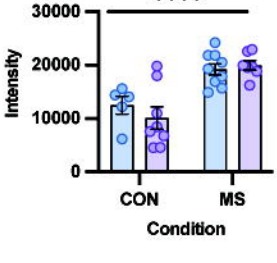

K.

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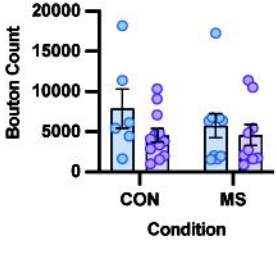

L.

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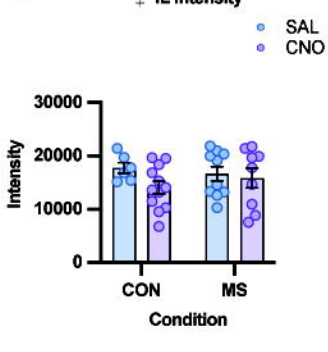

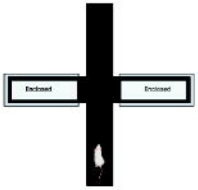
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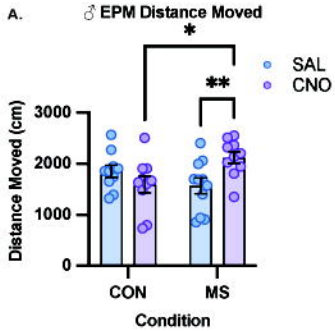
Enclosed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**
SAL
CNO
Distance Moved (cm)
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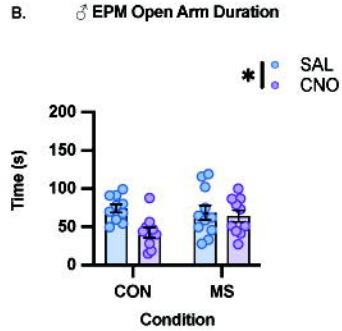
2000
1000
0
CON
MS
Condition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Time (s)
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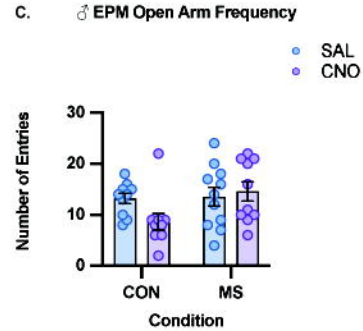
CON
MS
Condition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CNO
Number of Entries
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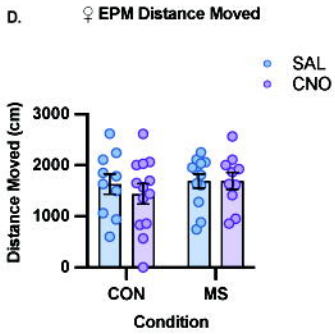
20
10
0
CON
MS
Condition


D.
♀ EPM Distance Moved
SAL
CNO
Distance Moved (cm)
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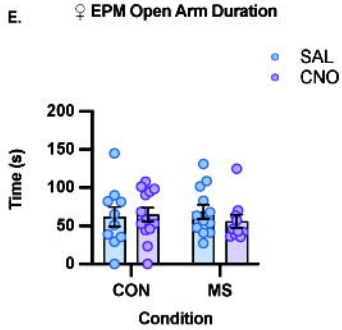
2000
1000
0
CON
MS
Condition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♀ EPM Open Arm Duration
SAL
CNO
Time (s)
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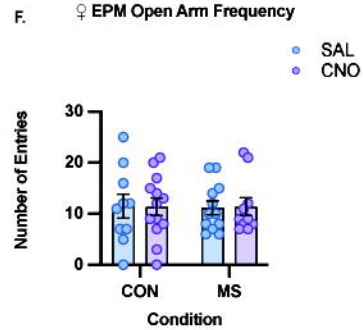
150
100
50
0
CON
MS
Condition


F.
♀ EPM Open Arm Frequency
SAL
CNO
Number of Entries
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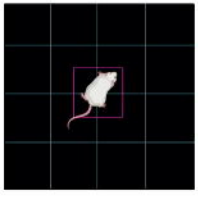
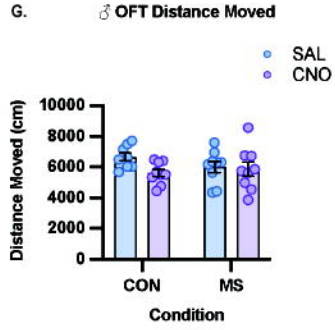
20
10
0
CON
MS
Condition




G.
♂ OFT Distance Moved
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CNO
Distance Moved (cm)
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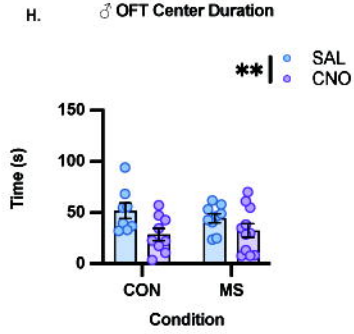
8000
6000
4000
2000
0
CON
MS
Condition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♂ OFT Center Duration
**
SAL
CNO
Time (s)
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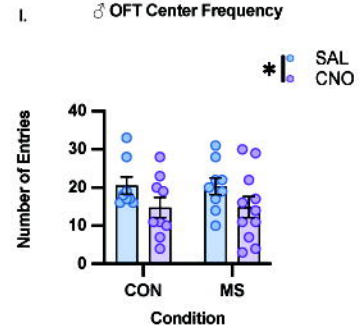
100
50
0
CON
MS
Condition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♂ OFT Center Frequency
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SAL
CNO
Number of Entries
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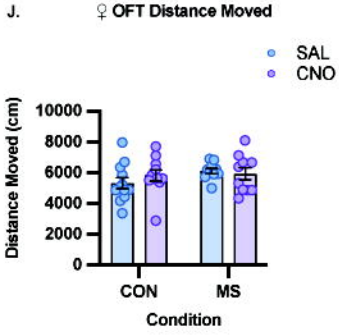
30
20
10
0
CON
MS
Condition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CNO
Distance Moved (cm)
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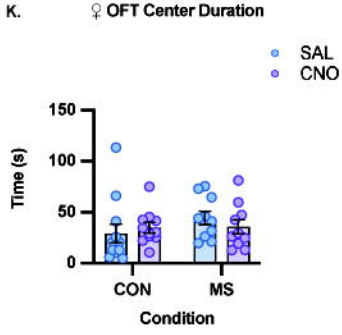
2000
0
CON
MS
Condition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CNO
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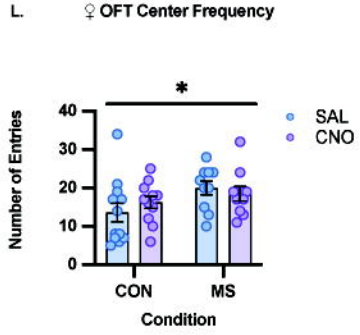
CON
MS
Condition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*
SAL
CNO
Number of Entries
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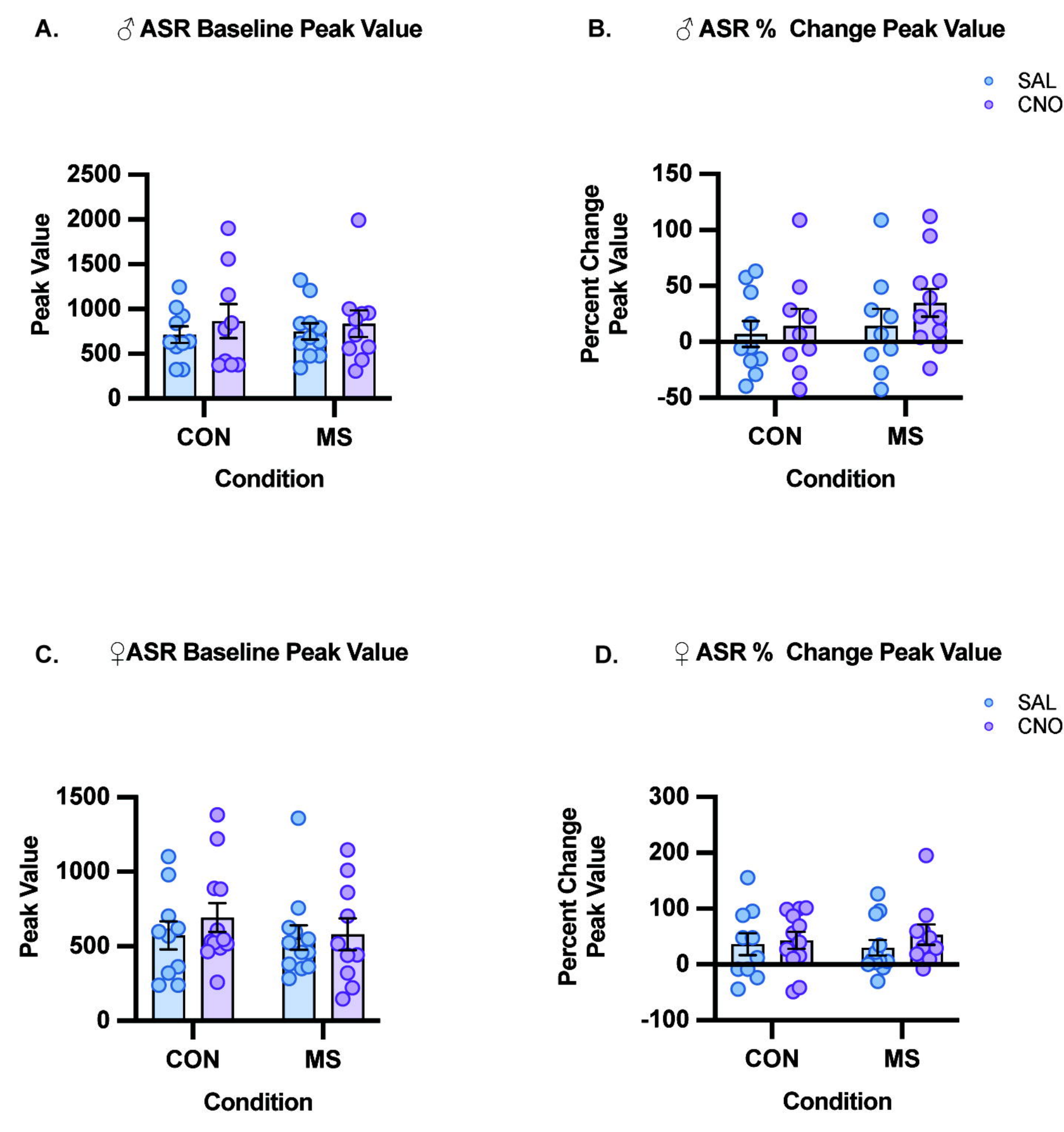
30
20
10
0
CON
MS
Condition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♂ ASR Baseline Peak Value
Peak Value
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500
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CON
MS
Condition
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♂ ASR % Change Peak Value
SAL
CNO
Percent Change Peak Value
150
100
50
0
-50
CON
MS
Condition
C.
♀ASR Baseline Peak Value
Peak Value
1500
1000
500
0
CON
MS
Condition
D.
♀ ASR % Change Peak Value
SAL
CNO
Percent Change Peak Value
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CON
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Condition