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

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Abstract

Early postnatal development of corticolimbic circuitry is shaped by the environment and is vulnerable to early life challenges. Prior work has shown that early life adversity (ELA) leads to hyperinnervation of glutamatergic basolateral amygdala (BLA) projections to the prefrontal cortex (PFC) in adolescence. While hyperinnervation is associated with later-life anxiety behaviors, the physiological changes underpinning corticolimbic and behavioral impacts of ELA are not understood. We tested whether postsynaptic BLA-driven PFC activity is enhanced in ELA-exposed animals, using the maternal separation (MS) model of ELA. PFC local-field potential following BLA stimulation was facilitated in MS-exposed adolescents. Since ELA increases activity of the early-developing BLA, while the PFC exhibits protracted development, we further examined impacts of glutamatergic BLA activity during early adolescence on later-life PFC innervation and heightened anxiety. In early adolescence, MS-exposed animals exhibited decreased anxiety-like behavior, and acute adolescent BLA inhibition induced behaviors that resembled those of MS animals. To examine long-lasting impacts of adolescent BLA activity on innervation, BLA-originating axonal boutons in the PFC were quantified in late adolescence after early adolescent BLA inhibition. We further tested whether late adolescent BLA-PFC changes were associated with anxious reactivity expressed as heightened acoustic startle responses. MS rearing increased BLA-PFC innervation and threat reactivity in late adolescence, however early adolescent BLA inhibition was insufficient to prevent MS effects, suggesting that earlier BLA activity or post-synaptic receptor rearrangement in the PFC drives altered innervation. Taken together, these

52 findings highlight both pre- and postsynaptic changes in the adolescent BLA-PFC circuit
53 following ELA.
54

55 **1. Introduction**

56 Early postnatal life is a dynamic period of brain development that involves a
57 coordinated symphony of circuit maturation. As many regions within the brain continue to
58 develop and mature postnatally, external stimuli impact this development and regulate
59 the degree of connectivity to other brain areas. Since subcortical limbic regions undergo
60 substantial synapse formation and pruning during early development while higher-order
61 areas such as the prefrontal cortex (PFC) mature throughout adolescence,[1–5] early
62 postnatal life and adolescence are two sensitive periods when limbic and prefrontal
63 regions form functional connections in response to external stimuli and neuronal activity.
64 Thus, activity within limbic regions over the course of development can affect the strength
65 and quantity of efferent connections to cortical areas.

66 The basolateral amygdala (BLA) has unique integration with aversion- and reward-
67 related circuitry and serves as the primary input nucleus of the amygdala.[6] Monosynaptic
68 BLA connectivity with the PFC[7–10] increases throughout adolescence in healthy rats,
69 facilitating the integration of emotional information with cognitive appraisals of the
70 environment.[11] Challenges to the early amygdala that alter local activity may therefore
71 induce activity changes within efferent projections, which may result in changes to
72 behavior when the circuit is recruited during adulthood.

73 Adolescence is a dynamic period when synapses undergo significant refinement
74 in the frontal cortex.[12,13] These reported changes are thought to involve glutamatergic

75 afferents with local remodeling of inhibitory-excitatory synaptic balance,[14,15] and coincide
76 with heightened emotional regulation.[16–18] Importantly, neuronal activity during sensitive
77 periods directly impacts pre- and post-synaptic plasticity within the PFC.[19] The present
78 study therefore investigated the impacts of early life environmental inputs and adolescent
79 BLA activity on the development of BLA-PFC circuitry and corresponding behaviors.

80 Theoretical frameworks regarding environmental influences on development posit
81 that postnatal brain circuitry has evolved to “expect” an appropriate quantity and quality
82 of caregiving stimuli in order to develop typically.[20] Disruptions to typical development can
83 be detrimental to the postnatal brain and can exert cascading effects on subsequent
84 maturation.[21–24] One such form of disruption includes early life adversity (ELA), where the
85 species-expectant environment is violated.[25,26] ELA can lead to several affective
86 consequences such as anxiety and major depressive disorder, which often emerge in
87 adolescence, and continue through adulthood.[13,27–34] The maternal separation (MS)
88 model of ELA in rodents induces corticolimbic and anxiety-related changes that resemble
89 those reported in ELA-exposed humans. Particularly, MS induces hyperinnervation of the
90 PFC from glutamatergic BLA projections,[18] as well as enhanced anxiety-like behavior as
91 measured by the acoustic startle response (ASR).[35] These effects were seen in
92 adolescent rodents as early as postnatal day (p)40 in male rats, and p30 in females, which
93 corresponds to early-mid adolescence. Therefore, we hypothesized that ELA leads to
94 pre- and post-synaptic changes to BLA-PFC connectivity via hyperactivity within the
95 developing BLA, and that preventing heightened presynaptic BLA activity during early-
96 mid adolescence would interrupt hyperinnervation and result in reduced anxiety
97 behaviors.

We first determined whether MS exposure led to facilitation of BLA-evoked local-field potentials (LFP) within the PFC in early-mid adolescence. Next, to test the role that BLA activity plays in the developing innervation to the PFC, BLA activity was manipulated via an inhibitory DREADD to reduce excitatory activity during early-mid adolescence. In an exploratory analysis, an open field test was performed following acute DREADD inhibition to test whether early adolescent BLA activity regulated anxiety-like behaviors differentially after MS exposure. Histology was performed in late adolescence to determine if earlier BLA inhibition prevented the establishment of long-range glutamatergic PFC hyperinnervation. Animals were also tested in late adolescence for enhanced acoustic startle response (ASR) at baseline or following an anxiety-provoking social threat cue (playback of 22-kHz ultrasonic vocalizations), to determine whether prevention of BLA-PFC hyperinnervation was associated with prevention of enhanced ASR.

2. Materials and 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 experimental subjects were bred in house (details in Supplemental Materials). Whole litters were randomly assigned to control (CON) or maternal separation (MS) rearing conditions. All separations lasted 4 hours each day beginning at 0900h with pups kept in

a separate room from the dam. Upon completion of MS 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

Early-mid adolescent (p30-45) rats were anesthetized and a concentric bipolar electrode was used to record within the prelimbic region while stimulating the BLA through a computer-controlled pulse generator (details in Supplemental Materials). 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). Viral injections are detailed in Supplemental Materials. Rats were allowed to recover for 1 week before behavioral testing. Daily from p33-39, rats were weighed and given an intraperitoneal (i.p) 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. Functional validation methods are detailed in Supplemental Materials.

2.4 Behavioral Assays

For each behavioral assay, rats were brought into the behavioral suite and allowed to habituate for 15 minutes.

2.4.1 Open Field Test (OFT)

For early adolescent testing (p33), 30 minutes after the first CNO or SAL injection, rats were placed into the open field (100 cm x 100 cm black acrylic arena). Video monitoring of behavior was done via an overhead CCTV camera (Panasonic WV-CP500, Secaucus, NJ), and behavioral scoring was done via Noldus Ethovision 9.0 Behavioral Tracking software. The central zone of the arena was defined as the inner 30x30 cm portion of the arena (Figure 4A).

2.4.2 Acoustic Startle Response (ASR)

On p54, rats 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. Twenty-four hours later, rats underwent a second ASR test, during which ASR was measured following exposure to a five-minute 22 kHz rat ultrasonic vocalization (USV) recording. For more details see Supplemental Materials.

2.5 Microscopy and Analysis

2.5.1 Viral Expression

159 Histological procedures are detailed in Supplemental Materials. In order to quantify the
160 amount of DREADD expression within and outside of the BLA, slices were imaged on a
161 Zeiss Imager M.2 microscope for bolus verification. Slices with dual hemispheric
162 expression in the BLA underwent volumetric analysis via the Cavalieri probe within
163 StereoInvestigator. Sections containing DREADD expression were analyzed at 2.5X
164 magnification to determine the extent of bolus infection within and spread outside of the
165 BLA. Average BLA volume was previously measured in a subset of animals, and the
166 volume of DREADD expression was quantified as a percent of the total BLA volume.
167 Animals with a total bolus volume that filled <50% of the BLA were excluded from
168 analysis.

169 *2.5.2 Axonal Innervation*

170 The corresponding PFC tissue of all included animals was processed using
171 immunohistochemistry (IHC) to identify mCherry expression. Sections containing the
172 infralimbic (IL) and preimbic (PL) regions were prepared for immunohistochemistry as
173 detailed in Falcy et al. 2020[36] (details in Supplemental Materials). Z-stacks were analyzed
174 for axonal bouton count and intensity.

175 *2.6 Statistical Analysis*

176 GraphPad Prism 10.0 Software was used for all data analyses and graphical
177 representation. Sample sizes were determined by power analyses using effect sizes from
178 prior experiments. Sex x rearing x treatment 3-way ANOVAs were conducted (with
179 frequency as repeated measures in BLA-evoked PFC LFP analyses), and when main

180 effects of sex or interactions with sex were observed, 2-way ANOVAs with factors of
181 treatment and rearing were conducted for each sex with Post-hoc Tukey's multiple
182 comparison's tests when appropriate. For simplicity, full statistical details are only
183 highlighted for significant comparisons in the Results section. For all statistical details,
184 see Supplemental Materials. Of note, three animals were removed from ASR analysis
185 due to data file corruption.
186
187 **3. Results**
188 *3.1 MS leads to facilitation of BLA-evoked local field potential in the early adult PFC.*
189 3-way ANOVA revealed a main effect of rearing ($F_{1,20} = 102.0$; $p < 0.0001$; partial $\eta^2 =$
190 0.732) and a rearing x intensity interaction ($F_{4,80} = 38.23$; $p < 0.0001$; partial $\eta^2 = 0.563$)
191 on PFC local field potential (Figure 1A-C; n=6/group), with MS exposure facilitating BLA-
192 evoked activity in both males (Figure 1B) and females (Figure 1C). These findings
193 indicate that exposure to MS results in increased BLA-evoked activity within the PFC in
194 early-mid adolescence.
195 *3.2 BLA inhibition during mid-adolescence does not prevent increased BLA-PFC*
196 *connectivity after MS*
197 Figure 2A summarizes the DREADD strategy used to inhibit the BLA. Ex-vivo recordings
198 of BLA pyramidal neurons show the effect of the inhibitory DREADD at 8-9 days post viral
199 injection (Figure 2B-D).
200 A subset of rats (n=6-8) were sacrificed following behavioral testing (p56-60) to
201 quantify BLA innervation to the PFC via axonal bouton count and fluorescent intensity.
202 Three-way ANOVA revealed a sex x rearing interaction with a moderate effect size on

203 bouton count in the PL ($F_{1,57}$ = 3.245; p=0.0770; partial η^2 = 0.054) and the IL ($F_{1,57}$ =
204 3.617; p=0.0623; partial η^2 = 0.06). Due to the presence of a moderate effect size for the
205 sex x rearing interaction, each sex was analyzed separately, with two-way ANOVAs
206 assessing effects of rearing condition and treatment (CNO or SAL). In males, MS
207 increased PL bouton count ($F_{1,25}$ = 4.661; p=0.0407; partial η^2 = 0.157; Figure 3E) and IL
208 bouton count ($F_{1,25}$ = 5.098; p=0.0329; partial η^2 = 0.169; Figure 3I). However, no effects
209 were observed in females.

210 Three-way ANOVA also revealed a main effect of rearing on bouton intensity in the
211 PL ($F_{1,57}$ = 10.87; p=0.0017; partial η^2 = 0.160) and IL ($F_{1,57}$ = 15.58; p=0.0002; partial η^2
212 = 0.215), as well as a sex x rearing interaction in bouton intensity within the PL ($F_{1,57}$ =
213 15.00; p=0.0003; partial η^2 = 0.208) and IL ($F_{1,57}$ = 13.19; p=0.0006; partial η^2 = 0.160).
214 Due to the presence of a sex x rearing interaction, each sex was analyzed separately,
215 revealing another male-specific main effect of rearing on PL bouton intensity ($F_{1,25}$ =
216 36.09; p=<0.0001; partial η^2 = 0.591, Figure 3G), and IL bouton intensity ($F_{1,25}$ = 29.53;
217 p=<0.0001; partial η^2 = 0.541; Figure 3K).

218 Treatment with CNO had no effects on either measure (count or fluorescent
219 intensity). Results of all statistical comparisons are summarized in Supplementary Tables
220 5-7. These findings indicate that MS rearing induces hyperinnervation of the PFC from
221 the BLA in late adolescent male animals only, and that BLA inhibition during early-mid
222 adolescence is insufficient to reverse these changes.

223

224

225 *3.3 Exposure to MS and acute BLA inhibition alters adolescent behavior in a sex*
226 *dependent manner*

227 Rats with inhibitory DREADD expression in the BLA were given an i.p injection of
228 CNO (2 mg/kg) or SAL 30 minutes before the open field test on p33. Animals (n=10-12
229 per condition) were assessed for time spent, latency to enter, and number of entries into
230 the center zone of the arena (Figure 4D-K).

231 A three-way ANOVA revealed a moderate effect size sex x treatment interaction
232 ($F_{1,79}$ = 3.061; p=0.081; partial η^2 = 0.037) on distance moved. Two-way ANOVAs within
233 each sex assessed effects of rearing condition and treatment (CNO or SAL). No effects
234 were seen in male animals (Figure 4D). In female animals, a main effect of treatment
235 ($F_{1,41}$= 4.526; p=0.0394; partial η^2 = 0.099) was found indicating that females treated with
236 CNO had increased locomotion (Figure 4E).

237 Three-way ANOVAs revealed main effects of sex ($F_{1,79}$ = 7.019; p=0.0098; partial
238 η^2 = 0.084) and treatment ($F_{1,79}$ = 7.148; p=0.0092; partial η^2 = 0.085) for the latency to
239 enter the center. Analyses also revealed a rearing x treatment interaction ($F_{1,79}$ = 5.098;
240 p=0.0268; partial η^2 = 0.062). Two-way ANOVAs assessed effects within each sex of
241 rearing condition and treatment. In male animals, a main effect of treatment ($F_{1,38}$ = 4.475;
242 p=0.0414; partial η^2 = 0.11) and a rearing x treatment interaction ($F_{1,38}$ = 4.235; p=0.0469;
243 partial η^2 = 0.105) were found for the latency to enter the center (Figure 4F). Post-hoc
244 analyses revealed differences between control SAL and control CNO treated animals
245 (p=0.0129) as well as control SAL and MS SAL animals (p=0.0258) indicating that control
246 animals treated with CNO as well as MS animals treated with SAL exhibited a decrease
247 in the latency to enter the center compared to control SAL animals. In female animals, a

248 significant main effect of treatment ($F_{1,41}$ = 4.185; p=0.0472; partial η^2 = 0.093) was found
249 in the latency to enter the center (Figure 4G), with CNO treated animals showing a
250 decreased latency to enter the center.

251 Three-way ANOVAs revealed a rearing x treatment interaction for center duration
252 ($F_{1,79}$ = 4.912; p=0.0296; partial η^2 = 0.059). Two-way ANOVAs further assessed effects
253 of rearing condition and treatment within each sex. No effects were seen in male animals
254 (Figure 4H). In female animals, analyses revealed a main effect of rearing ($F_{1,41}$ = 4.392;
255 p=0.0427; partial η^2 = 0.101) and a rearing x treatment interaction ($F_{1,41}$ = 4.412;
256 p=0.0422; partial η^2 = 0.101) on center duration (Figure 4I). Post-hoc analyses revealed
257 differences between control and MS SAL treated females (p=0.0045) such that MS
258 animals spent more time in the center compared to controls, and that this was not
259 mediated by CNO treatment.

260 A three-way ANOVA revealed a main effect of rearing ($F_{1,79}$ = 5.353; p=0.0234;
261 partial η^2 = 0.065) and a rearing x treatment interaction ($F_{1,79}$ = 6.176; p=0.0151; partial
262 η^2 = 0.074) for center frequency. Two-way ANOVAs within each sex assessed effects of
263 rearing condition and treatment. In male animals, a moderate effect sized rearing x
264 treatment interaction was observed ($F_{1,38}$ = 3.561; p=0.0677; partial η^2 = 0.087; Figure
265 4J). Female animals displayed a significant main effect of rearing ($F_{1,41}$ = 11.24;
266 p=0.0018; partial η^2 = 0.219; Figure 4K), such that MS females entered the center more
267 than control-reared females, mirroring the increased duration. These findings indicate that
268 MS rearing results in decreased anxiety-like behavior in the OFT, and that BLA inhibition
269 in early adolescence results in an MS-like phenotype in control animals, while having no
270 effect on MS animals.

3.4 Early adolescent BLA inhibition does not prevent ASR changes in MS-exposed late adolescence

On p54 (late adolescence), rats were tested for baseline startle levels to 30 white noise bursts. Latency to startle, peak startle time, and peak startle value were assessed with three-way ANOVAs. Previously established differences between males and females were observed and are likely related to animal size; main effects of sex were observed on baseline latency to startle ($F_{1,76}$ = 7.821; p=0.0065; partial η^2= 0.093), and baseline peak value ($F_{1,76}$ = 5.598; p=0.0205; partial η^2= 0.069). No effects of rearing or treatment were observed on baseline ASR in late adolescence (Figure 5A-C). On p55, rats were presented with a five-minute playback of a 22 kHz USV recording and then immediately tested for startle levels to 30 white noise bursts. Latency to startle, peak startle time, and peak startle value were assessed with three-way ANOVAs. Main effects of rearing were found for latency to startle ($F_{1,76}$ = 4.855; p=0.0306; partial η^2= 0.060; Figure 5D), and peak time ($F_{1,76}$ = 5.210; p=0.0252; partial η^2 = 0.064; Figure 5E), with MS-exposed animals startling more quickly than controls following USV playback. Main effects of sex were found for latency to startle on Day 2 ($F_{1,76}$ = 4.082; p=0.047; partial η^2= 0.051) as well as peak value on Day 2 ($F_{1,76}$ = 10.54; p=0.0017; partial η^2= 0.122). No effects of treatment were observed on USV mediated ASR. Rats were also evaluated for the percent change from baseline following USV presentation. A main effect of rearing was again found in latency to startle ($F_{1,76}$ = 4.081; p=0.0469; partial η^2 = 0.051; Figure 5G) and peak time ($F_{1,76}$ = 5.195; p=0.0255; partial η^2 = 0.064; Figure 5H), indicating that MS animals startled quicker than control animals following USV playback. A main effect of rearing was also observed for percent change in peak value ($F_{1,76}$ = 4.856; p=0.0306;

294 partial $\eta^2 = 0.060$) No main effects of treatment with CNO were found in any analyses,
295 and no interactions of rearing, sex, or treatment were present, therefore further analyses
296 were not conducted. These findings indicate that BLA inhibition via CNO in early-mid
297 adolescence had no overall effect on ASR in late adolescence.
298
299 **4. Discussion**
300 The current study identifies dissociable pre- and post-synaptic contributions to sex-
301 and age-specific effects of ELA on corticolimbic connectivity and behavior. We sought to
302 investigate if an adverse rearing environment altered BLA-PFC connectivity via aberrantly
303 high adolescent BLA activity, and if these changes drove anxiety-like behavioral
304 responses in early and late adolescence. We observed that MS rearing yielded
305 postsynaptic facilitation of PFC responsivity to BLA activation by mid-adolescence. We
306 hypothesized that presynaptic inhibition of the BLA during this period would prevent MS-
307 driven hyperinnervation from the BLA to the PFC, as well as MS-driven changes to
308 anxiety-like behaviors.
309 As previously observed by quantifying BLA axon terminals,[18] here, MS resulted in
310 hyperinnervation of the PFC as assessed by a more functionally relevant measure,
311 namely the number of axonal boutons. However, this increase was not attenuated by BLA
312 inhibition from p33-p39, suggesting that sustained adolescent activity of the BLA does not
313 regulate the development of presynaptic connectivity with the PFC. Additionally, no
314 increases in innervation were seen in females, indicating that by late adolescence, any
315 previously observed increases in BLA-PFC connectivity may have pruned back to control
316 levels. Both male and female rats exhibit significant pruning within the PFC between

317 adolescence and adulthood, however females display greater losses than males.[37] The
318 age at which peak synaptic pruning of the PFC occurs in males and females remains
319 unknown,[38] which allows for the possibility that inhibition of BLA activity in early-mid
320 adolescence had differential effects on BLA-PFC innervation across sexes. It is therefore
321 possible that the period of accelerated BLA-PFC innervation induced by MS rearing
322 occurred at an earlier timepoint in females, and thus by the time histology was performed
323 (p56), both control and MS females had reached adult innervation levels. Additionally,
324 previous work from our group showed increases in female BLA-PFC innervation that were
325 already apparent by p28; therefore, if BLA activity does provoke innervation to the PFC
326 in females, inhibition from p33-39 may have been too late to affect any MS-evoked
327 changes. Thus, it will be important to repeat this study with an earlier intervention time
328 point as well as earlier ages of assessment to capture any sex-specific effects of juvenile
329 BLA inhibition on adolescent innervation.

330 Since inhibiting presynaptic activity was not sufficient to reverse long-range
331 hyperinnervation, it is possible that postsynaptic plasticity within the PFC precedes and
332 drives these connectivity changes. For example, MS results in heightened expression of
333 GluN2A-containing NMDA receptors within the PL[39] as well as reduced GluA2-positive
334 AMPA receptors.[40] While the PFC displays late-maturing connectivity with regions such
335 as the BLA,[11,18] the postnatal environment is capable of directly impacting PFC activity
336 and receptivity,[41,42] and the nature of glutamate receptivity can influence development of
337 inputs.[43] Importantly, post synaptic NMDAR increases induced by MS have been shown
338 to correlate with anxiety-like behaviors,[44] and thus in concert with our findings here

339 showing a lack of structural changes following presynaptic inhibition, we posit that
340 postsynaptic mechanisms may be more critical for ELA induced outcomes in the PFC.

341 We observed anxiety-like ASR in late adolescence such that MS animals
342 responded to anxiogenic USV playback with elevated threat responsivity. Contrastingly,
343 MS-exposed animals tested in early adolescence exhibited faster center entry (males) or
344 increased center time (females), suggesting a decrease in anxiety-like behavior. Our
345 findings are largely consistent with other assessments of MS-reared animals in early
346 adolescence,[45–47] where by late adolescence, MS-reared animals display reduced center
347 time.[48] Importantly, the open field task has also been used as a measure of risk-seeking,[49]
348 and ELA is known to increase risk-seeking and impulsivity in adolescence.[50–59] It is
349 possible therefore that the anxiolytic behaviors seen during the OFT in early adolescence
350 may be attributable to increases in risk-seeking behaviors, and that this behavioral
351 phenotype shifts as animals continue to mature. Notably, acute BLA inhibition in
352 adolescent controls resulted in open field behavior that mirrored MS-exposed animals,
353 such that control animals treated with CNO had shorter latencies to enter the center of
354 the open field arena. Further work will tease apart the discrete mechanisms linking MS to
355 risk-seeking, and how developing BLA activity differentially drives these behaviors during
356 adolescence.

357 MS does not appear to alter later-life ASR via hyperactivity of the developing BLA,
358 as early-mid adolescent BLA inhibition did not prevent MS-induced changes. The
359 amygdala regulates anxiety-related enhancement of ASR reflex, with the BLA particularly
360 sensitive to ambiguous or distal threats from the environment.[60,61] Here we tested the
361 impacts of MS and early BLA manipulation on potentiation of ASR by a distal social threat,

namely playback of 22kHz USV.[57] Previous work has shown enhanced BLA activity following presentation of a 22 kHz USV,[62] indicating a role of the BLA in the processing of this fearful stimuli. Control-reared animals display a potentiated ASR following USV playback, while MS alters this potentiated response.[35] Here we observed that while MS-exposed animals exhibited increased potentiation of the ASR, early-mid adolescent BLA inhibition was not sufficient to rescue the heightened startle response induced by MS.

These findings should be considered with the limitation that 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 following MS exposure, which can alter typical stress responses.[63] We are also unable to causally link altered BLA-PFC innervation or functional connectivity with ASR. Taken together, we observed that postnatal ELA impacts development of both pre- and post-synaptic BLA-PFC connectivity as well as anxiety-related behaviors across adolescence. However, the insufficiency of pre-synaptic BLA inhibition to alter later BLA-PFC connectivity or anxiety-related behavior highlights the importance of investigating early post-synaptic changes in the PFC that may be driving MS-induced corticolimbic functional connectivity changes.

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Author Contributions
Heather C. Brenhouse and Kuei Y. Tseng and designed the study. Caitlyn R. Cody performed and analyzed the behavioral and axonal innervation data under the supervision of Heather C. Brenhouse. Janelle Lardizabal and Chaney McKnight assisted Caitlyn R. Cody with behavioral experiments, histology, and data collection. Emilce Artur de la Villarmois and Anabel Miguelez Fernandez performed all electrophysical experiments and data analysis under the supervision of Kuei Y. Tseng. Caitlyn R. Cody, Heather C. Brenhouse, and Kuei Y. Tseng wrote the manuscript. Caitlyn R. Cody, Emilce Artur de la Villarmois, and Kuei Y. Tseng prepared the figures.

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Competing Interests
The authors have nothing to disclose

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Figure Legends

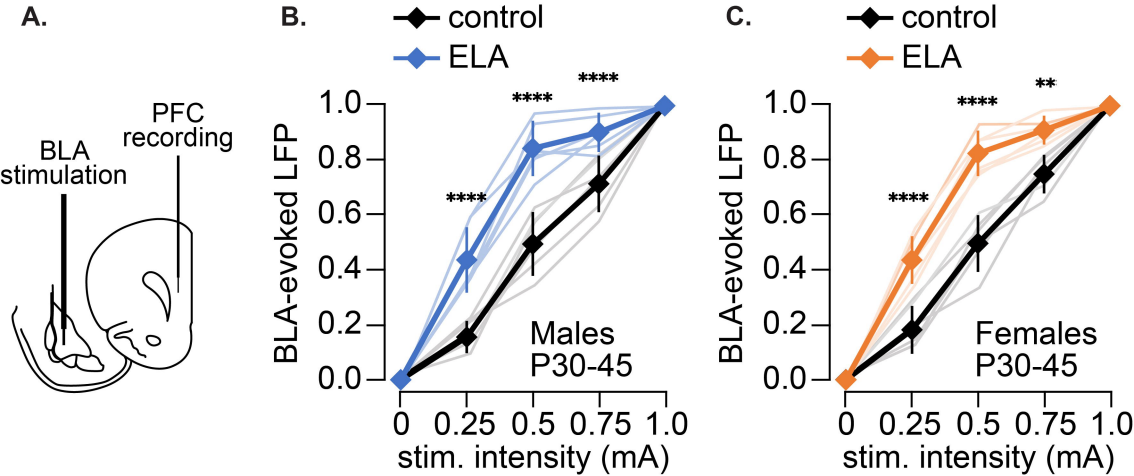
Figure 1. Effects of ELA on BLA-evoked PFC LFP. (**A**) Illustration of BLA stimulation and PFC recording sites. (B) Prelimbic PFC response to BLA stimulation at increasing intensities is enhanced by p30 as shown by local field potential (LFP) recordings in males (****p<0.0001, **p<0.01 difference between control and MS animals, after correction for multiple comparisons; N=6/group). (**C**) The prelimbic PFC LFP response to BLA stimulation is also enhanced by p30 in females (****p<0.0001, **p<0.01 difference between control and MS animals, after correction for multiple comparisons; N=6/group).

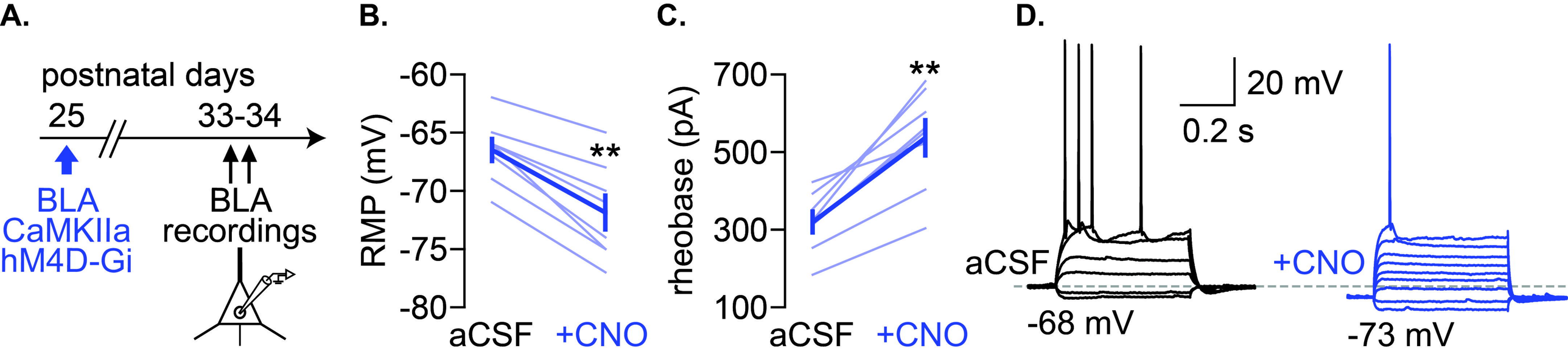
Figure 2. DREADD validation. (**A**) Timeline of DREADD delivery and ex-vivo recordings of BLA pyramidal neurons. (**B**) Bath application of CNO (10μM, 10 min) markedly hyperpolarized the resting membrane potential of all pyramidal neuron tested (**p<0.01, unpaired t-test, t=3.114, df=14). (**C**) Accordingly, a significant increase in the rheobase (minimal somatic current (pA) to elicit an action potential) was observed all neurons following bath application of CNO (**p<0.01, unpaired t-test, t=4.124, df=14). (**D**) Example traces of a BLA pyramidal neuron showing the inhibitory effect of CNO as revealed by the reduced current evoked action potential and hyperpolarized resting membrane potential.

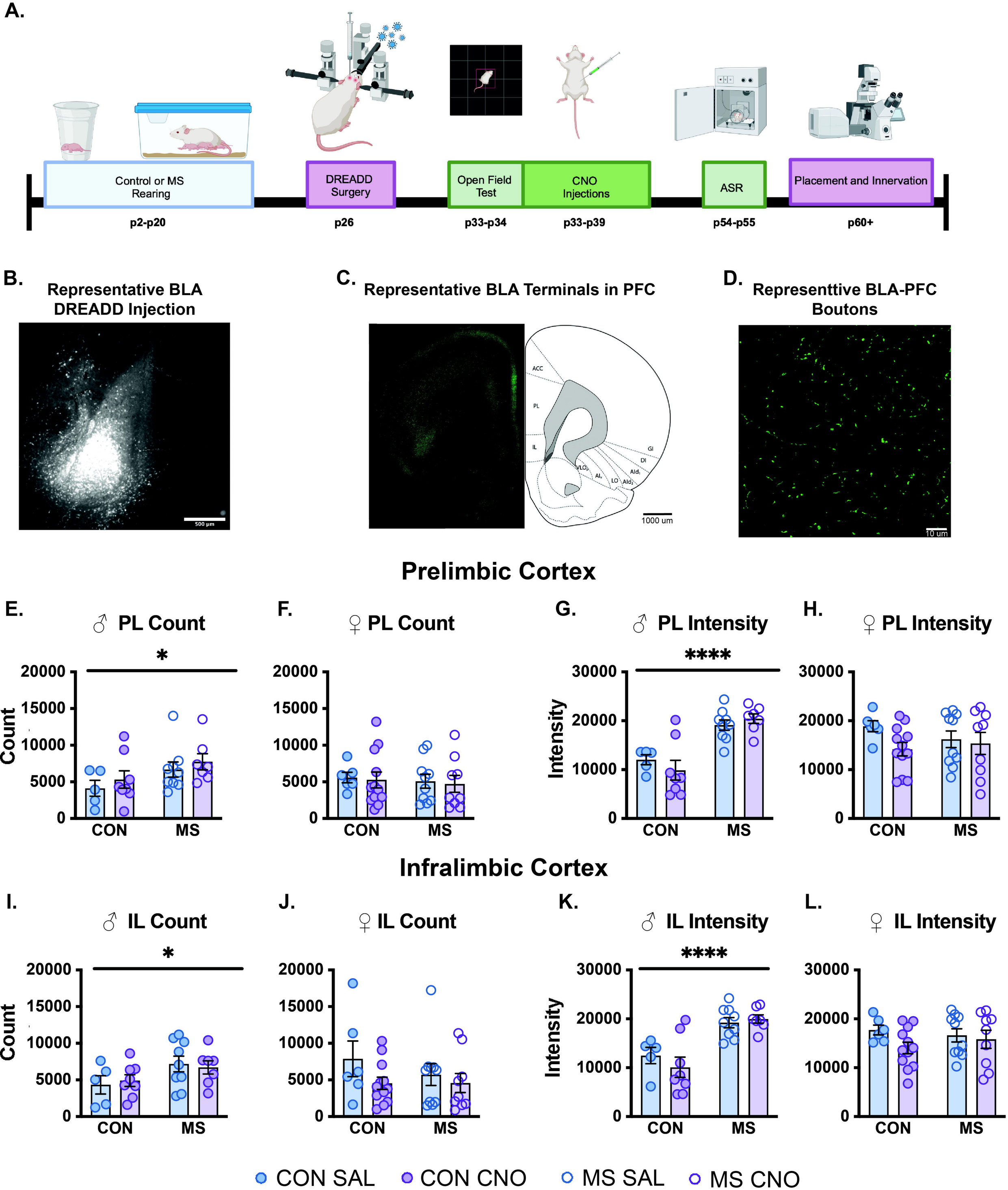
Figure 3. Effects of ELA with or without BLA inactivation during early-mid adolescence on late adolescent PFC innervation. **(A)** Timeline of DREADD experiment. **(B)** Representative DREADD injections in the BLA **(C-D)** BLA-PFC terminals in the PFC. **(E)** Male PL bouton count. **(F)** Female PL bouton count. **(G)** Male PL bouton intensity. **(H)** Female PL bouton intensity. **(I)** Male IL bouton count. **(J)** Female IL bouton count. (**K)** Male IL bouton intensity. **(K)** Female IL bouton intensity. Means ± SEM with individual data points are shown. (*p < 0.05 ****p<0.0001 rearing effect). n=5-12/group.

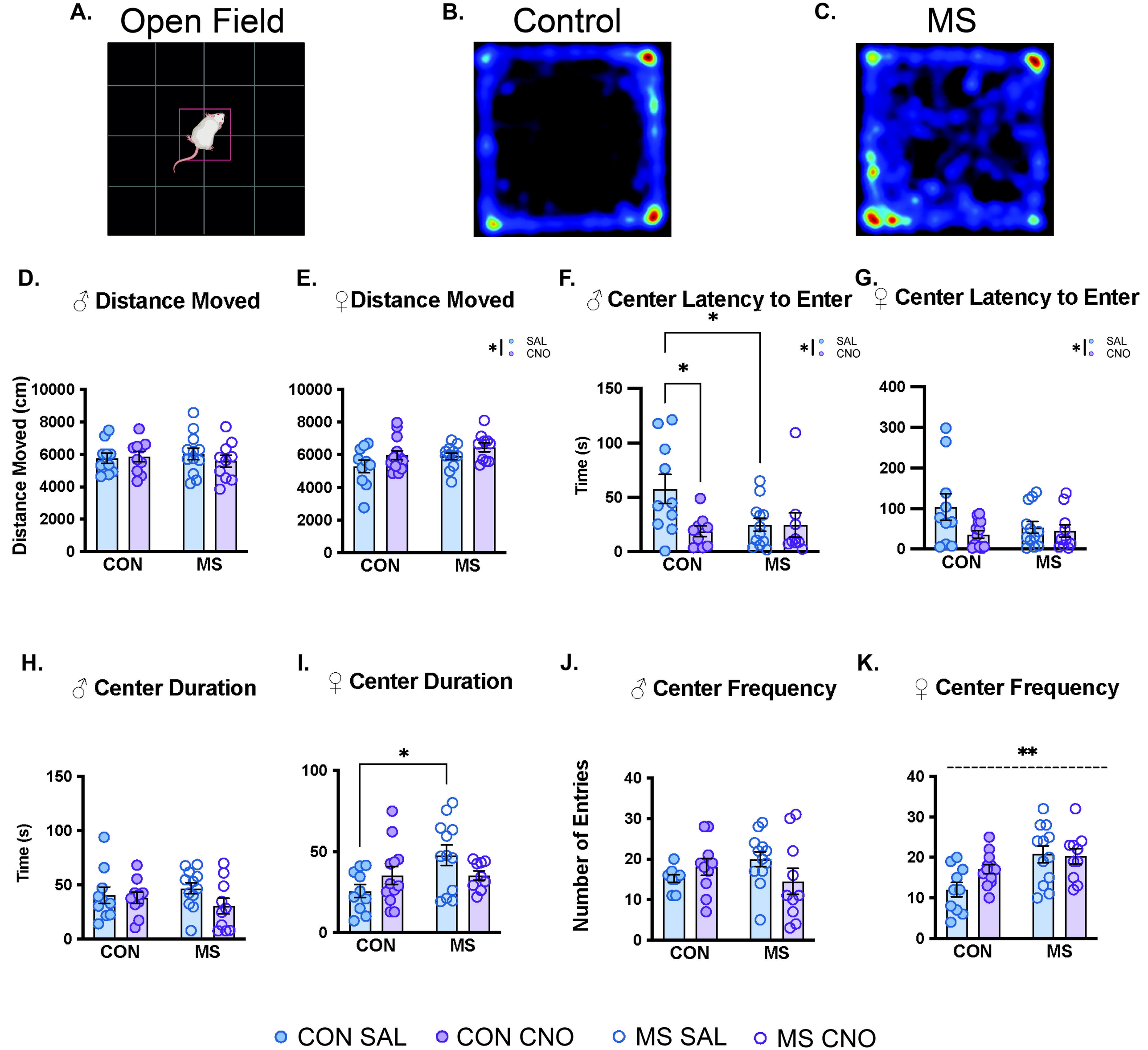
Figure 4. Effects of ELA with or without acute BLA inactivation on open field behavior during early adolescence. **(A)** Schematic of open field arena. **(B-C)** Representative heat maps depicting control and MS animal movement within the open field arena. OFT results for male **(D)** Distance Moved **(H)** Center Duration **(F)** Center latency to enter **(J)** Center frequency and female **(E)** Distance Moved **(I)** Center Duration **(G)** Center latency to enter **(K)** Center frequency. Means ± SEM with individual data points are shown. (*p < 0.05; **p<0.005; dashed line denotes main effect of rearing). n=10-13/group.

Figure 5. Effects of ELA with or without BLA inactivation during early-mid adolescence on late adolescent acoustic startle. (**A**) Day 1 latency to startle. (**B**) Day 1 latency to peak startle value. **(C)** Day 1 peak startle value. **(D)** Day 2 latency to startle. (**E**) Day 2 latency to peak startle value. **(F)** Day 2 peak startle value. **(G)** Percent change latency to startle. (**H**) Percent change latency to peak startle value. **(C)** Percent change peak startle value. Means ± SEM with individual data points are shown. (*p < 0.05 rearing effect). n=9-12/group.


A.
PFC recording
BLA stimulation
B.
control
ELA
BLA-evoked LFP
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
Males
P30-45
0
0.25
0.5
0.75
1.0
stim. intensity (mA)
C.
control
ELA
BLA-evoked LFP
1.0
0.8
0.6
0.4
0.2
0.0
****
****
**
Females
P30-45
0
0.25
0.5
0.75
1.0
stim. intensity (mA)

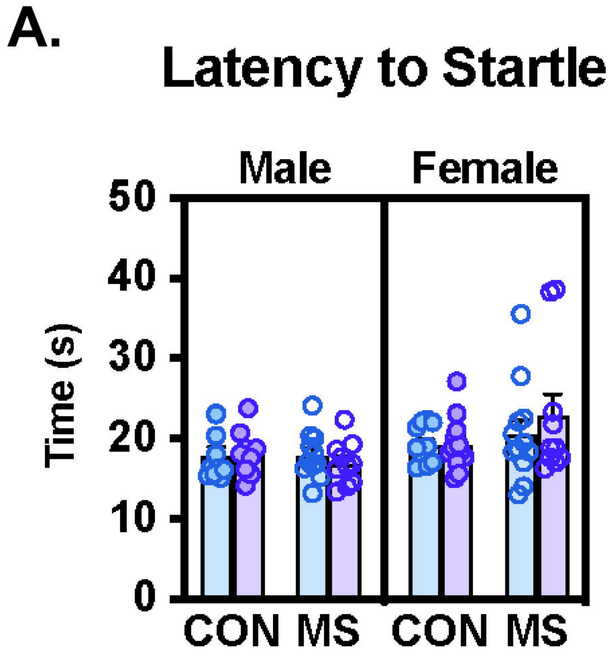

A.
postnatal days
25
33-34
BLA
CaMKIIa
hM4D-Gi
BLA
recordings
B.
RMP (mV)
-60
-65
-70
-75
-80
**
aCSF
+CNO
C.
rheobase (pA)
700
500
300
100
**
aCSF
+CNO
D.
20 mV
0.2 s
aCSF
+CNO
-68 mV
-73 mV


A.
Control or MS Rearing
DREADD Surgery
Open Field Test
CNO Injections
ASR
Placement and Innervation
p2-p20
p26
p33-p34
p33-p39
p54-p55
p60+
B.
Representative BLA DREADD Injection
500 µm
C.
Representative BLA Terminals in PFC
ACC
PL
IL
GI
DI
1000 um
D.
Representtive BLA-PFC Boutons
10 um
Prelimbic Cortex
E.
♂ PL Count
F.
♀ PL Count
G.
♂ PL Intensity
H.
♀ PL Intensity
Count
Intensity
CON
MS
Infralimbic Cortex
I.
♂ IL Count
J.
♀ IL Count
K.
♂ IL Intensity
L.
♀ IL Intensity
CON SAL
CON CNO
MS SAL
MS CNO

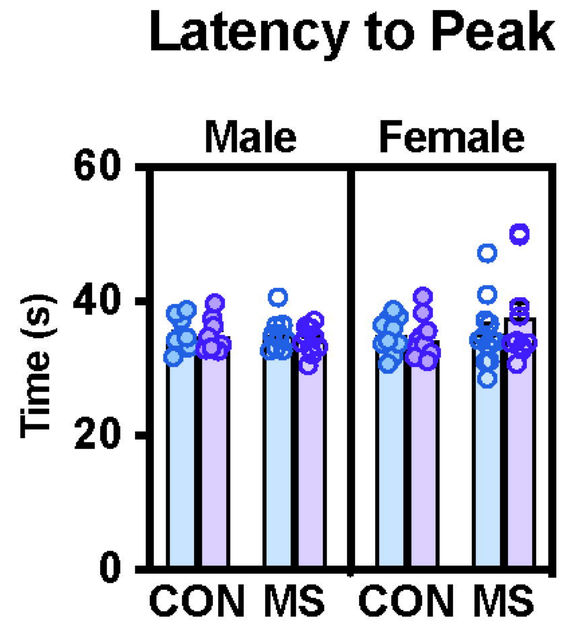

A.
Open Field
B.
Control
C.
MS
D.
♂ Distance Moved
Distance Moved (cm)
10000
8000
6000
4000
2000
0
CON
MS
E.
♀Distance Moved
*
SAL
CNO
F.
♂ Center Latency to Enter
Time (s)
150
100
50
0
G.
♀ Center Latency to Enter
400
300
200
100
H.
♂ Center Duration
I.
♀ Center Duration
J.
♂ Center Frequency
Number of Entries
40
30
20
10
K.
♀ Center Frequency
**
CON SAL
CON CNO
MS SAL
MS CNO

Day 1-Baseline Startle Response

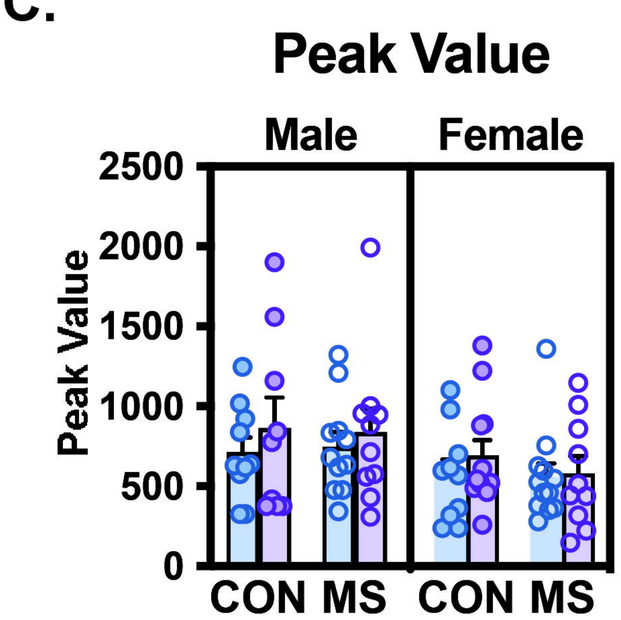
A.



B.

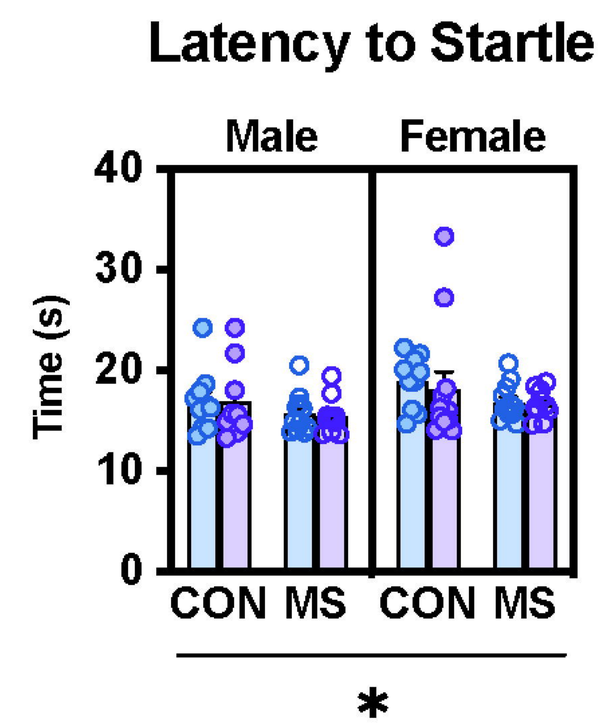


C.

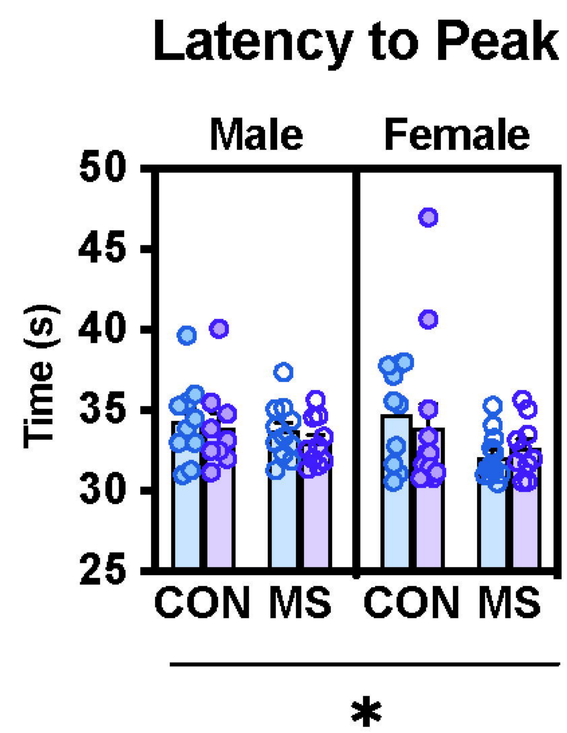


Day 2- USV Induced Startle Response

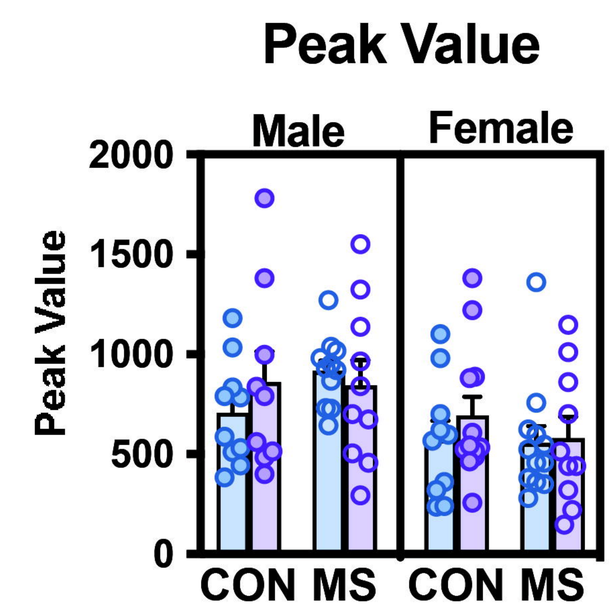
D.



E.

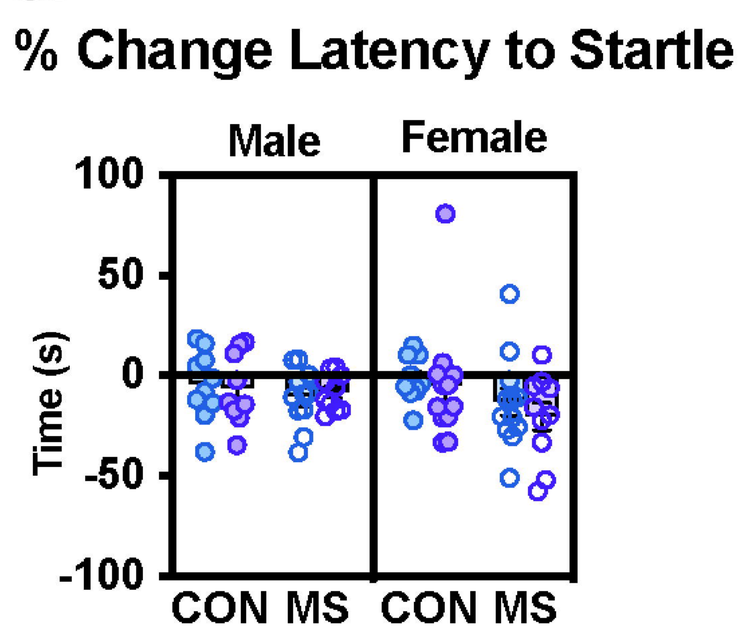


F.

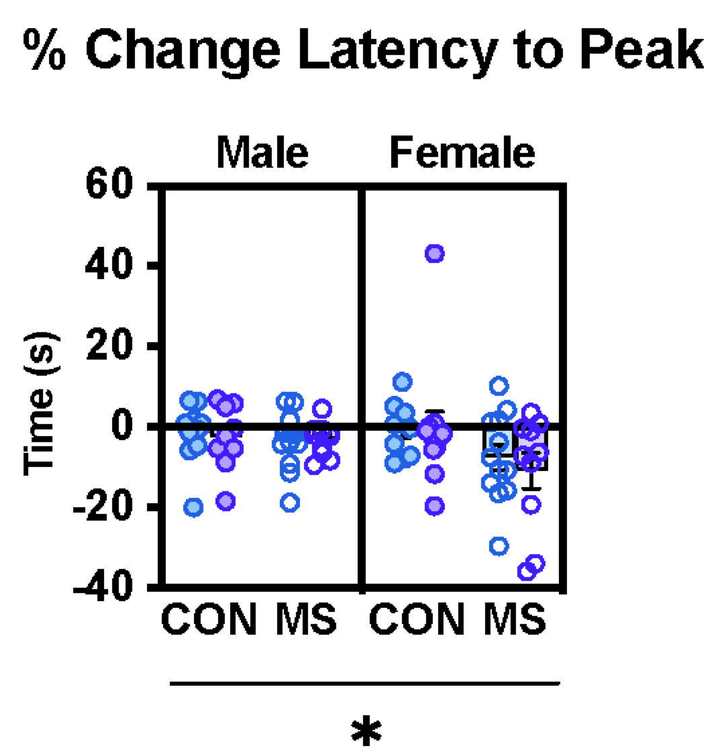


Percent Change- Day 1 to Day 2

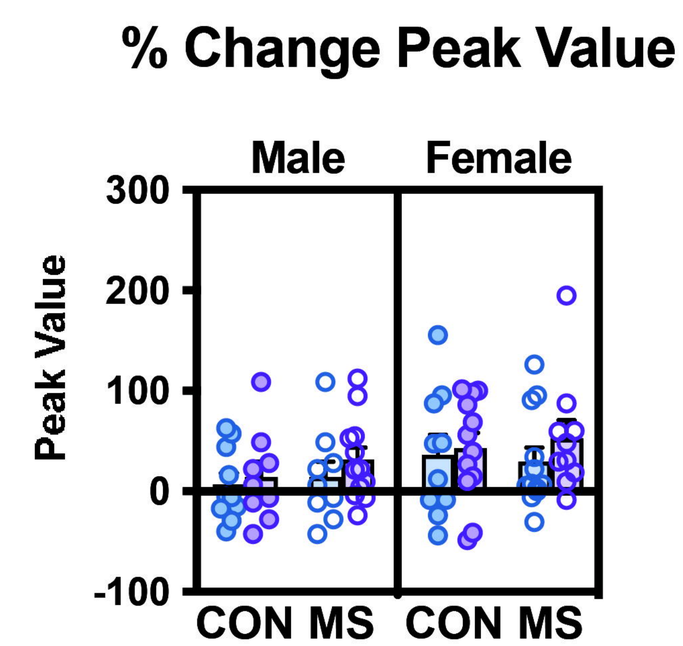
G.



H.



I.



CON SAL CON CNO MS SAL MS CNO