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Sex- and exposure age-dependent effects of adolescent stress on ventral tegmental area dopamine system and its afferent regulators

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Adolescent stress is a risk factor for schizophrenia. Emerging evidence suggests that age-dependent sensitive windows for childhood trauma are associated more strongly with adult psychosis, but the neurobiological basis and potential sex differences are unknown.

Using *in vivo* electrophysiology and immunohistology in rats, we systematically compared the effects of two age-defined adolescent stress paradigms, prepubertal (postnatal day [PD] 21–30; PreP-S) and postpubertal (PD41–50; PostP-S) foot-shock and restraint combined stress, on ventral tegmental area (VTA) dopaminergic activity, pyramidal neuron activity in the ventral hippocampus (vHipp) and the basolateral amygdala (BLA), corticoamygdalar functional inhibitory control, and vHipp and BLA parvalbumin interneuron (PVI) impairments. These endpoints were selected based on their well-documented roles in the pathophysiology of psychosis.

Overall, we found distinct sex- and exposure age-dependent stress vulnerability. Specifically, while males were selectively vulnerable to PreP-S-induced adult VTA dopamine neuron and vHipp hyperactivities, females were selectively vulnerable to PostP-S. These male selective PreP-S effects were correlated with stress-induced aberrant persistent BLA hyperactivity, dysfunctional prefrontal inhibitory control of BLA neurons, and vHipp/BLA PVI impairments. In contrast, female PostP-S only produced vHipp PVI impairments in adults, with the BLA structure and functions largely unaffected.

Our results indicated distinct adolescent-sensitive periods during which stress can sex-dependently confer maximal risks to corticolimbic systems to drive dopamine hyperactivity, which provide critical insights into the neurobiological basis for sex-biased stress-related psychopathologies emphasizing but not limited to schizophrenia. Furthermore, our work also provides a framework for future translational research on age-sensitive targeted interventions.

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INTRODUCTION

Exposure to early developmental stress (such as childhood trauma) is strongly associated with adult risks of psychotic disorders [1]. In this context, emerging studies implicate age-dependent sensitive periods during which developmental stress specifically associates with later-life psychosis risks [2, 3]. Childhood adversity is also associated with the adult manifestation of elevated presynaptic dopamine (DA) functions [4, 5], a pathophysiological hallmark of psychosis in schizophrenia [6]. Currently, the mechanisms that link early developmental stress exposure to adult DA dysfunctions are unclear.

Preclinical research indicates that stress exposure can induce various changes in presynaptic DA functions, indexed by

abnormal DA release upon stimulation and alterations in the firing of DA neurons in the ventral tegmental area (VTA) [7, 8]. Comparatively, more intense research effort has been given to DA release-related measures, and converging microdialysis evidence indicates that most acute stressors can immediately increase extracellular mesolimbic DA levels for tens of minutes [reviewed in 7]. This finding is consistent with increased DA neuron spontaneous firing during stress sessions or immediately after [9]. On the other hand, the effects of chronic and/or repeated stress on DA release [10, 11] and the underlying DA neuron activation appear to be more persistent, lasting for weeks [12, 13], albeit more heterogeneous and stressor-dependent [14, 15]. In particular, chronic stress can sensitize DA release to future

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challenges, although these results are inconsistent and again show stressor dependence [7]. Thus, these lines of evidence predict chronic stress to persistently modulate DA neuron firing. However, previous findings on the physiological effects of stress on DA neurons were obtained mainly from adult male animals, with developmental relevance and potential sex difference in stressor responses being largely unknown. In developing brains, although sporadic evidence suggests that early life stress during the first 2–3 postnatal weeks can lead to adult DA release cross-sensitization [16, 17] and/or increased VTA DA neuron excitability [18, 19], the ensuing windows such as during adolescence were largely unexplored. Given that in humans late childhood or adolescent trauma most strongly correlates with adult psychosis risk [2], studying how stress during the corresponding windows in rodents [i.e., postnatal day (PD), 21–60 [20]] modulate DA neuron firing could have substantial translational value.

We have reported recently that, in male rats, chronic stress during PD31–40 induced long-term tonic activation of VTA DA neurons [21]. Furthermore, this effect appears to be age- and sex-selective, as PD65–74 adult stress in males [22] and adolescent or adult stress in females [23] did not produce equivalent VTA firing abnormalities. Adolescence is a protracted period. Thus, based on behavioral and/or sexual development, rodent adolescence can be further divided into separate windows, defined by chronological age and relative temporal relationship to puberty (i.e., pre-adolescence/prepuberty vs. post-adolescence/postpuberty) [24]. Despite our recent substantial findings in PD31–40 rats [22, 23], it was unknown whether stress during other temporally defined adolescent windows may affect adult DA neuron firing.

In summary, based on the emerging human and animal literature linking developmental stress to adult DA dysfunctions, we sought to determine the long-term neurophysiological effects of two adolescent stress paradigms on adult VTA DA neuron firing. In particular, we emphasized age- and sex-dependence of stress exposure, and tested whether a 10-day stress paradigm can lead to distinct later-life schizophrenia-relevant electrophysiological alterations in VTA DA neurons and their afferent regulators, specifically the ventral hippocampus (vHipp) and the basolateral amygdala (BLA) [25]. Furthermore, we also determined the age- and sex-specific effects of adolescent stress on the maturation of parvalbumin interneurons (PVIs) in these regions, given their functional importance in regulating regional neurophysiological output and clinical relevance to schizophrenia [26].

MATERIALS AND METHODS

Male and female Sprague-Dawley rats were weaned at PD21 and randomly assigned to prepubertal stress (PreP-S), postpubertal stress (PostP-S), or their corresponding control groups. Similar to our previous studies [21–23], a 10-day combined footshock (FS) and restraint stress (RS) protocol started respectively at PD21 or PD41 in PreP-S or PostP-S rats, followed by *in vivo* extracellular recordings of VTA dopamine (together with behavioral tests), vHipp pyramidal, and BLA pyramidal neurons from different subsets of animals. Trajectories of PVI maturation after stress were determined by immunohistochemistry and fluorescent microscopy. Detailed experimental procedures are provided in *Supplementary Methods*.

RESULTS

PreP-S and PostP-S produced sex-selective effects on adult VTA DA neuron population activity

We first explored the effects of PD21–30 FS and RS combined stress on the adult VTA DA neuron activity and behaviors (Fig. 1A). A two-way ANOVA detected a significant sex and PreP-S interaction [$F(1,31) = 12.30, p < 0.01$] and a significant main effect of PreP-S [$F(1,31) = 9.852, p < 0.01$] on DA neuron population activity. Tukey's post hoc analyses revealed that PreP-S selectively increased DA population activity in males ($p < 0.01$) (Fig. 1B). This effect is limited to

the medial aspect of the VTA, a subregion projecting predominantly to the reward- and affect-related ventral striatum [27] [two-way repeated measure (RM) ANOVA; sex:stress condition: $F(3,93) = 7.165, p < 0.001$; PreP-S:Males, $p < 0.05$] (Fig. 1C). At PD65–71, PreP-S-induced behavioral changes were evaluated using elevated plus maze (EPM), novel object recognition (NOR), and amphetamine-induced hyperlocomotion (AIH). PreP-S:Males selectively exhibited less time spent in the open arms [interaction: $F(1,38) = 6.228, p < 0.05$; PreP-S:Males, $p < 0.05$] in EPM (Fig. 1D) and reduced discrimination index [interaction, $F(1,37) = 6.884, p < 0.01$; PreP-S:Males, $p < 0.01$] in NOR (Fig. 1F). These sex differences in stress-induced anxiety-like behaviors are broadly consistent with previous studies [28–31], and the observed male-biased cognitive effects of stress are consistent with accumulating evidence from humans [reviewed in [32]]. Although a two-way RM ANOVA on post-amphetamine locomotion revealed significant main effects of time [$F(5,031,181.1) = 16.51; p < 0.0001$] and stress:sex conditions [$F(3,36) = 9.573, p < 0.0001$] (Fig. 1G), Tukey's post hoc analysis did not reveal any significant stress-related differences within each sex.

Next, we determined the long-term effects of PD41–50 postpubertal stress (PostP-S) (Fig. 1H). PostP-S selectively increased DA population activity in females [interaction: $F(1,31) = 11.47; p < 0.01$; sex: $F(1,31) = 13.32, p < 0.01$; PostP-S:Females, $p < 0.01$] (Fig. 1I), an effect again localized to the medial VTA [stress:sex condition: $F(3,93) = 6.714, p < 0.001$; PostP-S:Females, $p < 0.05$] (Fig. 1J). Behaviorally, no significant effect was found in EPM or NOR (Fig. 1K–M). In AIH, despite significant main effects of time [$F(4,326,164.4) = 17.93; p < 0.0001$] and stress:sex conditions [$F(3,38) = 11.49; p < 0.0001$], post hoc comparisons did not reveal significant stress-related effects (Fig. 1N). Altogether, this dataset indicates marked sex and exposure-time differences in stress-induced adult DA neuron firing alterations.

Neither PreP-S nor PostP-S altered the average firing rate and burst firing of DA neurons in adults (Fig. S1). Body weight development was largely unaffected by stress (Table S1), except for a transient body weight decrease measured 10 days after PostP-S. To control for potential confounding factors from behavioral tests and differential recovery time, we performed DA recordings at 1–2 or 5–6 weeks after stress in another set of behavioral testing-naïve rats (Fig. S2). Whereas both PreP-S and PostP-S uniformly increased DA neuron population activity in 1–2 weeks (Fig. S2A and C), the long-term stress impact was sex- and exposure-age-specific (Fig. S2B and D), a pattern similar to the observations from the animals previously tested for behaviors. Furthermore, consistent with previous reports [21, 23], we found that prepubertal or postpubertal RS alone did not alter adult DA neuron firing (Fig. S3). Interestingly, prepubertal FS alone recapitulated the above described male-selective stress vulnerability to PreP-S (Fig. S3). This result contrasted with our previous finding that during PD31–40, only the combined FS and RS are sufficient to alter adult DA neuron firing [21]. Thus, this FS-selective effect suggest that at younger ages (i.e., PD21–30) male rats are increasingly vulnerable to a broader range of stressor types. Given that prominent sex differences were only observable in the long-term DA neuron responses, in subsequent experiments we focused on the combined stress and adult stress consequences.

Sex and exposure-age differences in adult vHipp pyramidal neuron firing stress responses

VTA DA neuron tonic and phasic firing modes are regulated by distinct afferent circuits. The ventral subiculum (vSub) of the vHipp predominantly controls DA neuron tonic activity (i.e., population activity) via a polysynaptic, disinhibitory circuit that involves the nucleus accumbens (NAc) and the ventral pallidum (VP) [25]. Emerging evidence suggests that preweaning early-life stress can produce enduring activity changes in hippocampal pyramidal

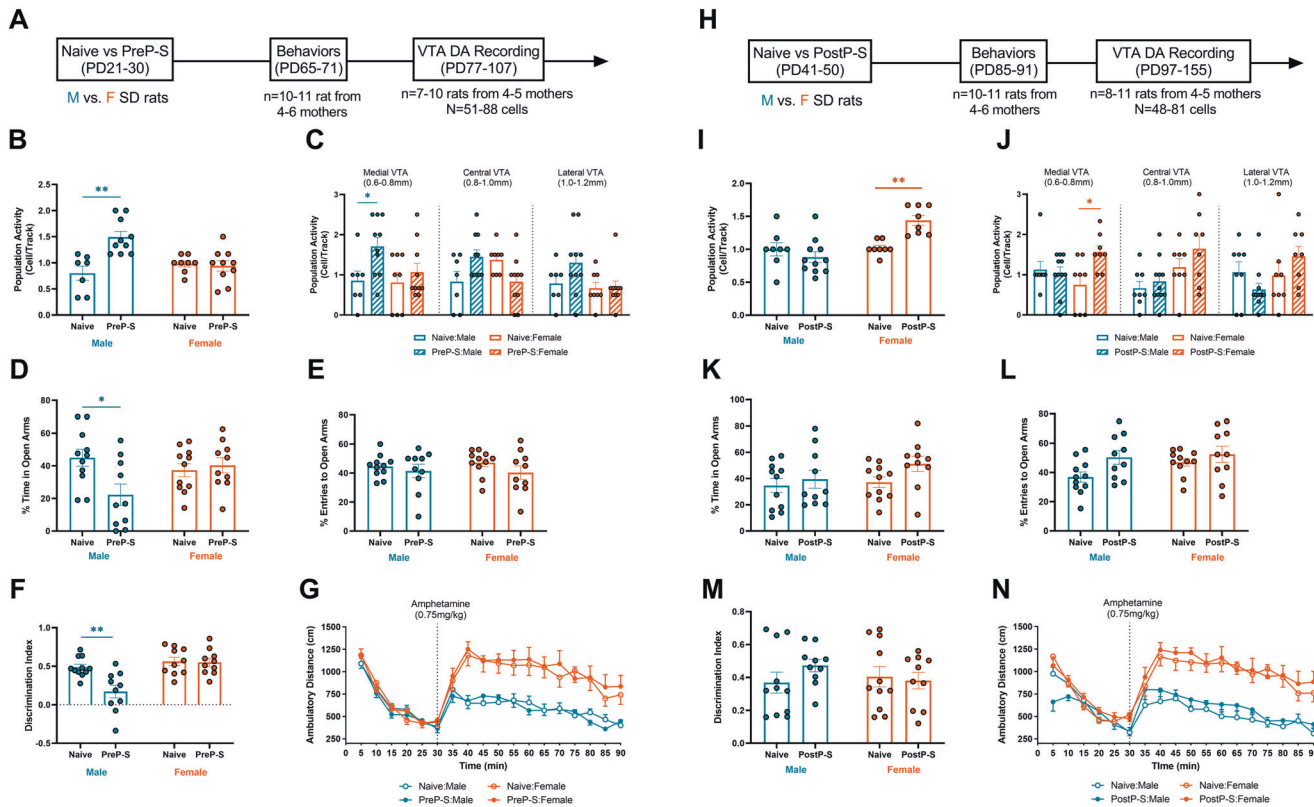


Fig. 1 Effects of prepubertal stress (PreP-S) and postpubertal stress (PostP-S) on adult ventral tegmental area (VTA) dopamine (DA) neuron activity and behaviors in males vs. females. **A** Experimental timeline for PreP-S effects. Male (blue) and female (orange) Sprague-Dawley rats were exposed to repeated footshock and restraint combined stress (see *Supplementary Methods* for details) during PD21–30. During PD65–71, rats were evaluated using elevated plus-maze (EPM), novel object recognition (NOR) and amphetamine-induced hyperlocomotion (AIH) tests. After at least a week of recovery, rats were subsequently tested for DA neuron activity via *in vivo* single-unit electrophysiology. **B** PreP-S produced a persistent increase in DA neuron population activity in males ($p < 0.01$) but not in females. **C** The male-specific effect of PreP-S was preferentially in the medial aspect of VTA (i.e., 0.6–0.8 mm from the midline). **D** In addition to the DA dysfunction, concomitant anxiety-like behaviors were observed only in PreP-S male rats, indexed by less time spent in the open arms, whereas females displayed resilience to PreP-S in this measure. **E** No significant difference was found in the percentage of open entries. **F** PreP-S:Male rats displayed a reduced discrimination index that reflects memory/cognitive impairments, an effect absent in PreP-S females. **G** In the AIH test, although females showed significantly greater post-amphetamine locomotion (0.75 mg/kg; *i.p.*) than males (main effect: $p < 0.0001$), post hoc Tukey's tests did not detect any significant differences between stress vs. naive groups within each sex. This indicates no effects of PreP-S on adult locomotor sensitivity to amphetamine. **H** Experimental timeline for PostP-S effects. **I** PostP-S produced a persistent increase in DA neuron population activity in females ($p < 0.01$) but failed to alter that in males. **J** PostP-S-induced DA population activity increase in females was preferentially in the medial VTA. **K–M** No behavioral difference was found in EPM and NOR. **N** In AIH, despite significant main effects in the post-amphetamine locomotion, no within-sex PostP-S effect was found. Data are presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$.

neurons (PN) [33, 34]. Consistently, our previous study shows that PD31–40 stress leads to increased PN firing rates in the adult vHipp [22]. However, the precise temporal boundaries and sex dependencies of such stress vulnerability in vHipp PNs are unknown. To characterize potential vHipp neuron stress vulnerability, we recorded from PNs in adult PreP-S (age: PD76–124), PostP-S (age: PD92–146), and control rats (age: PD81–131) of both sexes.

Putative vHipp PNs were recorded in adults and identified by electrophysiological signatures as previously described [22] (Fig. 2A). No effect of stress on vHipp PN population activity was detected (Fig. 2B). Significant group median differences were detected in adult PN firing rate after stress (data non-normally distributed, Kruskal–Wallis test, $H = 24.80$, $p < 0.001$), characterized by a male-selective increase in by PreP-S ($p < 0.05$; Dunn's test) and a female-selective increase by PostP-S ($p < 0.01$) (Fig. 2C). In addition, significant group median differences in bursting firing were also observed (Kruskal–Wallis, $H = 16.34$, $p < 0.01$), with post hoc tests revealing a sex-selective increase only in PreP-S:Males ($p < 0.05$) (Fig. 2D). Altogether, this dataset indicates distinct sex- and expose-age-dependent stress effects on adult vHipp PN firing, a vulnerability pattern corresponding highly to VTA DA neurons.

Sex and exposure-age differences in BLA pyramidal neuron firing response to stress

The BLA sends dense excitatory projections to modulate the vHipp [35], and chronic stress can age-dependently increase BLA PN firing [36] and excitability [37] that persists for days. Notably, aberrant BLA activities can induce deleterious effects on the hippocampus, characterized by interneuron deficits [38] and synaptic plasticity disruptions [39]. Moreover, across stress exposure and different exposure time, amygdala alterations from developmental stress tend to precede hippocampal alterations, suggesting that amygdala stress response represents a “temporal prerequisite” for hippocampal alterations [reviewed in ref. [40]]. Thus, we posit that, before the observed adult vHipp PN and VTA DA neuron activation, developmental stress might age- and sex-dependently activate the BLA [26]. Prior studies have shown that restraint stress can age-dependently increase BLA PN spontaneous firing rate and excitability [36, 41]. Consistently, another study reported that continuous social isolation beginning in adolescence can increase adult BLA excitability [42]. However, whether stress in adolescent substages can have persistent effects on the firing of BLA PN and whether there is sex dependency have not been examined systematically.

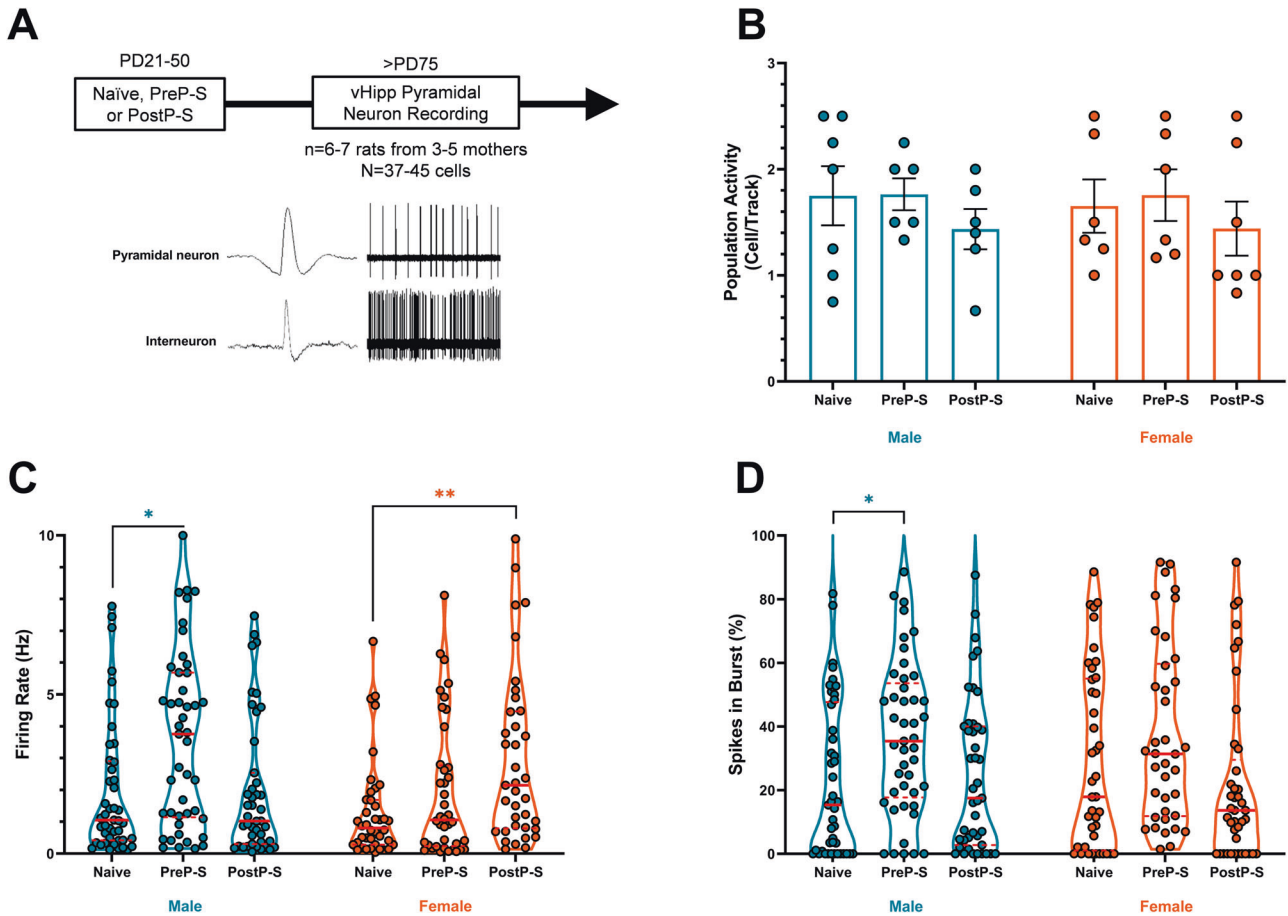


Fig. 2 Sex-selective and exposure age-dependent effects of PreP-S vs. PostP-S on adult ventral hippocampus (vHipp) pyramidal neuron activity. **A** Experimental timeline, representative spike waveform, and 10-s sample firing of identified vHipp neurons. vHipp pyramidal neurons (PNs) were identified based on low firing rate (<10 Hz) and long spike duration (>2.2 ms). **B** No change in the number of vHipp putative PNs detected per electrode track. **C** PreP-S selectively increased vHipp PN firing rate in males whereas PostP-S selectively increased PN firing rate in females. **D** PreP-S selectively increased percent of vHipp spikes occurring in bursts (defined by interspike interval <80 ms) in males. Violin plots were provided (here and in subsequent figures) to indicate datasets that contain non-normally distributed group data. Accordingly, Kruskal–Wallis analysis of group median differences followed by Dunn’s multiple-comparison test was used. Data are presented as median (solid red line) and quartiles (dashed red line). * $p < 0.05$; ** $p < 0.01$.

To address this, we recorded from putative BLA PNs both 1–2 weeks (short-term effect) and >5 weeks (long-term effect) after stress. BLA PNs were identified based on reported criteria [36, 43] (Fig. 3A and B). For the short-term effect of PreP-S, a significant group median difference was detected (Kruskal–Wallis test, $H = 13.33$, $p < 0.01$), and Dunn’s tests revealed a selective increase in PreP-S:Males ($p < 0.01$) (Fig. 3C). This effect was persistent, as a significant group median difference was also selectively observed in PreP-S:Males at >5 weeks after stress (Kruskal–Wallis test, $H = 24.69$, $p < 0.0001$; Dunn’s test, PreP-S:Males, $p < 0.0001$) (Fig. 3D). In contrast, no stress-related effect was observed in the short-term response to PostP-S. For the long-term PostP-S effect, although significant group median differences were observed (Kruskal–Wallis test, $H = 13.64$, $p < 0.01$), Dunn’s analysis did not reveal any stress-related change. Instead, only between-sex differences were found in PostP-S:Males vs. Naïve:Females ($p < 0.05$) and PostP-S:Males vs. PostP-S:Females ($p < 0.05$) comparisons. Altogether, this dataset indicates that while PreP-S chronically activated the BLA specifically in males, PostP-S failed to affect BLA PN firing in either sex.

Sex- and exposure age-dependent stress effects on pIPFC-BLA inhibitory control

The dorsomedial PFC exerts “top-down” control of stress-induced BLA activation [44, 45]. This modulatory action of PFC can be

studied by spike plasticity recordings. We have demonstrated previously that for PFC-evoked BLA PN extracellular activity, high-frequency electric stimulation (HFS) in the prelimbic PFC (pIPFC) can lead to spike long-term depression (LTD) [46]. Such inhibitory pIPFC-BLA regulation has been independently demonstrated by chemogenetic activation [47] and attributed to a feedforward inhibition mechanism driven by BLA interneurons [44]. Functionally, PFC-BLA control is thought to be adaptive to cope with the stress-induced BLA hyperactivity [46], a mechanism proposed to underlie cognitive regulation of emotion [48]. Notably, PFC inhibitory modulation over BLA neurons is prone to severe stress [46, 47] and is not developed in adolescence (PD39) [48]. Altogether, these previous findings predict that PFC-BLA inhibitory regulation may have heightened responsiveness to stress during early life (e.g., adolescence) and is potentially age- and sex-dependently regulated [49]. To test this, we next evaluated the longitudinal impacts of PreP-S and PostP-S stress on the pIPFC-BLA inhibitory control pathway.

The impact of pIPFC HFS on BLA PN evoked spiking was assessed at <3 weeks or >6 weeks after stress. HFS-induced spike plasticity was recorded as described previously [46] (Fig. 4A, B). We did not observe any difference in the baseline evoked firing properties (Table S2). For the short-term PreP-S effect (tested at PD37-51), in the time course response a two-way RM ANOVA in

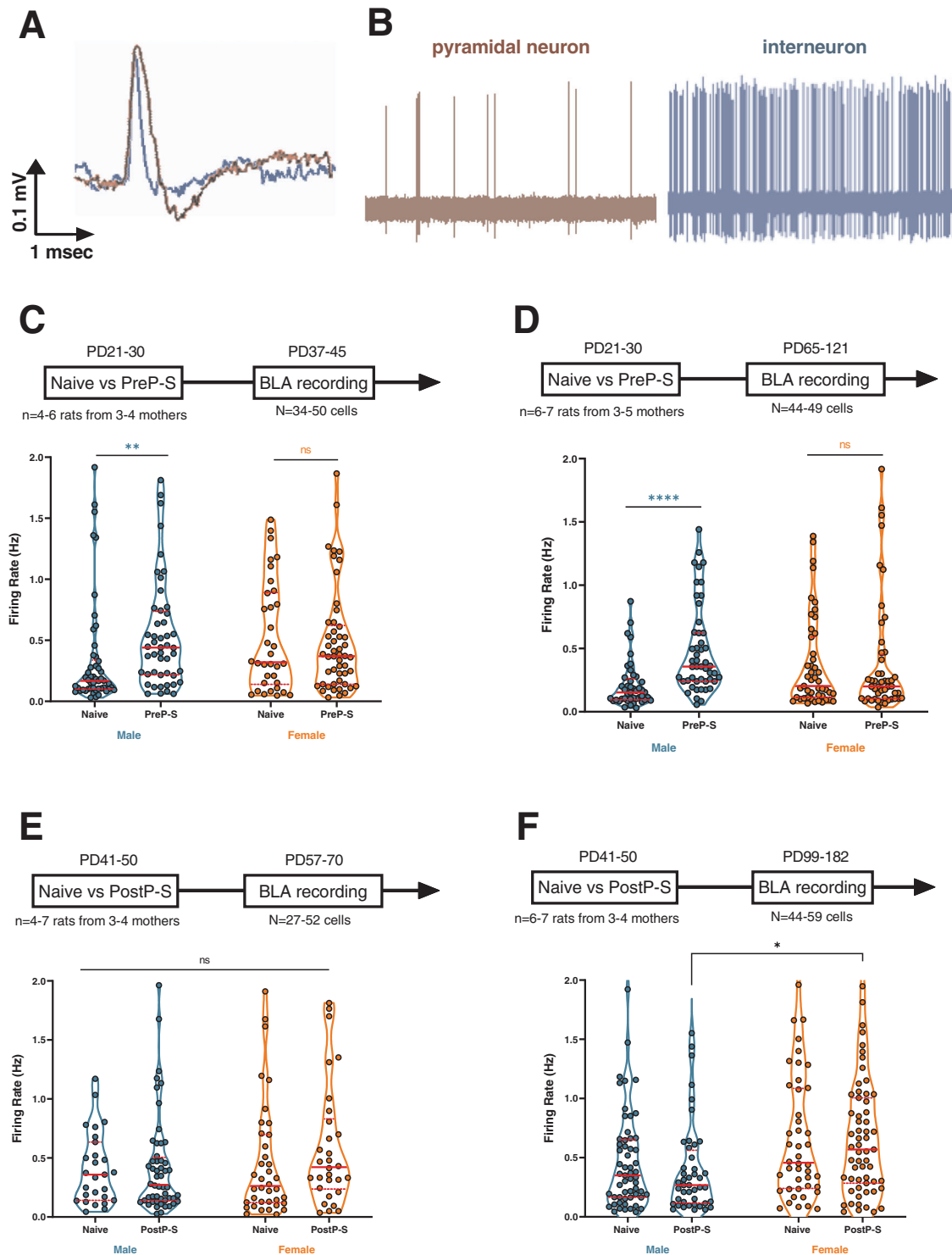
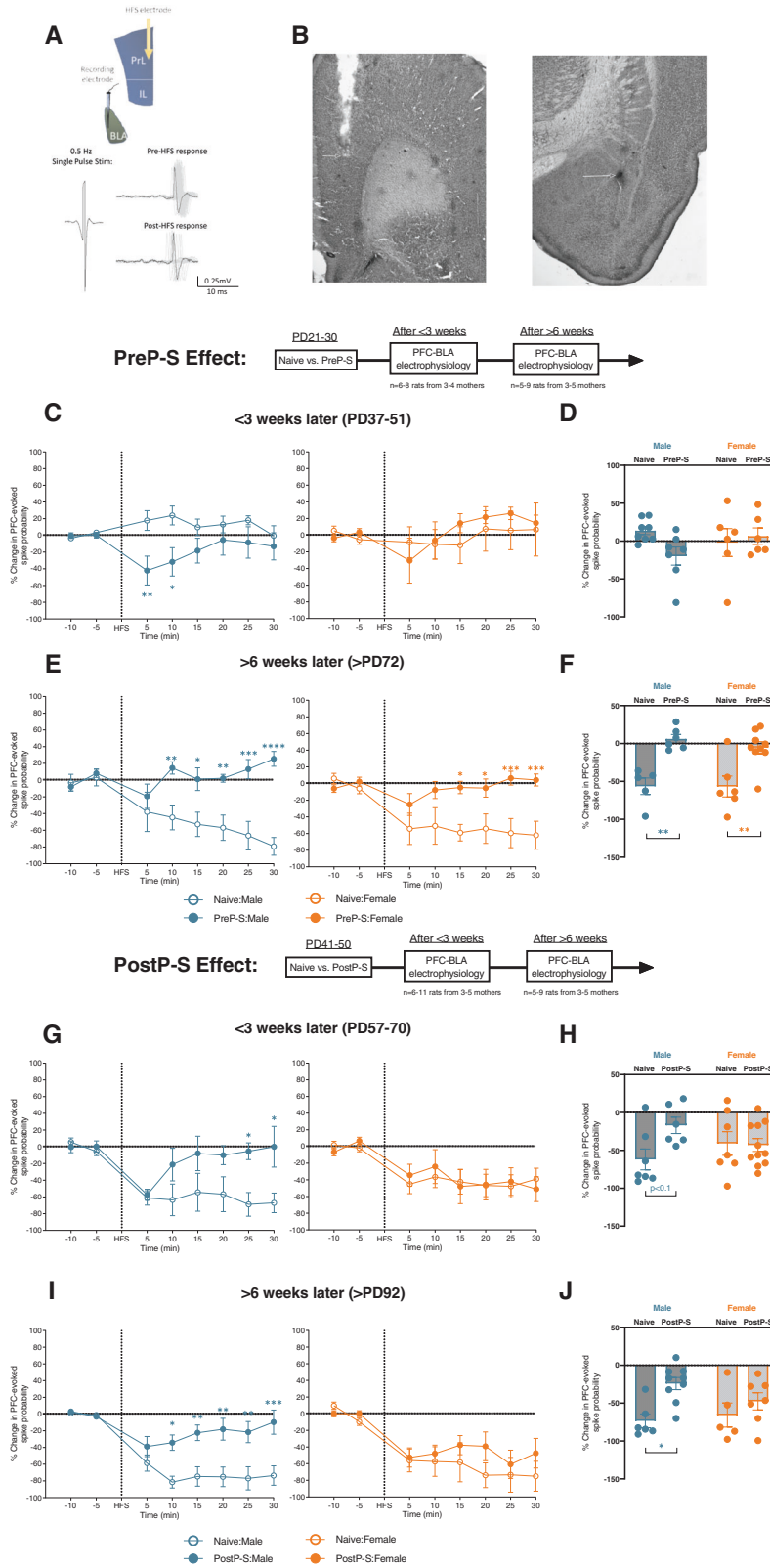


Fig. 3 Sex differences in amygdala neuron firing rate following stress. **A** and **B** Representative spike waveform and 10-s sample of spontaneous activity of basolateral amygdala (BLA) neurons. BLA pyramidal neurons were defined by spike duration >2.2 ms and mean firing rate <2 Hz. **C** PreP-S-induced BLA pyramidal neuron hyperactivity (i.e., increased firing rate), measured 1–2 weeks post-stress, was observed only in males, whereas females showed resilience to these short-term effects. **D** Male-specific, PreP-S-induced BLA hyperactivity persisted into adulthood; no effect was observed in adult females. **E** No effect of sex or PostP-S was found in the 1–2 week responses. **F** No within-sex PostP-S effect was found in the 5–6 week responses, although females showed higher stress activation compared to stressed males. Firing rate data were analyzed by Kruskal–Wallis test followed by Dunn’s post hoc multiple comparisons. Data are presented as median (solid red line) and interquartile range (dashed red line). ** $p < 0.01$; **** $p < 0.0001$.



males revealed significant effects of PreP-S [$F(1,13) = 7.283$, $p < 0.05$] and PreP-S and time interaction [$F(7,91) = 2.269$, $p < 0.05$]. Bonferroni's post hoc analyses indicated significant spike depression at 5 ($p < 0.01$) and 10 min ($p < 0.05$) post-HFS, whereas these effects were absent in PreP-S:Females (Fig. 4C).

Notably, the negative main effect of time suggested an overall immature circuit response to HFS at this age (PD37-51), consistent with previous findings [46] and with the minimal response (<20%) observed in mean post-HFS spike probability alteration (Fig. 4D). This male-specific emergence in HFS-induced spike depression

Fig. 4 Sex-specific effects of stress on PFC-BLA inhibitory control. A, Top A diagram illustrating the recording paradigm (see *Supplementary Methods* for details). A stimulating electrode and a recording electrode were implanted in the prelimbic prefrontal cortex (pPFC) and the BLA, respectively. BLA pyramidal neurons responsive to repeated pPFC single-pulse stimulation (0.5 Hz; 0.1–1.5 mA; square wave with 0.25 ms duration) were searched and recorded for 10 min to obtain baseline measures (spike probability maintained at ~50%). High-frequency stimulation (HFS; 20 Hz; 10 s, suprathreshold) was given immediately following the baseline recording, and spike probability response to HFS was recorded for an additional 30 min (Post-HFS). **A, Bottom** BLA pyramidal neurons typically respond to pPFC HFS with a long-term depression (LTD) of spiking, i.e., exhibiting fewer action potential responses to PFC stimuli after HFS. **B** Representative photomicrographs of pPFC stimulating electrode and BLA recording electrode placement. **C** In <3 weeks after stress, PreP-S induced a male-specific precocious form of PFC-BLA spike LTD in the first 10 min of post-HFS recordings (left), whereas in females PreP-S did not induce an effect (right) (time-course data). **D** No short-term effect of PreP-S was found across the test intervals in the mean Post-HFS spike probability change. **E** At >6 weeks after PreP-S, stressed rats (solid circles) of both sexes failed to show HFS-induced LTD (time-course data). **F** PreP-S induced impaired mean LTD averaged across test intervals in both sexes. **G** In <3 weeks post-stress, PostP-S induced a male-specific LTD impairment at 25–30 min post-HFS. **H** When averaged across the entire Post-HFS testing period, there was a trend toward LTD impairment only in males. **I** In >6 weeks post-stress, PostP-S induced a robust LTD deficit in the time course response only in males. **J** In >6 weeks post-stress, averaging across the entire test interval showed significant LTD impairments only in PostP-S males. Time-course data were analyzed by repeated measure two-way ANOVA with Bonferroni tests at each time point. Mean post-HFS response was analyzed by two-way ANOVA with Tukey's post hoc test. * $p < 0.05$; ** $p < 0.01$.

was similar to the adult-like response, which we tentatively labeled as precocious maturation of plasticity. For the long-term effects of PreP-S, in the time course response we found significant effects of PreP-S [$F(1,9) = 28.42, p < 0.001$], time [$F(7,63) = 2.574, p < 0.05$] and interaction [$F(7,63) = 5.674, p < 0.0001$] in males, with significant LTD deficits at 10–30 min post-HFS. Similar LTD deficits were also observed in females, in which impaired spike depression was observed at 15–30 min post-HFS [interaction: $F(7,91) = 4.773, p < 0.001$]; Fig. 4E]. Consistently, significant long-term PreP-S effects were observed in the mean spike response in both sexes [PreP-S: $F(1,22) = 32.47, p < 0.0001$] (Fig. 4F).

After PostP-S, males displayed LTD deficits in the time course response at <3 weeks [interaction: $F(7,77) = 2.663, p < 0.05$; 25 and 30 min Post-HFS, $p < 0.05$; Fig. 4G] and >6 weeks [interaction: $F(7,91) = 4.296, p < 0.001$; 10–30 min Post-HFS, $p < 0.05$; Fig. 4I]. In contrast, no effect was found in females at both testing ages (Fig. 4G and I). Consistently, in the mean spike response, despite a trend for a short-term effect [interaction: $F(1,27) = 3.698, P = 0.0651$; Fig. 4H], after 6 weeks post-stress, only PostP-S:Males showed PFC-BLA spike LTD deficits [PostP-S: $F(1,23) = 9.589, p < 0.01$; PostP-S:Males>Naïve:Males, $p < 0.05$; Fig. 4J].

In conclusion, these findings suggest distinct stress vulnerability at the level of pPFC-BLA inhibitory control. Whereas females are vulnerable to the long-term PreP-S effects, males displayed more pronounced vulnerability to both stress paradigms. Moreover, after PreP-S males displayed an accelerated pPFC-BLA maturation, a putative concomitant adaptive response to BLA hyperactivity (Fig. 3C), albeit insufficient.

Age- and sex-dependent stress vulnerability of parvalbumin interneurons in the BLA and the vHipp

Throughout extended adolescence, corticolimbic PVIs adopt perineuronal nets (PNNs), which are extracellular matrix structures that enhance GABAergic inhibition and support fast-spiking ability [50]. Developmental stress can induce diverse PVI cellular alterations, including decreased PV or PNN expression and their co-localization [51]. Given the critical role of PVIs in regulating PN firing [52], PVI impairments could underlie the observed PN hyperactivity in both the BLA and the vHipp. To explore this, we next determined the longitudinal impact of stress on PVIs, emphasizing co-localization with PNNs.

PV, PNN cell counts and their co-labeling were assessed by immunohistochemistry and fluorescent microscopy in the basal nucleus of BLA (Fig. 5A, B). Substantial PVI impairments were found mostly in males after PreP-S (Fig. 5C, D). Thus, at PD75 PreP-S:Males displayed reduced PV + (Welch t-test; $t(8.863) = 4.349, p < 0.01$), PNN + [$t(8.913) = 2.555, p < 0.05$], and co-labeled cells counts [$t(5.634) = 2.934, p < 0.05$]. Reduced co-labeled cell count was also detected at PD41, suggesting persistent deficits. All these

effects were absent in females. Instead, PreP-S:Females displayed transient reductions of PNN + [$t(7.309) = 2.819, p < 0.01$] and PV/PNN co-labeled cells [$t(9.997) = 2.495, p < 0.05$] at PD31. Notably, these effects were exposure age-dependent, as PostP-S failed to change these markers in either sex (Fig. 5E, F).

We next determined vHipp PVI impairments (Fig. 6A, B). In males, PreP-S reduced PV + [$t(9.120) = 4.939, p < 0.001$] and co-labeled cell counts [$t(5.832) = 3.773, p < 0.01$] only at PD95. In contrast, PreP-S:Females were largely unaffected, except for a transient decrease of PV + cell count at PD41 [$t(9.561) = 2.291, p < 0.05$] (Fig. 6C, D). A reversed sex dependence was found after PostP-S. Thus, only females at PD95 displayed significant reductions of PV + [$t(9.573) = 6.918, p < 0.001$] and co-labeled cell counts [$t(10.00) = 3.845, p < 0.01$] (Fig. 6E, F).

Collectively, BLA and vHipp PVIs are sex- and age-dependently disrupted by developmental stress. While vHipp PVI impairments are strongly correlated with sex- and exposure-age-dependent heightened PN activity (Fig. 2), in the BLA stress-induced PVI impairments appeared to be male-dominant.

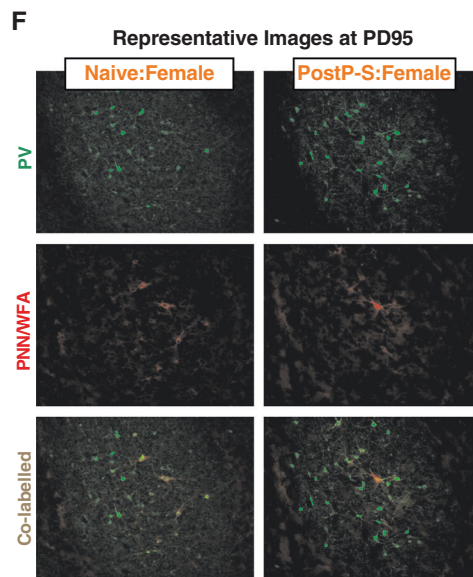
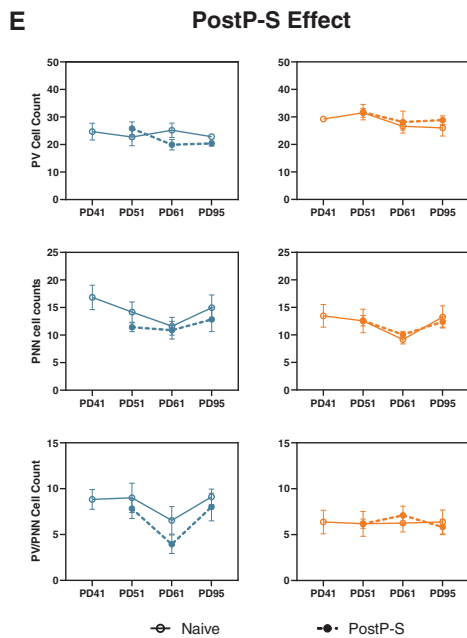
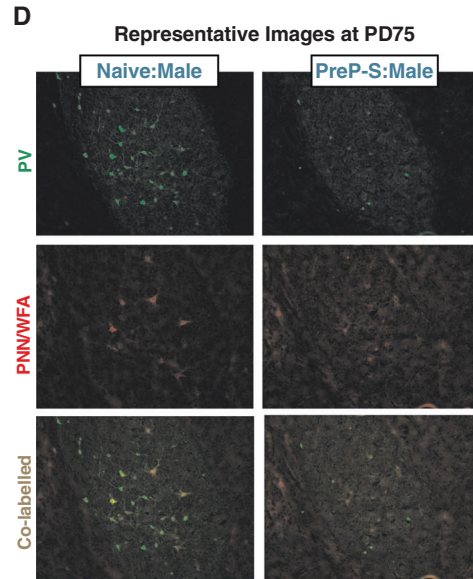
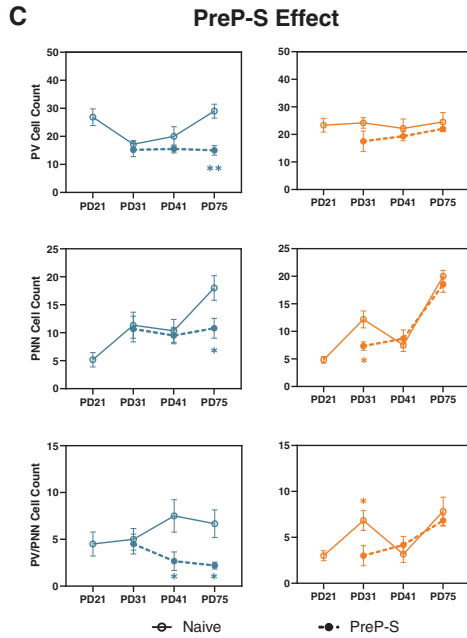
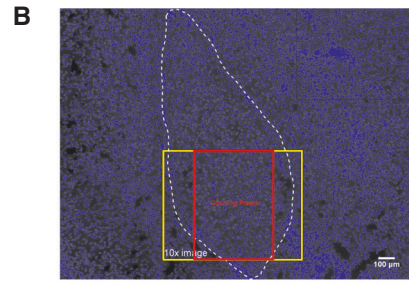
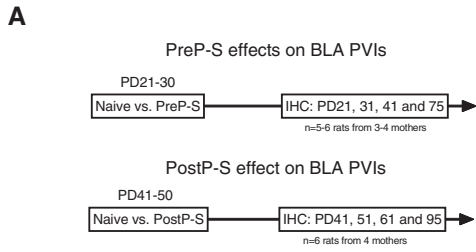
DISCUSSION

Summary of findings

This is the first systematic study to examine sex differences in the electrophysiological effects of two adolescent stress paradigms on VTA DA neurons and related regulatory regions. Our main findings are that adolescent stress during age- and sex-selective sensitive windows is sufficient to persistently increase adult DA system activities, potentially by differentially modulating activity states and PVI maturation of the vHipp and the BLA. See Supplementary Table S3 for a summary of findings.

Age group and stress paradigm

Rodent adolescence is a prolonged period containing several substages [53]. In this study, we grouped rats according to chronological age and defined stressors in PD21–30 and PD41–50 as “prepubertal stress” and “postpubertal stress”, respectively. We did not individually control for pubertal onset age, as techniques that reliably measure this (e.g., repeated vaginal lavage) are stressful [54], which would confound our analyses. Notably, our operational definition of “prepuberty” is consistent with several previous reports [for example [55, 56]], and the designation of PD41–50 as “postpuberty” was based on documented average pubertal onset age at PD36 in Sprague-Dawley female rats and at approximately six weeks of age in males [57]. Importantly, we selected these temporally distant windows to investigate the exposure age-dependent effects of adolescent stress. PD21–30 and PD41–50 in rodents correspond approximately to childhood and adolescence in humans [20, 58, 59], during which stress



experience age-dependently associates with adult psychopathology risks (e.g., schizophrenia [2, 3]) [60] and with differential structural/functional stress consequences [e.g., [61]]. However, in preclinical research neurophysiological stress impacts during distinct childhood and adolescent windows have been seldom

compared systematically. Moreover, we purposefully selected PD21-30 and PD41-50 to complement our recent studies using the same stress protocol during PD31-40 separately in males and females [21, 23], with the goal to generalize conclusions within broader contexts. Noteworthy, recovery duration (i.e., stress

Fig. 5 Sex differences in the longitudinal impact of PreP-S and PostP-S on BLA parvalbumin (PV) interneurons. **A** After stress exposure, immunohistochemistry and fluorescent microscopy were performed in the BLA at various post-stress time points. Specifically, the BLA was sampled before the stress paradigms (in naïve rats) and subsequently at 1, 10, or 45 days after stress termination. **B** A 4× image with DAPI staining showing BLA anatomical borders and the counting frame. Cell counts of PV+ neurons, perineuronal-net+ (PNN+, labeled by WFA) neurons, and their co-labeling were measured in a 550 × 690 μm counting frame on 10× images (909 × 692 μm). **C** PreP-S-induced changes were detected primarily in males, including delayed reductions of PV+ and PNN+ cell counts only at PD75 and chronically reduced PV+/PNN+ co-labeled cell counts at PD41 and PD75. In the female BLA, PreP-S resulted in immediate yet transient reductions of PNN+ and PV+/PNN+ co-labeled cells only at PD31 (i.e., one day after stress), suggesting potential recovery at later time points. **D** Representative 10× images from PreP-S:Males at PD75. **E** PostP-S did not impair BLA PV interneurons in either sex. **F** Representative images from PostP-S:Females at PD95. Data are presented as mean ± SEM. Unpaired t-test with Welch correction was conducted at each post-stress time point. * $p < 0.05$, ** $p < 0.01$, indicating stress-related changes.

recency) determines stress outcomes [62]. Thus, to ensure comparable recovery timelines, the age of our adult recordings was different between experiments for PreP-S and for PostP-S, although we intentionally created some overlap. Given that VTA, vHipp, and BLA activities appear overall stable in young and mature adult rats, we do not believe that a 20-day delay in the adult testing age of PostP-S rats could drive the observed activity differences across multiple regions.

Sensitive periods for stress are defined by regions and outcome measures [63], and here we defined stress vulnerability based on electrophysiological consequences in VTA DA system and its afferent regulators. We used 10-day FS + RS combined stress as reported previously [21], as its duration is suitable for being placed within rodent adolescent substages, allowing us to infer developmental stage specificity. Notably, current literature on adolescent stress impact on adult VTA DA neuron activity has been almost exclusively conducted in social stress models [8, 58], despite in humans childhood adversity with no apparent social element has also been associated with risks for DA-related psychopathologies [64]. Childhood adversities are often repeated and involve complex experiences. Considering all these factors, we selected a non-social and heterotypic stress paradigm to study exposure age- and sex-dependent effects.

Stress-induced DA neuron hyperactivity: relevance to previous studies

Our VTA recordings showed that stress during chronologically discrete, sex-dependent adolescent-sensitive periods induced long-term DA neuron tonic activation in adults, without affecting phasic firing parameters (Fig. 1 and S1). Tonic spontaneous DA neuron firing is a physiological prerequisite for phasic, behaviorally relevant DA release [25]. Thus, this study complemented the vast microdialysis literature on stress-induced long-term increase in DA release [reviewed in refs. [8, 65]]. Interestingly, although the exact vulnerability windows shifted between sexes, PreP-S and PostP-S appeared to converge to induce persistent VTA hyperdopaminergic activities (Figs. 1 and S2). This markedly contrasts with the adult stress responses we recently reported, which are characterized by transient VTA hypodopaminergic activities [22, 23]. Furthermore, the chronicity of stress effects on tonic dopaminergic activities itself is a novel finding. This is because, although studies using social defeat stress have shown that DA neuron phasic activation can last 28 days [12, 13], to our knowledge, no other works have used long post-stress delay or comprehensively assessed both the tonic and the phasic DA neuron activity.

Relevance to schizophrenia

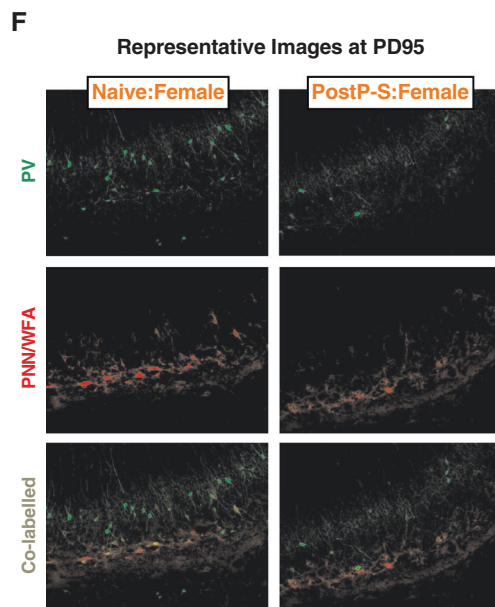
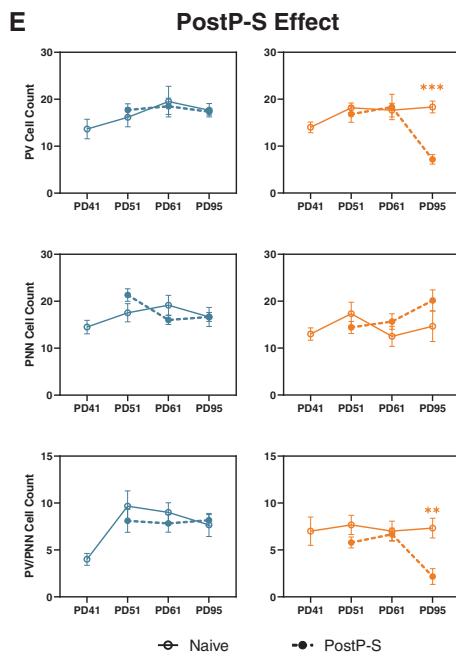
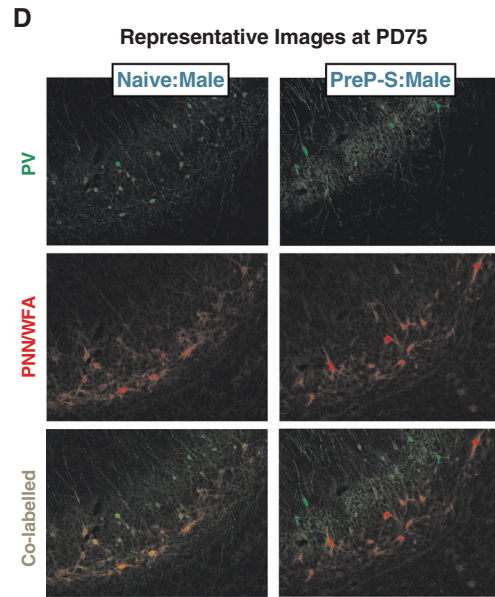
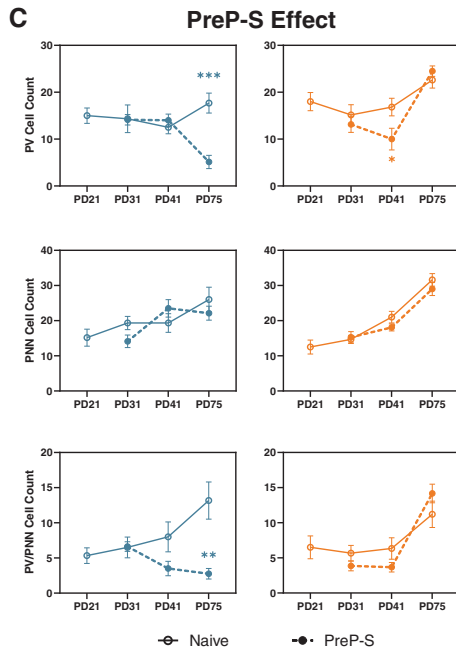
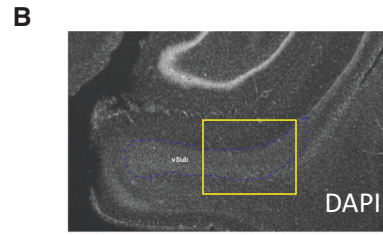
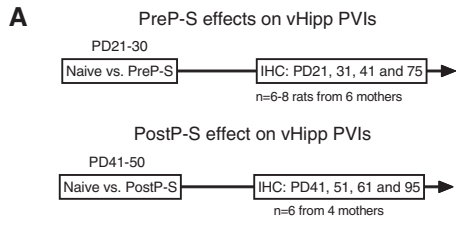
Correlating with hyperdopaminergic states, PreP-S male and PostP-S female rats also exhibited vHipp hyperactivity and PVI impairments. These results are consistent with schizophrenia clinical observations [66], a neurodevelopmental model of schizophrenia risks (i.e., the MAM model [26, 66]), and our previous findings with PD31-40 stress [22]. In particular, the stress-induced increase in VTA

DA neuron spontaneous firing is consistent with human molecular imaging evidence showing elevated presynaptic striatal DA functions in schizophrenia patients [67], which implicates increased active DA terminals driven by more active neurons. The increased vHipp and BLA PN firing rates are consistent with the increased cerebral perfusion in corresponding regions of schizophrenia patients [68, 69]. Therefore, although the exact sensitive period timing is sex-dependent, upon adolescent stress exposure the adult stress outcomes closely recapitulate neurophysiological disruptions observed in schizophrenia. Intriguingly, our previous studies show that adult (PD65-74) rats of both sexes are resilient to stress-induced persistent DA neuron firing alterations [22, 23]. Thus, the current study extended our previous findings and further indicated that adult DA neuron activity states are dynamically modulated by adolescent stress history. Notably, PD21-30 male rats were also susceptible to singular FS-induced hyperdopaminergic states (Fig. S3), a phenotype reminiscent of early PFC-lesioned rats [21]. This may indicate a relatively immature PFC in prepubertal males, which could contribute to increased vulnerability to a broader range of stressor types.

Potential role of vHipp PVIs in age-dependent stress impacts

The vHipp regulates VTA DA neuron tonic activity via a well-established vSub-NAC-VP polysynaptic circuit [25]. Thus, the observed vHipp firing increase after sex- and age-selective stress exposure is upstream to the stress-induced VTA hyperdopaminergic activities. The observed vHipp PVI impairments are likely an important mediator of the vHipp/VTA pathophysiology [26], as PVIs are a major inhibition source and are highly stress-sensitive [51]. Notably, direct vHipp PV knockdown upregulates vHipp PN output and VTA DA neuron population activity [70], similar to the stress effects in the current study. Thus, we posit that the stress-induced vHipp physiological alterations are likely secondary to PVI impairments.

The developmental trajectories of vHipp PVIs could potentially explain the observed stress-sensitive period timing differences. Developmental stress can impair PVIs via activating oxidative stress-related processes [71–73]. During early adolescence in rodents, anti-oxidative PNNs are not fully developed [74, 75]. Thus, vHipp PVIs might be particularly vulnerable during early adolescence, as demonstrated by stress during PD21-30 (this study) or PD31-40 [22], as well as several other studies [reviewed in [51]]. The present data in male rats demonstrated that only PreP-S was sufficient to reduce adult PV, PNN, and co-labeled cell counts in vHipp, whereas PostP-S failed to do so (Fig. 6). PVIs lacking PNNs are incapable of fast-spiking inhibitory activities [50]. Moreover, enzymatically dissolving vHipp PNNs can activate vHipp PNs and increase downstream VTA DA population activities [76]. Considering these previous findings, our results suggest that male PreP-S might impair PVIs by disrupting their PNN association, which could lead to loss of PVI-mediated inhibition and the observed vHipp hyperactivity. Notably, the above-described line of evidence has been almost exclusively established in males. In females, emerging studies (albeit in other brain regions) suggest



that PV and PNNs develop following sexually divergent trajectories [77–80], which is proposed to contribute to differential PVI stress vulnerability [81]. Whether adolescent vHipp PV/PNN developments are sexually divergent is unknown. Nonetheless, our data clearly indicated that PostP-S females at PD95 showed

PV/PNN pathologies similar to those of PreP-S males (Fig. 6). Thus, it is possible that instead of a continuous increase in PV/PNN expression as observed in males [82], female vHipp PVIs might develop following a more dynamic trajectory, contributing to the selective postpubertal vulnerability.

Fig. 6 Sex differences in the longitudinal impact of stress on vHipp PV interneurons. **A** vHipp was sampled at different time points after PreP-S and PostP-S to examine potential stress-induced PV interneuron (PVI) impairments. **B** A 4x image with DAPI staining showing the relative location of the 10x imaging site targeting the proximal portion of the ventral subiculum of the vHipp. **C** PreP-S induced male-dominant PVI impairments in the vHipp. The PreP-S effect in males was delayed, as stress-related reductions of PV+ and PV+/PNN+ co-labeled cell counts were only observed at PD75 (i.e., 45 days after stress). Female vHipp responded to stress with a minor PVI deficit, characterized by a PV reduction at PD41 (i.e., 10 days after stress), which did not persist into PD75. **D** Representative images showing PreP-S effect in males on PVIs measured at PD75. **E** In PostP-S females, stress-related reductions of PV and PV/PNN co-labeled cell count were only observed at PD95 (i.e., 45 days after stress), whereas in males, PostP-S did not induce any change in any examined marker. Data are presented as mean \pm SEM. Unpaired t-test with Welch correction was conducted at each post-stress time point. $^{**}p < 0.01$; $^{***}p < 0.001$, indicating stress-related changes.

Potential involvement of the BLA

Chronic stress can activate BLA PNs [83]. Under normal conditions, BLA PNs are potently inhibited by local PVIs, exhibiting low spontaneous firing rates [52, 84]. This activity state could be disrupted by BLA PVI impairments. Importantly, the BLA sends dense glutamatergic projections to the vHipp [35], and pharmacological BLA activation (e.g., via picrotoxin infusion) disrupts schizophrenia-related hippocampal GABAergic neurotransmission [68], including reduced PV expression [38]. Thus, early BLA GABAergic deficits are proposed to drive hippocampal PVI pathology and VTA hyperactivity in schizophrenia [25, 68]. In our male BLA recordings, high spontaneous firing occurred within 1–2 weeks after PreP-S (i.e., PD37–45; Fig. 3C), which preceded the adult emergence of vHipp PVI impairments (PD75; Fig. 6C). Therefore, these male observations supported that vHipp PVI deficits might be consequences of BLA hyperactivity [38, 85]. The BLA directly projects to limbic hippocampal PNs but also presumably to PVIs [86, 87]. Thus, whether the observed BLA activation directly confers excitotoxicity to PVIs, or by PN activation, is worth future investigations using projection-specific approaches. Since our data found concurrent BLA PVIs impairments (reduced PV/PNN co-labeling at PD41; Fig. 5C) and BLA hyperactivity (PD37–45; Fig. 3C), it is plausible that persistent BLA PVI disruption drives BLA hyperactivity to further lead to impairments in vHipp PV networks. Alternatively, a direct, long-lasting increase in BLA PN excitability is also possible, which would be consistent with previous findings with continuous social isolation stress beginning in adolescence [42] and with the emerging findings that stress can specifically increase glutamatergic neurotransmission in BLA neurons projecting to vHipp [88]. Only in PreP-S males, BLA hyperactivity persisted into adulthood, which might directly contribute to the adult vHipp hyperactivity via monosynaptic projections and in turn cause VTA hyperdopaminergic activity. Whether such male-selective adult BLA hyperactivity is an upstream therapeutic target or if some of the observed changes can mediate the downstream stress sequelae warrants further investigation.

We also tested the effects of PreP-S and PostP-S on pPFC-BLA spike plasticity. Previous studies indicated that mPFC inhibitory control over BLA PNs is immature during rat adolescence [48]. In this study, within 3 weeks after PreP-S, in males HFS can already induce an adult-like spike LTD (Fig. 4C and D). This male-selective BLA accelerated maturation after PreP-S might emerge as an adaptive process to ameliorate BLA PN hyperactivity. Interestingly, PreP-S did not trigger this adaptive process in females, which is expected due to the normal firing of the BLA after stress (Fig. 3). In adulthood, while naïve animals of both sexes achieved mature PFC-BLA LTD, both male and female PreP-S rats showed LTD deficits. These adult changes suggest that long after PreP-S, the PFC is still incapable of properly gating BLA activity, consistent with the observed long-lasting BLA hyperactivity in males (Fig. 3C). Intriguingly, despite PFC-BLA control being disrupted (Fig. 4C), adult PreP-S females did not display either BLA hyperactivity or PVI deficits. Thus, it appears that PFC-BLA dysfunctional connectivity alone might represent the inability of the PFC to regulate excessive BLA firing rather than a direct cause. On the other hand,

we observed a male-selective PostP-S effect on PFC-BLA control within 3 weeks (PD57–70; i.e., early adulthood) and after 6 weeks after stress, with no effect observed in females. Collectively, these results indicated male-dominant vulnerability in PFC-BLA inhibitory control circuit regardless of stress exposure age. The behavioral implications of this sex difference remained to be thoroughly determined by future studies, which putatively pertain to the processing of fear stimuli [46].

Potential mediators of sex differences

The observed resilience of female VTA DA neurons to PreP-S (Fig. 1B) extended our previous findings with PD31–40 stress [23]. These findings together suggest a sudden emergence of stress vulnerability in the female VTA DA system during late adolescence (i.e., PD41–50). Thus, the temporal profile of female stress vulnerability is distinct from that of males, which encompasses PD21–30 (this study) and PD31–40 [22]. Speculatively, the observed female prepubertal resilience to chronic stress is potentially related to more mature neuroendocrine stress responses at this age [characterized by more rapid negative feedback [89]], whereas in males stress hormone (e.g., corticosterone) feedback modulation does not reach adult capacity until PD30–40 [90]. Future studies are necessary to determine if the observed electrophysiological stress consequences are linked to sex-biased neuroendocrine responses after stress [91].

Conclusion and translational relevance

Using a rat model, we examined adolescent sensitive periods for stress to induce adult hyperdopaminergic states. While male rats were vulnerable to stress during the early, prepubertal period of adolescence, females were more vulnerable in postpuberty. During these distinct sensitive periods measured by DA system responses, age- and sex-dependent stress exposure also elicited vHipp PN hyperactivity and PVI impairments, which with the DA system dysfunctions collectively recapitulated schizophrenia-related circuit disruptions. Despite the overall sex-convergent activating effects on VTA DA neurons and vHipp PNs, stress-induced changes in BLA spontaneous activity, PFC-BLA functional connectivity, and BLA PVIs structural integrity are primarily observed in PreP-S males. In females, however, these stress outcome measures were largely unaffected by either stress paradigm. Altogether, our findings are consistent with the emerging clinical evidence that childhood and adolescence may represent distinct sensitive periods for stress to specifically increase psychosis risks [2], and further pinpoint vHipp and VTA DA system hyperactivities as potential neurobiological mediators. In humans, histories of childhood adversity are associated with elevated DA synthesis capacity [4], sensitized DA release to acute amphetamine/stressor challenges [92, 93], and heightened mid-brain activation in response to emotional stimuli [94]. These observations are consistent with the increased VTA DA population activity observed in the present study, supporting the hypothesis that environmental stressors sensitize the dopamine system which in turn leads to development of psychosis [6]. Furthermore, early adversity/trauma can induce differential impairments in human limbic structures based on exposure age and sex [40].

Nevertheless, due to methodological and ethical challenges, how these abnormalities interact temporally and/or whether they are due to differential vulnerability in humans are unknown. Thus, the present study offered several novel testable hypotheses for clinical research. Noteworthy, individuals at clinical high risks for schizophrenia tend to experience trauma more likely before early adolescence (before age 12 years) [95], which approximately corresponds to the prepuberty in this study. Thus, the observed male-selective PreP-S effects might explain the well-known male-biased risks for more severe schizophrenia symptoms and earlier psychosis onset [96, 97]. Although relatively protected from DA hyperactivity induced by earlier stress, females are vulnerable to stress post-pubertally (this study) and in adulthood [23], which would be consistent with the human observation that adult females are at increased susceptibility to affect-related psychopathologies [98]. Therefore, our findings may have strong implications for the etiology and pathophysiology of stress-related developmental disorders, with a particular emphasis on schizophrenia. Beyond this, the discovered sex-specific stress effects on DA neuron firing might provide a framework for understanding other sex-biased, DA-related psychopathologies [60, 63, 99].

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AUTHOR CONTRIBUTIONS

XZ: conceptualization, data acquisition, formal analysis, interpretation, writing—original draft. AAG: conceptualization, resources, interpretation, writing—review & editing, supervision, and funding acquisition.

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