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Adolescent Stress Confers Resilience to Traumatic Stress Later in Life: Role of the Prefrontal Cortex

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Abstract:

Background: Stress during adolescence is usually associated with psychopathology later in life. However, under certain circumstances, developmental stress can promote an adaptive phenotype, allowing individuals to cope better with adverse situations in adulthood, thereby contributing to resilience.

Methods: Sprague Dawley rats (50 males, 48 females) were subjected to adolescent chronic variable stress (adol CVS) for 2-weeks at PND45. At PND 85, a group was subjected to single prolonged stress (SPS). After a week, animals were evaluated in an auditory-cued fear conditioning paradigm and neuronal recruitment during reinstatement was assessed by Fos expression. Patch clamp electrophysiology (17-35 cells/group) was performed in male rats to examine physiological changes associated with resilience.

Results: Adol CVS blocked fear potentiation evoked by SPS. We observed that SPS impaired extinction (males) and enhanced reinstatement (both sexes) of the conditioned freezing response. Prior adol CVS prevented both effects. SPS effects were associated with a reduction of infralimbic (IL) cortex neuronal recruitment after reinstatement in males and increased engagement of the central amygdala in females, both also prevented by adol CVS, suggesting different neurocircuits involved in generating resilience between sexes. We explored the mechanism behind reduced IL recruitment by studying the intrinsic excitability of IL pyramidal neurons. SPS reduced excitability of IL neurons and prior adol CVS prevented this effect.

Conclusion: Our data indicate that adolescent stress can impart resilience to the effects of traumatic stress on neuroplasticity and behavior. Our data provide a mechanistic link behind developmental stress induced behavioral resilience and prefrontal (IL) cortical excitability.

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Introduction:

Understanding factors that affect the brain during adolescence has substantial health relevance given the onset of numerous affective conditions (e.g., depression, anxiety disorders) during this developmental period (1–3). In general, chronic stress during development is associated with the emergence of pathology, particularly when occurring during early life (4–8). However, mild to moderate stress during development period may promote an adaptive response to adverse situations later in life, contributing to stress resilience (9–13). While the mechanisms implicated in developmental vulnerability to stress dysregulation are widely studied, resiliency after stress is poorly understood.

Memories that are acquired under stressful situations are usually strongly consolidated and can be retrieved more easily than those acquired in neutral situations (14). Prior exposure to stress can further enhance the acquisition and/or expression of the fear related behaviors (15), processes linked to the prelimbic (PL) and infralimbic (IL) divisions of the rodent medial prefrontal cortex (16). Learned fear has an obvious adaptive value, increasing the chance of survival in life threatening situations (16). However, traumatic experiences can lead to exaggerated and prolonged fear responses, as seen in posttraumatic stress disorder (PTSD). Here, individuals experience recurring episodes of involuntary memories associated with an intense stress response, resulting in hyper alertness and avoidance of situations that remind them of the traumatic event (15,17). The aberrent fear response in PTSD is iassociated with ventromedial PFC (homolog to the rodent IL) hypoactivity and loss of top-down control over the amygdala (18). Interestingly, although there is a high chance of experiencing trauma in the

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population, only about 7% of people develop PTSD (2,19,20), suggesting that resilience or vulnerability to development of PTSD may be determined by experiential and/or genetic factors.

Rodents are widely used to study how stress affects learned fear memories, and as a means of gaining insight into possible mechanisms underlying PTSD. Stress-enhanced fear models usually combine exposure to one or more stressors, with fear responses tested in a conditioning paradigm (15). One of the most widely-used and reproduced models is the single-prolonged stress protocol (SPS) developed by Liberzon (21,22). Exposure to SPS impairs extinction and extinction recall of a fear conditioned response one week later (23–25), comprising a late-emerging enhancement of fear, as is characteristic of PTSD. SPS also reduces neuronal activation in the IL , which may play a role in the abnormal fear extinction deficits associated with SPS (26).

Previous work from our lab shows that chronic variable stress (CVS) during adolescence can evoke specific effects later in life which may determine either risk or resilience (27,28). In the present study we assess the impact of adolescent CVS on stress vulnerability or resilience to subsequent SPS in adulthood Our data indicate that the experience of stress during adolescence blocks fear potentiation following SPS and prevents SPS-induced decreases in excitability of IL pyramidal neurons, suggesting a a key role for this cortical region in conferring resilience to traumatic stress

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Materials and Methods

Detailed methods described in supplemental material.

Animals: Male and female Sprague Dawley rats were bred in-house, with group assignments controlled for litter. All procedures and care performed in the animals were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Experimental design: Experiment 1: experimental groups were Control (No CVS – No SPS), Adol CVS (only chronic stress in adolescence), SPS (only single prolonged stress in adulthood) and CVS-SPS or double-hit group (adol CVS + SPS in adulthood) of both sexes. Experiment 2: Control (No adol CVS) and adol CVS, both sexes. Experiment 3: Control, adol CVS, SPS and CVS-SPS, only male rats.

Adolescent chronic variable stress (adol CVS): Rats were subjected to 14 days of CVS exposure during late adolescence (Postnatal day (PND) 45 $\pm$ 2). CVS consisted of a set of unpredictable variable stressors applied twice daily (AM and PM). Following CVS, animals were allowed to recover for 4 weeks to be then subjected to the single prolonged stress (SPS) protocol evaluated during adulthood. Timeline shown in Fig. 1.

Single prolonged stress (SPS): 4 weeks after recovering from adol CVS, half of the CVS and Control animals were subjected to SPS following Liberzon's protocol (24) consisting of 2h restraint, 20 minute swim (25 + 2°C) and ether anesthesia until loss of consciousness. The other half served as controls (no SPS).

Cued Fear Conditioning Paradigm: A week after SPS animals were subjected to an auditory tone cued fear conditioning protocol to evaluate the performance during the conditioning,

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extinction, and reinstatement sessions. The conditioned response evaluated was freezing behavior, considered as general absence of movement, which was scored using a video tracking system (EthovisionXT-Noldus).

Context fear conditioning test: As a way of quantifying the conditioned response to the context, we evaluated the initial freezing response exerted every day of the extinction procedure before the first tone was presented and analyzed the progression of this response over 3 days.

Immunohistochemistry: 90 minutes after reinstatement, rats were euthanized to obtain their brains for immunodetection of Fos, a marker of neuronal activation. We quantified the number of Fos positive nuclei/unit area in the IL and PL subdivisions of the mPFC , the basolateral amygdala complex (BLA) and the central lateral (CeL) and central medial (CeM) divisions of the central amygdala (CeA).

Cognitive function assessment: Following the same timeline for CVS onset, duration, and age of testing, control and adol CVS animals coming from the same litters used in the previous experiment were subjected to the novel object recognition (NOR) test and then the Morris water maze (MWM) a week later.

Electrophysiology: Following the same timeline as the behavioral studies, whole-cell patch clamp recordings were obtained from layer V pyramidal neurons in the IL PFC. A warm slicing protocol was used to prepare healthy adult rat brain slices as previously described (30). All

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measurements of intrinsic membrane excitability were taken from the resting membrane potential (RMP) in the current clamp mode approximately 1 min after whole-cell configuration was established.

Statistical analysis

Fear conditioning data were analyzed by repeated measurements ANOVA (adol CVS x SPS x Sex x time), with a level of significance of $p < 0.05$. Novel object recognition and Morris water maze data were analyzed by 2x2 ANOVA (Adol CVS x Sex). Fos data were analyzed by a 2-way ANOVA (2×2 design: adol CVS x SPS) within each sex with a level of significance $p <0.05$. Electrophysiology data were analyzed by 2x2 ANOVA (adol CVS x SPS). Details of number of cells used for electrophysiology data analysis are outlined in Table 1. In the cases where significant differences and interactions were found, the Bonferroni test was used for post hoc analysis. In the case there were only main effects of the factors but no significant interaction between them, we performed planned comparisons to evaluate individual differences. Data were analyzed using STATISTICA 7.0 (Statsoft, Inc.,Tulsa, USA) and Prism 8 (GraphPad Software, La Jolla California USA). Data not following a normal distribution were log transformed for analysis. Outliers were detected using the Grubbs' test (GraphPad Software) and removed from analysis.

Results:

Experiment 1: Cued conditioned response: Fig. 1.C-F illustrates the conditioned freezing response throughout the different sessions of the fear conditioning paradigm in animals that were submitted to chronic variable stress during adolescence (adol CVS) and later subjected to

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single prolonged stress (SPS) in adulthood. Animals were submitted to a tone-conditioned paradigm as shown in Fig 1.B. Fig 1.C shows the effects for each phase of paradigm evaluated. None of the treatments had effects on the conditioning phase, although male rats showed higher freezing than females in general. When assessing extinction over 3 days, SPS groups exhibited higher freezing, with no effect of sex. When analyzing within sex effects, we observed a significant impairment of extinction learning after SPS in males (but not females). This effect was blocked in the SPS group subjected to prior adol CVS. These results also contrast with the mild deficit on extinction learning following adolescent CVS alone. This paradoxical effect observed in the group subjected to the double-hit of CVS and SPS lead us to posit that the adol CVS prevented the effects of SPS, generating a resilient phenotype. The levels of extinction attained were stable for both sexes as tested in the recall phase. In this case, five days after extinction, animals received a brief extra extinction session (3 tones) to test for possible spontaneous recovery of the conditioned response and to corroborate that the levels of freezing in all the groups were equal before reinstatement. Lastly, we submitted the animals to a reinstatement procedure and observed enhanced reinstatement of the freezing response to the tone in both males and females exposed to SPS. Enhanced reinstatement was not observed in males or female exposed to both adolescent CVS and SPS, again suggesting a possible beneficial effect of developmental stress exposure in fear processing.

Context conditioned response: To quantify the conditioned response to the conditioning context, we evaluated the freezing response evoked every day of the extinction procedure before the first tone was presented and analyzed the progression over 3 days (Fig 1.E). We

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observed that, during presentation of the tone, SPS groups exhibited higher freezing in general. In the case of male rats, the group that was subjected to prior adol CVS did not show enhanced freezing to the context on any of the days evaluated.

Fos expression after reinstatement: Figure 2 shows the Fos immunoreactivity (Fos-ir) in the mPFC and amygdala of the animals, tested in response to the reinstatement trial (90 min after the onset of the session). In the case of males, SPS decreased Fos induction only in the IL cortex (Fig 2.A), while the other groups remained at control levels. Effects of SPS on IL Fos were not observed in animals exposed to CVS in adolescence. Conversely, in females, effects of adol CVS and SPS were only observed in the central nucleus of the amygdala, particularly in lateral portion (CeL), and this was also prevented in the double-hit group (Fig 2.B).

Experiment 2: Cognitive effects of adolescent CVS: With the purpose of detecting the possible impact of adolescent CVS on learning or mnemonic processes, which could confound the interpretation of the results observed in the double-hit experiment, we subjected a cohort of littermates to alternative behavioral tests to assess the integrity of cognitive functions (Fig 3). In this case we only observed effects of adol CVS on the MWM probe test, with no effects during the training phase. Exposure to stress in adolescence enhanced recall of platform location, indicated by increased time spent on the target quadrant, regardless of sex.

Experiment 3: Electrophysiology: We next investigated the potential cellular mechanisms underlying how adolescent stress can prevent SPS-induced changes in fear behavior and Fos

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activation in IL in male rats. We measured the intrinsic membrane properties and firing frequency of IL pyramidal neurons in layer V, the major source of subcortical output from the IL (29). We found that prior experience of adol CVS prevented SPS-mediated changes in intrinsic excitability of IL pyramidal neurons. Specifically, we observed that SPS increased rheobase and decreased membrane resistance, whereas prior experience of adol CVS was able to prevent these effects (Fig 4D & 4E respectively). Additionally, SPS increased the threshold for action potential (AP) firing and decreased AP amplitude, both of which were prevented by prior adol CVS (Fig 4F and 4G respectively). SPS also reduced the duration of AP (AP 50), which was also blocked by prior adol CVS. It should be noted that adol CVS alone increased AP duration (Fig 4H). Finally, adol CVS was also able to attenuate the reduction in peak firing frequency observed following SPS (Fig 4K). Together these data indicate that prior experience of adolescent stress is able to prevent the reduction in intrinsic excitability and firing rate of IL layer V pyramidal neurons following SPS. We did not observe changes in membrane capacitance (Fig 4J), indicating that treatments likely did not affect cell size.

Discussion

Our results strongly suggest that prior experience with stress during adolescence evokes a resilient phenotype in the adult, characterized by the prevention of the effects of SPS in a fear conditioning paradigm and on IL pyramidal cell excitability. Our data indicate that the adaptations resulting from exposure to chronic stress during adolescence buffer the behavioral impact of a model of traumatic stress in adulthood, blocking known effects of SPS on subsequent fear potentiation. This was accompanied by reversal of SPS-induced IL hypoactivity

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(males) and CeL recruitment (females) following reinstatement of the conditioned response. The IL plays a major role in promoting extinction of conditioned fear, whereas the CeA is involved in expression of fear behaviors: either may drive pathological fear responses, albeit by different mechanisms. Subsequent electrophysiological analysis indicates that SPS induces a profound hypoactivity of layer V pyramidal cells in the IL that was blocked by prior adolescent CVS, suggesting that altered excitability of IL glutamatergic pyramidal neurons may explain resilience to the behavioral effects of SPS, at least in male rats (Figure 5).

Stress during development is generally thought to evoke negative behavioral effects later in life (27,28,30–35). However, prior studies also support the ability of adolescent stress to confer stress resilience in adulthood, using a number of stress models (reviewed in 11,36,37). Salutary effects of adolescent stress on subsequent severe stress reactivity may be related to stressor modality, timing or duration. For instance, intermittent predator stress from PND 33 to PND 56 increases active coping two weeks after the stress (38). Predictable chronic mild stress (PCMS) encompassing all adolescence improves outcomes related to anhedonia, coping and anxiety, and prevents behavioral effects after chronic unpredictable stress (CUS) in adulthood (39). Adolescent PCMS also enhances extinction and prevents reinstatement and spontaneous recovery in a fear conditioning model evaluated immediately and one week following PCMS (40). In combination with our observation of stress resilience 4 weeks following CVS exposure, these studies suggest that adolescent stress effects enhancing the mechanisms for resilience endure for several weeks, if not persisting throughout life. It is notable that the impact of

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adolescent stress differs from that imposed earlier in life, where the data generally report detrimental effects of stress (41–45).

Although some authors proposed that the resilient phenotype is promoted by the predictability of the stressors (40), the unpredictable nature of CVS suggests that the resilience mechanism might be independent of response habituation. Timing combined with stressor modality seem to be an important factor as well. In this sense, prior work indicates adult resilience even after a single intense stressor protocol at PND37 or following 3 days of predator related stressors at PND33-35 (46). In contrast, a 3-day pre-pubertal exposure to variate stressors failed to attenuate exaggeration of fear responses in adulthood, indicating that developmental timing is critical for establishment of resilience.

Stress modality may also play a role in the development of resilience. For instance, complete social isolation during adolescence increases anxiety-like behavior, acoustic startle and impaired extinction in adulthood (47), whereas social instability (PND 30-45), that entails poor social structure but no isolation, evokes a reduction of conditioned fear expression in adulthood (48). The same protocol of social instability proved to be less effective in female rats (49), pointing to possible sex-dependent mechanisms for the programming of the long-term effects of adolescent stress, consistent with our observed sex differences.

Morris water maze and novel object recognition tests were used to assess whether adolescent CVS causes lasting cognitive deficits that could explain poor retention of the conditioned memory or faster extinction of the fear response. Our data indicate that general cognitive

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function was not negatively affected in the animals with prior CVS, confirming specificity of the resilience phenotype to fear-related memories. Interestingly, adol CVS improved the retention of spatial memory in both sexes in the MWM, an effect reported in other models of juvenile stress (50,51). However, CVS encompassing the whole duration of adolescence decreased the time on the target platform of the Morris water maze when tested after 3 weeks of recovery from CVS (52), pointing again to the importance of timing, duration and intensity of adolescence stress for resilience.

Hypoactivity of the medial PFC is observed in several mental health disorders, including PTSD (53). Results from our group and others indicate that stress during adolescence reduces neuronal recruitment (Fos expression) to adult stressors in the mPFC (27,28,54). In humans, PTSD has been associated with a reduction in prefrontal drive, leading to abnormal extinction of conditioned fear (18,55–57). Similarly, reduced IL mPFC activity following SPS in male rats may underlie abnormal extinction of fear responses (26). Consistent with these data, our results indicate reduced IL recruitment following SPS in male rats accompanied by higher freezing during extinction and reinstatement. The reduced engagement of the IL after SPS in response to conditioned cues during the reinstatement procedure also suggests a possible reduction of IL activity occurring during the extinction procedure, which would explain the impairment of extinction learning previously observed in the same group. Remarkably, in the case of the female rats, Fos expression was only affected by SPS in the CeL division of the central amygdala (with no changes in the PFC) and this area also exhibited resilience in the double-hit group. CeL is associated with expression of conditioned fear responses (58,59). Our data indicate that SPS

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females exhibit enhanced freezing expression during reinstatement with minimal effect during extinction (consistent with 60), further aligning with the involvement of CeL and lack of involvement of the IL in the resilient effect in female rats. These results indicate sex differences in response to stress and fear related behaviors and highlight involvement of sex-specific brain regions, particularly the IL in males and CeL in females, in evoking a resilient phenotype (Figure 5).

Our electrophysiology studies indicate that adolescent CVS counteracts the inhibitory effects of SPS on intrinsic excitability of layer V pyramidal neurons in the IL (Figure 5). Our data also align with our fear conditioning findings (Figure 1), where robust resilience effects are only observed following the double-hit of both stressors. Our results suggest that plastic changes in the PFC following adol CVS may serve to prime the PFC to prevent the decrements in excitability observed with SPS, which eventually leads to the behavioral resilience observed in fear conditioning.

Neurons in the mPFC are specifically activated during stressful situations and modulate their responses to subsequent exposure to the same stressor experience (61), thus playing a critical role in eliciting adaptive responses to aversive stimuli (62). Modification of PFC responses to the same stimulus can be mediated through altered glutamatergic or dopaminergic drive onto the mPFC projection neurons (63,64). Adolescent social defeat decreases adult NMDA receptor expression in the IL PFC, and also reduces freezing to fear conditioning (65). Thus, the enhanced excitability we observed in SPS rats with prior history of adol CVS might be a long-term

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adaptation to the reduced excitatory drive that may occur following adol CVS. Our data indicate that the intrinsic excitability changes do not manifest at baseline conditions under adol CVS alone. Consistent with our findings, resilient mice show control level firing activity (66,67). Thus it is possible that prior adol CVS may serve to prime the pyramidal cells to react appropriately when faced with the second hit of SPS by modulating their responses to buffer the reduction in excitability associated with SPS.

Prior work indicates that resilience or vulnerability to stress-induced emotional dysregulation may be modulated by changes in intrinsic excitability of prefrontal neurons (68–70). Intrinsic membrane properties play a critical part in determining the prefrontal excitatory/inhibitory balance, as they directly shape neuronal output by influencing the probability of a neuron firing an action potential in response to excitatory synaptic inputs and modulate firing capacity. Animals resilient to social defeat display increased neural activity in the mPFC and optogenetic drive of the mPFC can promote resilience (71). However, the cell type (GABAerigc versus glutamatergic) exhibiting this hyperactivity is not known (71,72). Similar studies exploring the role of mPFC glutamatergic pyramidal neurons in other models of psychiatric disorders have been inconsistent (73–75). Our findings provide significant addition to the resilience literature by indicating that alteration in intrinsic excitability of IL mPFC glutamatergic pyramidal neurons might promote a resilient phenotype following adolescent stress.

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The exact mechanism underlying the altered excitability of IL pyramidal neurons observed in our study is yet to be determined. Alterations in ion channels can influence the excitability of neurons. For example, G protein-gated inwardly rectifying K+ channels (GIRKs) play a crucial role in maintaining the excitability of layer V/VI pyramidal neurons in the prelimbic cortex (76,77). Other studies have implicated the role of hyperpolarization-activated cyclic nucleotide–gated (HCN) channels in modulating the intrinsic membrane excitability of cortical neurons (78). Further work is needed to identify the specific ionic mechanisms by which adolescent stress can protect against future stressors during adulthood. (79)

Conclusion:

Our results support the idea that certain combinations of stressful situations during adolescence can be beneficial, evoking resilience to stress in adult life. We propose that, in rats, chronic variable stress during late adolescence determines differential activation or recruitment of the IL in response to intense stress in adulthood. This rearrangement of prefrontal activity results in a phenotype that is resilient to stress-enhanced fear learning, reducing contextual response, facilitating extinction and preventing reinstatement of the fear conditioned response, following trauma, which is of potential translational value for the study of PTSD. Furthermore, our data indicate sex specific effects in behavioral resilience following adolescent stress and identifies sex differences in stress reactive brain regions. It would be important to determine which stressor type might result in a positive emotional valence and whether that ultimately evokes a resilience response. For example, the exercise (swim) or social component (crowding) of the adolescent CVS regimen might help individuals cope with

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subsequent stress in adulthood (80,81). The next challenge is to find the most efficient developmental triggers for the generation of resilience to the effects of adult stress, possibly including positive developmental interventions, with the goal of reducing the incidence of stress-related affect conditions, including PTSD.

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The data have been partially presented as abtracts and posters at various conferences and the manuscript has been published in Bioarxiv.

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Bibliography

1. Andersen SL, Teicher MH (2008): Stress, sensitive periods and maturational events in adolescent depression. *Trends Neurosci* 31: 183–91.

2. Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005): Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV Disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry* 62(6):593–602.

3. Paus T, Keshavan M, Giedd JN (2008): Why do many psychiatric disorders emerge during adolescence? *Nat Rev Neurosci* 9: 947–957.

4. de Kloet ER, Joëls M, Holsboer F (2005): Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6: 463–475.

5. Oitzl MS, Champagne DL, van der Veen R, de Kloet ER (2010): Brain development under stress: Hypotheses of glucocorticoid actions revisited. *Neurosci Biobehav Rev* 34: 853–866.

6. Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB (2008): The link between childhood trauma and depression: Insights from HPA axis studies in humans. *Psychoneuroendocrinology* 33: 693–710.

7. Riboni FV, Belzung C (2017): Stress and psychiatric disorders: from categorical to dimensional approaches. *Current Opinion in Behavioral Sciences* 14:72-77.

8. Tost H, Champagne FA, Meyer-Lindenberg A (2015): Environmental influence in the brain, human welfare and mental health. *Nature Neuroscience* 18 (10): 1421–1431.

9. Southwick SM, Charney DS (2012): The Science of Resilience: Implications for the Prevention and Treatment of Depression. *Science* 338 (6103): 79–82.

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10. Ricon T, Toth E, Leshem M, Braun K, Richter-Levin G (2012): Unpredictable chronic stress in juvenile or adult rats has opposite effects, respectively, promoting and impairing resilience. *Stress* 15: 11–20.

11. Romeo RD (2015): Perspectives on stress resilience and adolescent neurobehavioral function. *Neurobiology of Stress* 1: 128–133.

12. Schmidt M (2011): Animal models for depression and the mismatch hypothesis of disease. *Psychoneuroendocrinology* 36: 330–338.

13. Ordoñes Sanchez E, Bavley CC, Deutschmann AU, Carpenter R, Peterson DR, Karbalaei R, *et al.* (2021): Early life adversity promotes resilience to opioid addiction-related phenotypes in male rats and sex-specific transcriptional changes. *Proc Natl Acad Sci* 118: e2020173118.

14. Meir Drexler S, Wolf OT (2017, July 1): The role of glucocorticoids in emotional memory reconsolidation. *Neurobiology of Learning and Memory*, vol. 142. Academic Press, pp 126–134.

15. Blouin AM, Sillivan SE, Joseph NF, Miller CA (2016): The potential of epigenetics in stress-enhanced fear learning models of PTSD. *Learn Mem* 23: 576–86.

16. Giustino TF, Maren S (2015): The Role of the Medial Prefrontal Cortex in the Conditioning and Extinction of Fear. *Front Behav Neurosci* 9: 298.Giustino TF, Maren S (2015): The Role of the Medial Prefrontal Cortex in the Conditioning and Extinction of Fear. *Front Behav Neurosci* 9. https://doi.org/10.3389/fnbeh.2015.00298

17. Sareen J (2014): Posttraumatic stress disorder in adults: Impact, comorbidity, risk factors, and treatment. *Can J Psychiatry* 59: 460–467.

18. Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, *et al.* (2009): Neurobiological

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Basis of Failure to Recall Extinction Memory in Posttraumatic Stress Disorder. *Biol Psychiatry* 66: 1075–1082.

19. Benjet C, Bromet E, Karam EG, Kessler RC, McLaughlin KA, Ruscio AM, *et al.* (2016): The epidemiology of traumatic event exposure worldwide: results from the World Mental Health Survey Consortium. *Psychol Med* 46: 327–43.

20. Website: Epidemiology of PTSD - PTSD: National Center for PTSD. Last accessed March 15, 2021 https://www.ptsd.va.gov/professional/treat/essentials/epidemiology.asp.21. Liberzon I, Krstov M, Young EA (1997): Stress-restress: Effects on ACTH and fast feedback. *Psychoneuroendocrinology* 22: 443–453.

22. Liberzon I, Krstov M, Young EA (1997): Stress-restress: Effects on ACTH and fast feedback. *Psychoneuroendocrinology* 22: 443–453.

23. Knox D, Nault T, Henderson C, Liberzon I (2012): Glucocorticoid receptors and extinction retention deficits in the single prolonged stress model. *Neuroscience* 223: 163–173.

24. Knox D, George SA, Fitzpatrick CJ, Rabinak CA, Maren S, Liberzon I (2012): Single prolonged stress disrupts retention of extinguished fear in rats. *Learn Mem* 19: 43–9.

25. Kohda K, Harada K, Kato K, Hoshino A, Motohashi J, Yamaji T, *et al.* (2007): Glucocorticoid receptor activation is involved in producing abnormal phenotypes of single-prolonged stress rats: A putative post-traumatic stress disorder model. *Neuroscience* 148: 22–33.

26. Piggott VM, Bosse KE, Lisieski MJ, Strader JA, Stanley JA, Conti AC, *et al.* (2019): Single-prolonged stress impairs prefrontal cortex control of amygdala and striatum in rats. *Front Behav Neurosci* 13.

27. Cotella EM, Scarponi Gómez A, Lemen P, Chen C, Fernández G, Hansen C, *et al.* (2019):

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Long-term impact of chronic variable stress in adolescence versus adulthood. *Prog Neuro-Psychopharmacology Biol Psychiatry* 88: 303–310.

28. Cotella EM, Morano RL, Wulsin A, Martelle SM, Lemen P, Fitzgerald M, *et al.* (2020): Lasting Impact of Chronic Adolescent Stress and Glucocorticoid Receptor Selective Modulation in Male and Female Rats. *Psychoneuroendocrinology* 112: 104490.
29. Baker A, Kalmbach B, Morishima M, Kim J, Juavinett A, Li N, Dembrow N (2018): Specialized subpopulations of deep-layer pyramidal neurons in the neocortex: Bridging cellular properties to functional consequences. *J Neurosci* 38: 5441–5455.
30. Green MR, Barnes B, Mccormick CM (2013): Social instability stress in adolescence increases anxiety and reduces social interactions in adulthood in male long-evans rats. *Dev Psychobiol* 55: 849–859.
31. Negrón-Oyarzo I, Pérez MÁ, Terreros G, Muñoz P, Dagnino-Subiabre A (2014): Effects of chronic stress in adolescence on learned fear, anxiety, and synaptic transmission in the rat prelimbic cortex. *Behav Brain Res* 259: 342–353.
32. Wulsin A, Wick-Carlson D, Packard BA, Morano R, Herman JP (2016): Adolescent chronic stress causes hypothalamo-pituitary-adrenocortical hypo-responsiveness and depression-like behavior in adult female rats. *Psychoneuroendocrinology* 65: 109–17.
33. Wilkin MM, Waters P, McCormick CM, Menard JL (2012): Intermittent physical stress during early- and mid-adolescence differentially alters rats' anxiety- and depression-like behaviors in adulthood. *Behav Neurosci* 126: 344–360.
34. Bourke CH, Neigh GN (2011): Behavioral effects of chronic adolescent stress are sustained and sexually dimorphic. *Horm Behav* 60: 112–120.

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35. Begni V, Zampar S, Longo L, Riva MA (2020): Sex Differences in the Enduring Effects of Social Deprivation during Adolescence in Rats: Implications for Psychiatric Disorders. *Neuroscience* 437: 11–22.

36. Bock J, Rether K, Gröger N, Xie L, Braun K (2014, February 5): Perinatal programming of emotional brain circuits: An integrative view from systems to molecules. *Frontiers in Neuroscience* 8:11.

37. Suri D, Vaidya VA (2015): The adaptive and maladaptive continuum of stress responses – a hippocampal perspective. *Rev Neurosci* 26. 3

38. Kendig MD, Bowen MT, Kemp AH, McGregor IS (2011): Predatory threat induces huddling in adolescent rats and residual changes in early adulthood suggestive of increased resilience. *Behav Brain Res* 225: 405–414.

39. Suo L, Zhao L, Si J, Liu J, Zhu W, Chai B, *et al.* (2013): Predictable chronic mild stress in adolescence increases resilience in adulthood. *Neuropsychopharmacology* 38: 1387–1400.

40. Deng JH, Yan W, Han Y, Chen C, Meng SQ, Sun CY, *et al.* (2017): Predictable Chronic Mild Stress during Adolescence Promotes Fear Memory Extinction in Adulthood. *Sci Rep* 7: 1–15.

41. Johnson DC, Casey BJ (2015): Easy to remember, difficult to forget: The development of fear regulation. *Dev Cogn Neurosci*. 11: 42-55.

42. Lukkes JL, Mokin M V, Scholl JL, Forster GL (2009): Adult rats exposed to early-life social isolation exhibit increased anxiety and conditioned fear behavior, and altered hormonal stress responses. *Horm Behav* 55: 248–56.

43. Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002): Chronic stress induces

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contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* 22: 6810–8.

44. Yee N, Schwarting RKW, Fuchs E, Wöhr M (2012): Juvenile stress potentiates aversive 22-kHz ultrasonic vocalizations and freezing during auditory fear conditioning in adult male rats. *Stress* 15: 533–544.

45. McEwen BS (2007): Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev* 87: 873–904.

46. Chaby LE, Sadik N, Burson NA, Lloyd S, O'Donnel K, Winters J, *et al.* (2020): Repeated stress exposure in mid-adolescence attenuates behavioral, noradrenergic, and epigenetic effects of trauma-like stress in early adult male rats. *Sci Rep* 10: 17935.

47. Skelly MJ, Chappell AE, Carter E, Weiner JL (2015): Adolescent social isolation increases anxiety-like behavior and ethanol intake and impairs fear extinction in adulthood: Possible role of disrupted noradrenergic signaling. *Neuropharmacology* 97: 149–159.

48. Morrissey MD, Mathews IZ, McCormick CM (2011): Enduring deficits in contextual and auditory fear conditioning after adolescent, not adult, social instability stress in male rats. *Neurobiol Learn Mem* 95: 46–56.

49. McCormick CM, Mongillo DL, Simone JJ (2013): Stress The International Journal on the Biology of Stress Age and adolescent social stress effects on fear extinction in female rats Age and adolescent social stress effects on fear extinction in female rats. *Stress* 16: 678–688.

50. Frisone DF, Frye CA, Zimmerberg B (2002): Social isolation stress during the third week of life has age-dependent effects on spatial learning in rats. *Behav Brain Res* 128: 153–160.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.16.435691; this version posted March 17, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

51. Li J, Li Y, Sun Y, Wang H, Liu X, Zhao Y, *et al.* (2019): Chronic mild corticosterone exposure during adolescence enhances behaviors and upregulates neuroplasticity-related proteins in rat hippocampus. *Prog Neuro-Psychopharmacology Biol Psychiatry* 89: 400–411.

52. Isgor C, Kabbaj M, Akil H, Watson SJ (2004): Delayed effects of chronic variable stress during peripubertal-juvenile period on hippocampal morphology and on cognitive and stress axis functions in rats. *Hippocampus* 14: 636–648.

53. Hains AB, Arnsten AFT (2008): Molecular mechanisms of stress-induced prefrontal cortical impairment: implications for mental illness. *Learn Mem* 15: 551–64.

54. Ishikawa J, Nishimura R, Ishikawa A (2015): Early-life stress induces anxiety-like behaviors and activity imbalances in the medial prefrontal cortex and amygdala in adult rats. *Eur J Neurosci* 41: 442–453.

55. Milad MR, Rauch SL, Pitman RK, Quirk GJ (2006): Fear extinction in rats: Implications for human brain imaging and anxiety disorders. *Biol Psychol* 73: 61–71.

56. Rauch SL, Shin LM, Phelps EA (2006): Neurocircuitry Models of Posttraumatic Stress Disorder and Extinction: Human Neuroimaging Research-Past, Present, and Future. Biol Psychiatry 60(4): 376–382.

57. Milad MR, Orr SP, Pitman RK, Rauch SL (2005): Context modulation of memory for fear extinction in humans. *Psychophysiology* 42: 456–464.

58. Marek R, Strobel C, Bredy TW, Sah P (2013): The amygdala and medial prefrontal cortex: partners in the fear circuit. *J Physiol* 591: 2381–2391.

59. Marek R, Sah P (2018): Neural Circuits Mediating Fear Learning and Extinction BT - Systems Neuroscience. In: Cheung-Hoi Yu A, Li L, editors. Cham: Springer International Publishing,

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pp 35–48.

60. Keller SM, Schreiber WB, Staib JM, Knox D (2015): Sex differences in the single prolonged stress model. *Behav Brain Res* 286: 29–32.

61. Jackson ME, Moghaddam B (2006): Distinct patterns of plasticity in prefrontal cortex neurons that encode slow and fast responses to stress. *Eur J Neurosci* 24: 1702–1710.

62. Milad MR, Quirk GJ (2002): Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature* 420: 70–74.

63. Bagley J, Moghaddam B (1997): Temporal dynamics of glutamate efflux in the prefrontal cortex and in the hippocampus following repeated stress: Effects of pretreatment with saline or diazepam: *Neuroscience* 77: 65–73.

64. Jackson ME, Moghaddam B (2004): Stimulus-specific plasticity of prefrontal cortex dopamine neurotransmission. *J Neurochem* 88: 1327–1334.

65. Novick AM, Mears M, Forster GL, Lei Y, Tejani-Butt SM, Watt MJ (2016): Adolescent social defeat alters N-methyl-d-aspartic acid receptor expression and impairs fear learning in adulthood. *Behav Brain Res* 304: 51–59.

66. Han MH, Nestler EJ (2017): Neural Substrates of Depression and Resilience. Neurotherapeutics 14: 677–686.

67. Friedman AK, Walsh JJ, Juarez B, Ku SM, Chaudhury D, Wang J, *et al.* (2014): Enhancing depression mechanisms in midbrain dopamine neurons achieves homeostatic resilience. *Science* 344: 313–319.

68. Vialou V, Bagot RC, Cahill ME, Ferguson D, Robison AJ, Dietz DM, *et al.* (2014): Prefrontal cortical circuit for depression- and anxiety-related behaviors mediated by cholecystokinin:

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.16.435691; this version posted March 17, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Role of ΔFosB. *J Neurosci* 34: 3878–3887.

69. Labonté B, Engmann O, Purushothaman I, Menard C, Wang J, Tan C, *et al.* (2017): Sex-specific transcriptional signatures in human depression. *Nat Med* 23: 1102–1111.

70. Kumar S, Hultman R, Hughes D, Michel N, Katz BM, Dzirasa K (2014): Prefrontal cortex reactivity underlies trait vulnerability to chronic social defeat stress. *Nat Commun* 5: 4537.

71. Covington HE, Lobo MK, Maze I, Vialou V, Hyman JM, Zaman S, *et al.* (2010): Antidepressant Effect of Optogenetic Stimulation of the Medial Prefrontal Cortex. *J Neurosci* 30: 16082–16090.

72. Russo SJ, Murrough JW, Han MH, Charney DS, Nestler EJ (2012): Neurobiology of resilience. *Nature Neuroscience* 15:1475–1484.

73. Lee E, Hong J, Park YG, Chae S, Kim Y, Kim D (2015): Left brain cortical activity modulates stress effects on social behavior. *Sci Rep* 5: 13342.

74. Warden MR, Selimbeyoglu A, Mirzabekov JJ, Lo M, Thompson KR, Kim SY, *et al.* (2012): A prefrontal cortex-brainstem neuronal projection that controls response to behavioural challenge. *Nature* 492: 428–432.

75. Yizhar O, Fenno LE, Prigge M, Schneider F, Davidson TJ, Ogshea DJ, *et al.* (2011): Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature* 477: 171–178.

76. Anderson EM, Gomez D, Caccamise A, McPhail D, Hearing M (2019): Chronic unpredictable stress promotes cell-specific plasticity in prefrontal cortex D1 and D2 pyramidal neurons. *Neurobiol Stress* 10: 100152.

77. Hearing M, Kotecki L, MarronFernandezdeVelasco E, Fajardo-Serrano A, Chung HJ, Luján R,

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.16.435691; this version posted March 17, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Wickman K (2013): Repeated Cocaine Weakens GABAB-Girk Signaling in Layer 5/6 Pyramidal Neurons in the Prelimbic Cortex. *Neuron* 80: 159–170.

78. Shah MM (2014): Cortical HCN channels: Function, trafficking and plasticity. *J Physiol* 592: 2711–2719.

79. Matovic S, Ichiyama A, Igarashi H, Salter EW, Sunstrum JK, Wang XF, *et al.* (2020): Neuronal hypertrophy dampens neuronal intrinsic excitability and stress responsiveness during chronic stress. *J Physiol* 598: 2757–2773.

80. Herring MP, O'Connor PJ, Dishman RK (2010): The effect of exercise training on anxiety symptoms among patients: A systematic review. *Arch of Intern Med* 170(4): 321–331.

81. Ozbay F, Johnson DC, Dimoulas E, Morgan CA, Charney D, Southwick S (2007): Social support and resilience to stress: from neurobiology to clinical practice. *Psychiatry (Edgmont)* 4: 35–40.

Figure legends:

Figure 1: A) Experimental timeline. **B)** Fear conditioning paradigm **C, D)** Previous adolescent stress enhanced extinction in male rats and prevented reinstatement of tone-conditioned freezing after SPS in both male and female rats. **Conditioning:** There was an interaction adol CVS x SPS ($F_{(1,79)}$= 5.075, p=0.027) but no individual differences in the Bonferroni test. The significant effect of time($F_{(5,395)}$= 469.308, p<0.0001) confirmed the conditioning of the response. There was main effect of sex ($F_{(1,79)}$= 7.724, p=0.007), with Bonferroni comparisons indicating a general higher expression of freezing in male rats (ϕ). **3-day extinction**: there were significant effects of SPS ($F_{(1,76)}$= 6.698, p=0.012),

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interaction adol CVS x SPS ($F_{(1,76)}$= 7.414, p= 0.008) and time ($F_{(2,152)}$= 475,661 , p<0.0001), with no effect of sex. The CVSxSPS post hoc analysis over confirmed that, in general, SPS groups had higher freezing levels compared to controls over the whole extinction procedure regardless of sex. When performing planned comparisons by sex, only male rats showed statistical effects, the SPS group had higher freezing than the control group on all three days (p<0.05 *) while the CVS only group had enhanced freezing only on day 2 (p<0.05 *). Prior adol CVS prevented the SPS effects, as the double-hit group remained at control levels on all testing days and had significantly less freezing than the SPS group on day 2 (p<0.05 #). Only on day 3 we observed sex differences, SPS evoked higher freezing in male rats (p<0.05 ϕ). **Recall:** We observed a main effect of sex ($F_{(1,76)}$=7.958, p=0.006) with males expressing more freezing in general (ϕ), and a triple interaction sex x adol CVS x SPS ($F_{(1,76)}$=4.792, p=0.032) with no difference for any individual Bonferroni comparison. **Reinstatement:** : We observed a significant adol CVS x SPS interaction $F_{(1,76)}$= 11.8095, p=0.001. Planned comparisons regardless of sex showed that both SPS and CVS groups expressed higher freezing than the control group (p<0.05 respectively), while the double-hit group prevented the effect of SPS, remaining at control freezing levels and expressing significantly less freezing time than the SPS group (p<0.05). When analyzing the individual responses by sex, we observed that in female rats, only the SPS group differed from control (p<0.05*). In the case of male rats, the SPS group had higher freezing than control (p<0.05*) and the adol CVS-SPS group (p<0.05#). **Context conditioned response:** Tested as freezing during the 2 minutes prior to the first tone each day of the extinction procedure. We observed a main effect of adol CVS $F_{(1,76)}$=4.097, p=0.046 and significant adol CVS x SPS interaction $F_{(1,76)}$=11.729, p=0.001. The posthoc analyses indicated that animals subjected to

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SPS expressed higher freezing when re-exposed to the conditioning context compared to all the other groups ($p<0.05$ respectively). There was a sex effect $F_{(1,76)}=6.971$, $p=0.01$, with male rats having more freezing time ($p<0.05$ ϕ). There was also an effect of time $F_{(2,152)}=172.946$, $p<0.00001$, indicating reduction of freezing to subsequent exposure. Finally, there was an interaction of time x sex x SPS $F_{(2,152)}=9.253$, $p=0.0005$. Planned comparisons showed that male rats subjected to the SPS model expressed higher freezing than the control group on day 2 (*) while the group subjected do the double-hit model of stress had less context freezing compared to all the other groups on day 1 ($p<0.05$ respectively). This difference was maintained against the SPS group on the rest of the days tested ($P<0.05$ respectively #). In the case of females, SPS group had higher freezing to the context than all the other groups on day 1 ($p<0.05$ respectively °) and the adol CVS-SPS group also had more freezing compared to controls on that day ($p<0.05$ *).

Data represented as Mean±SEM. Image created with BioRender.com

Figure 2 : Fos immunoreactivity (Fos-ir) in the medial prefrontal cortex **(A)** and amygdala **(B)** of the animals 90 min after reinstatement (see timeline figure 1.B). In the case of males, we observed a significant effect of SPS in the infralimbic (IL) cortex of male rats, $F_{(1, 16)}=7.706$, $p=0.0135$. Planned comparisons confirmed that the SPS groups had significantly less Fos-ir than the control and CVS animals ($p<0.05$ *), while the other groups did not differ from controls. In females, effects of adol CVS and SPS were only observed in the central nucleus of the amygdala, particularly, in lateral portion (CeL) and not on the central medial division (CeM) (SPS $F_{(1, 23)}= 5.945$, $p=0.0229$, adol CVS x SPS $F_{(1, 23)} = 7.241$, $p=0.0130$). Bonferroni's test

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confirmed that SPS significantly increased neuronal recruitment ($p<0.05$ *) and this was prevented in the double-hit group ($p<0.05$ #). Data as Mean+SEM.

Figure 3: Previous adolescent stress does not affect novel object recognition nor locomotion in both sexes. **Novel object Recognition:** There were no effects of adolescent CVS on the discrimination ratio **(A)** between novel and familiar object in both sexes. There was a main effect of sex $F_{(1, 29)}=6.170$, $p=0.0192$ indicating that recognition memory is enhanced in females ($p<0.05$ *). Importantly, there were no differences in locomotion **(B)** caused by adol CVS, although the main effect of sex $F_{(1, 28)}=11.96$, $p=0.0018$, confirmed that females had increased locomotion ($p<0.05$ *).

Morris Water maze: there were no effects of adol CVS nor sex during training **(C)**. Adol CVS had a main effect on test day **(D)** $F_{(1, 27)}=16.80$, $p=0.0003$. Planned comparisons showed that CVS group of both sexes had better retention of the location of the platform during the probe test ($p<0.05$ respectively *). Data as Mean+SEM.

D: The panel displays the collapsed heat maps for the probe test by group. The arrow indicates the position of platform during the training phase of the test.

Figure 4: Adolescent CVS prevents SPS effects on membrane properties and intrinsic excitability of IL pyramidal neurons. **(A)** Experimental timeline; **(B)** Schematic of coronal brain sections through PFC where recordings were performed, blue boxes indicate infralimbic region of the PFC;**(C)** Pyramidal neurons were identified based on somal morphology, presence of prominent apical dendrite and capacitance within the range of 70-90pF. Arrows indicate pyramidal

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neurons; **(D)** There was a significant main effect of SPS ($F_{(1,99)}$= 32.3, p<0.0001[a (Sup. table 1)]) and adol CVS ($F_{(1,99)}$= 8.5, p=0.005[a]) on rheobase. SPS significantly increased rheobase compared to the control group (p<0.05), which was prevented by prior adol CVS (p<0.05 compared to SPS); **(E)** There was a significant main effect of SPS ($F_{(1,98)}$= 41.96, p<0.0001[b]), adol CVS ($F_{(1,98)}$= 21.7, p=0.0002[b]) and a significant adol CVS X SPS interaction ($F_{(1,98)}$= 6.7, p=0.01[b]) on membrane resistance. Bonferroni's test indicated that SPS significantly decreased membrane resistance (p<0.05), which was prevented by prior adol CVS (p<0.05 compared to SPS). Statistical analysis for membrane resistance was performed on log transformed data. **(F)** There were significant main effects of SPS ($F_{(1,92)}$=19, p<0.0001[c]), adol CVS ($F_{(1,92)}$=14.3, p=0.0003[c]) and a significant adol CVS x SPS interaction ($F_{(1,92)}$=5.0, p=0.02[c]) on action potential (AP) threshold. Bonferroni's test indicated that SPS significantly increased AP threshold compared to controls (p<0.05), with prior adol CVS preventing the effect (p<0.05 compared to SPS); **(G)** There were main effects of SPS ($F_{(1,97)}$=20, p<0.001[d]), adol CVS ($F_{(1,97)}$=25.9, p<0.0001[d]) and significant adol CVS x SPS interaction ($F_{(1,97)}$= 6.5, p=0.01[d]) on action potential amplitude. Bonferroni's test indicated that SPS significantly lowered AP amplitude compared to controls (p<0.05), and prior experience adol CVS prevented it (p<0.05 compared to SPS); **(H)** There was a significant effect of SPS ($F_{(1,97)}$=8.3, p=0.005[e]) and adol CVS ($F_{(1,97)}$= 42.2, p<0.0001[e]) on AP50. Planned comparisons indicated a decrease in AP50 following SPS compared to control (p<0.05) and prior adol CVS prevented that effect (p<0.05 compared to SPS). Increase in AP50 was also observed following adol CVS only (p<0.05 compared to control). Statistical analysis for AP50 were performed on log transformed data. **(I)** Resting membrane potential was unaltered among the groups. 2 way ANOVA revealed no significant main effect of SPS ($F_{(1,95)}$=1.3, p=0.3), adol CVS ($F_{(1,95)}$=0.02,

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p=0.9) or SPSx adol CVS interaction ($F_{(1,95)}$=0.1, p=0.7); **(J)** Membrane capacitance was unaltered among groups. 2 way ANOVA revealed no significant main effect of SPS ($F_{(1,97)}$=1.8, p=0.2), Adol CVS ($F_{(1,97)}$=3.6, p=0.06) or SPSx adol CVS interaction ($F_{(1,97)}$=2.9, p=0.09); **(K)** Analysis of peak firing frequency revealed a significant main effect of SPS ($F_{(1,36)}$=13.4, p=0.0008[h]). Planned comparisons indicated that SPS significantly reduced peak firing frequency compared to controls (p<0.05), whereas the prior adol CVS+SPS group did not differ from the control group; **(L)** representative traces of action potentials evoked by 20pA current injection for the respective groups; For D,H,K * and # represents planned comparison effects compared with control and SPS respectively. For E, F and G* and # represents post hoc Bonferroni effects compared with control and SPS respectively. Data represented as Mean±SEM.

Figure 1

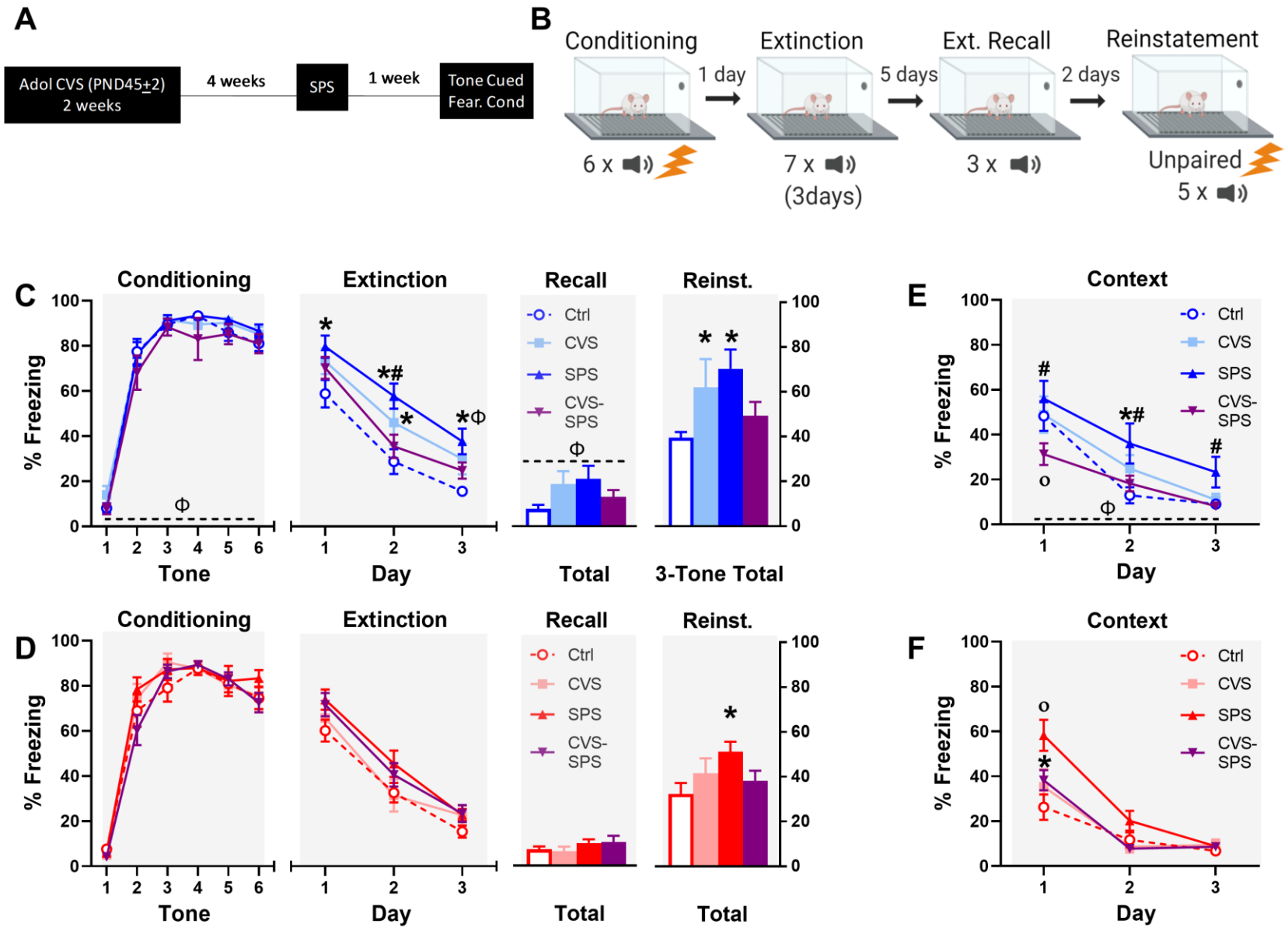

A
Adol CVS (PND45±2)
2 weeks
4 weeks
SPS
1 week
Tone Cued
Fear. Cond
B
Conditioning
1 day
Extinction
5 days
Ext. Recall
2 days
Reinstatement
6 x
7 x
(3days)
3 x
Unpaired
5 x
C
Conditioning
Extinction
Recall
Reinst.
% Freezing
Tone
Day
Total
3-Tone Total
Ctrl
CVS
SPS
CVS-SPS
D
E
Context
F

Figure 2

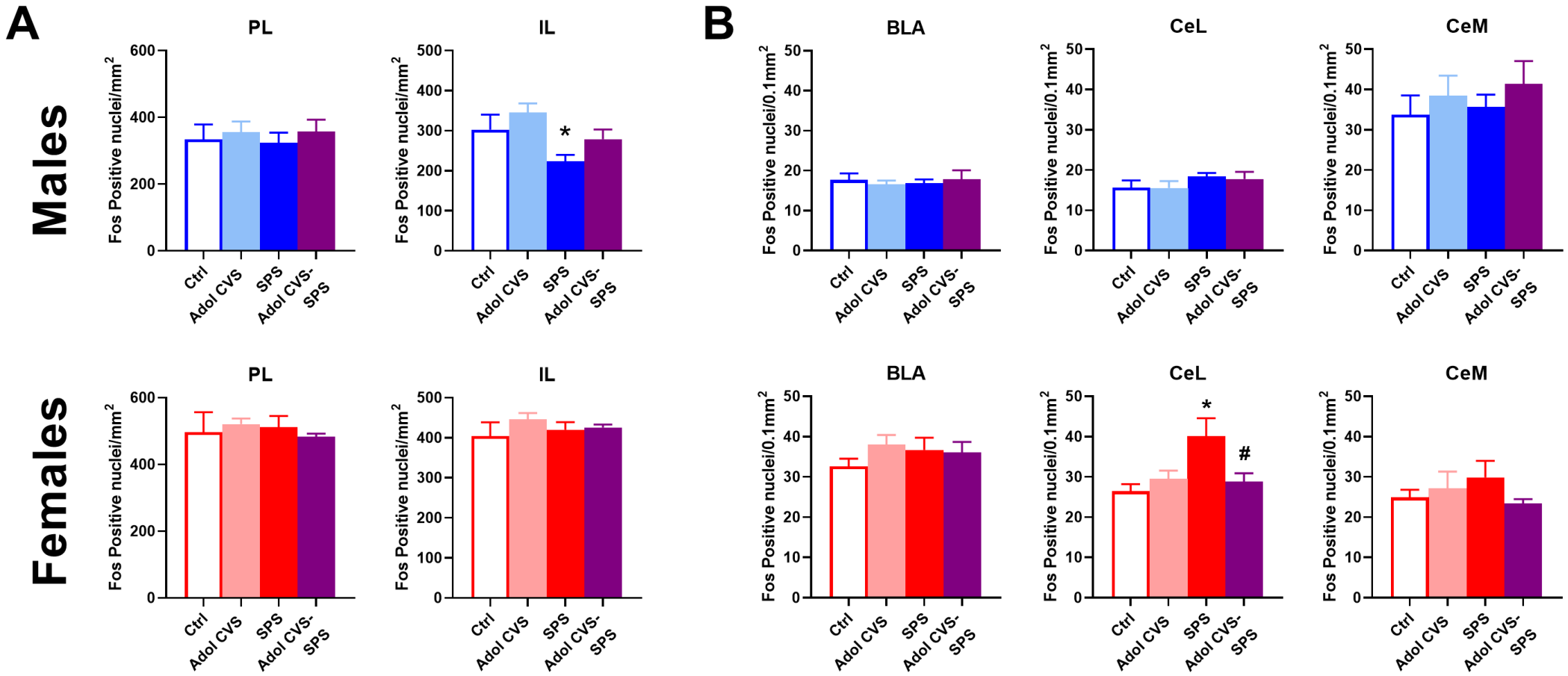

A
Males
Females
PL
IL
Fos Positive nuclei/mm2
Ctrl
Adol CVS
SPS
Adol CVS-SPS
*
B
BLA
CeL
CeM
Fos Positive nuclei/0.1mm2
#

Figure 3

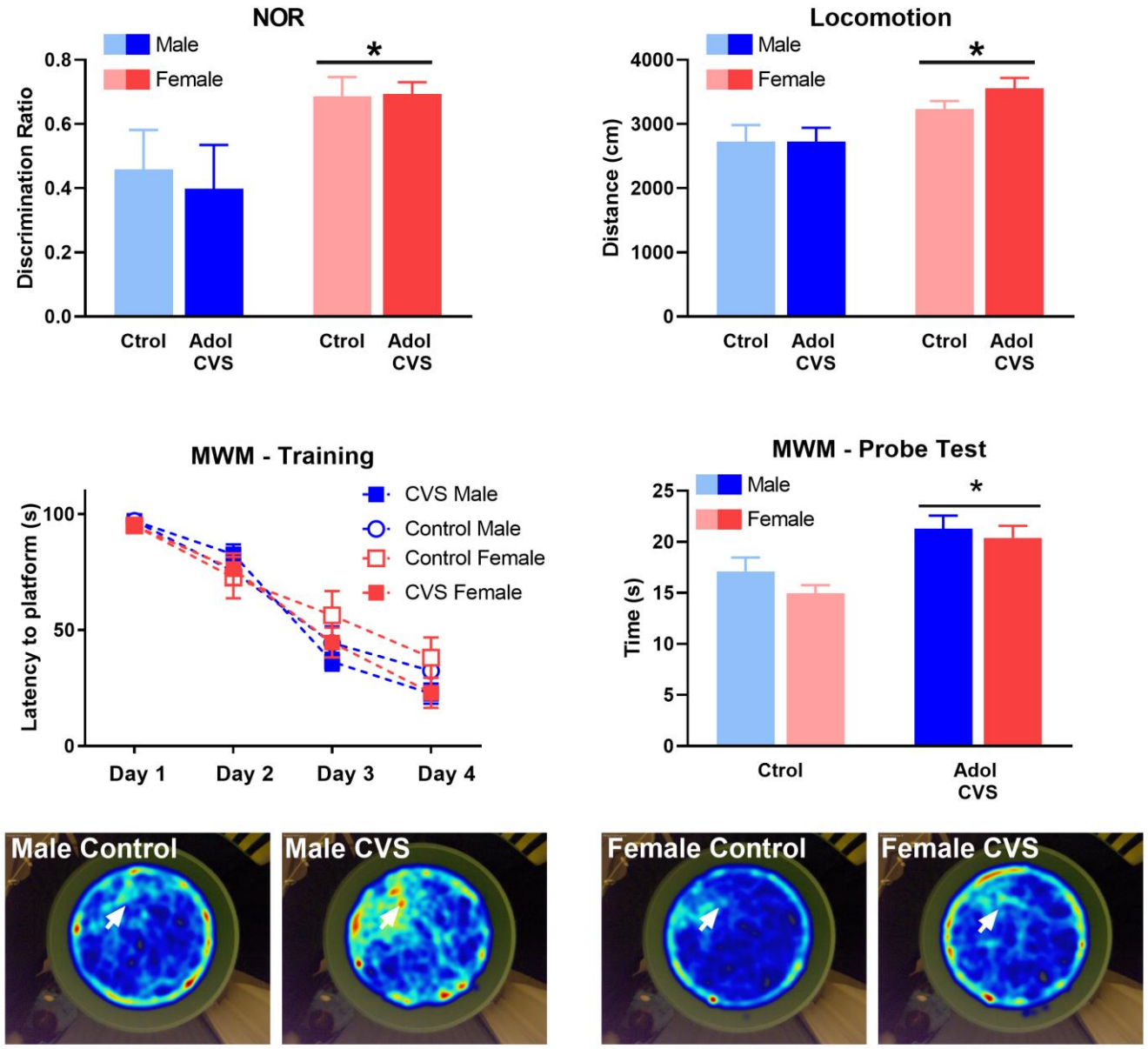

NOR
Male
Female
Discrimination Ratio
0.8
0.6
0.4
0.2
0.0
*
Ctrol
Adol CVS
Ctrol
Adol CVS
Locomotion
Male
Female
Distance (cm)
4000
3000
2000
1000
0
*
Ctrol
Adol CVS
Ctrol
Adol CVS
MWM - Training
CVS Male
Control Male
Control Female
CVS Female
Latency to platform (s)
100
50
0
Day 1
Day 2
Day 3
Day 4
MWM - Probe Test
Male
Female
Time (s)
25
20
15
10
5
0
*
Ctrol
Adol CVS
Male Control
Male CVS
Female Control
Female CVS

Figure 4

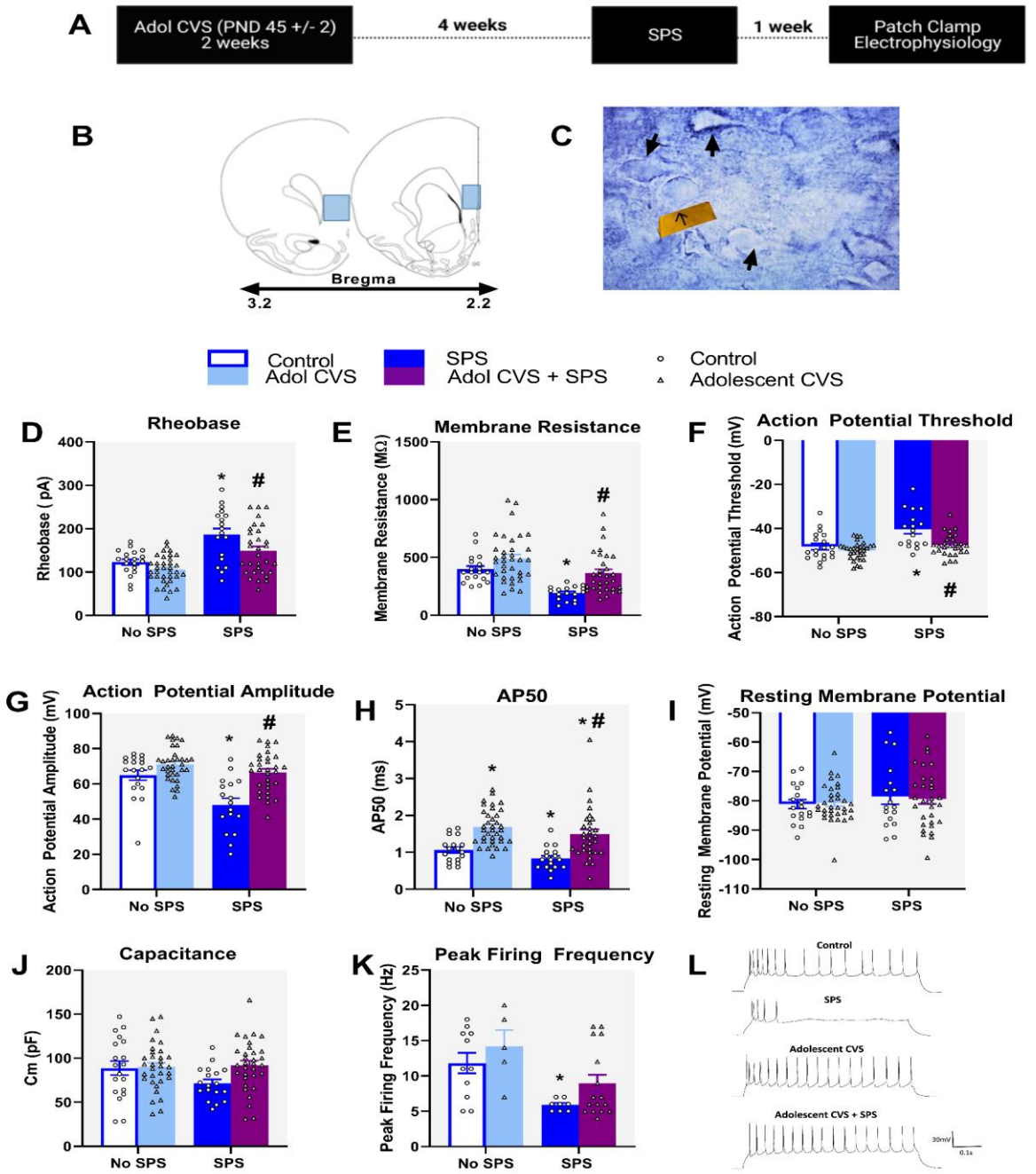
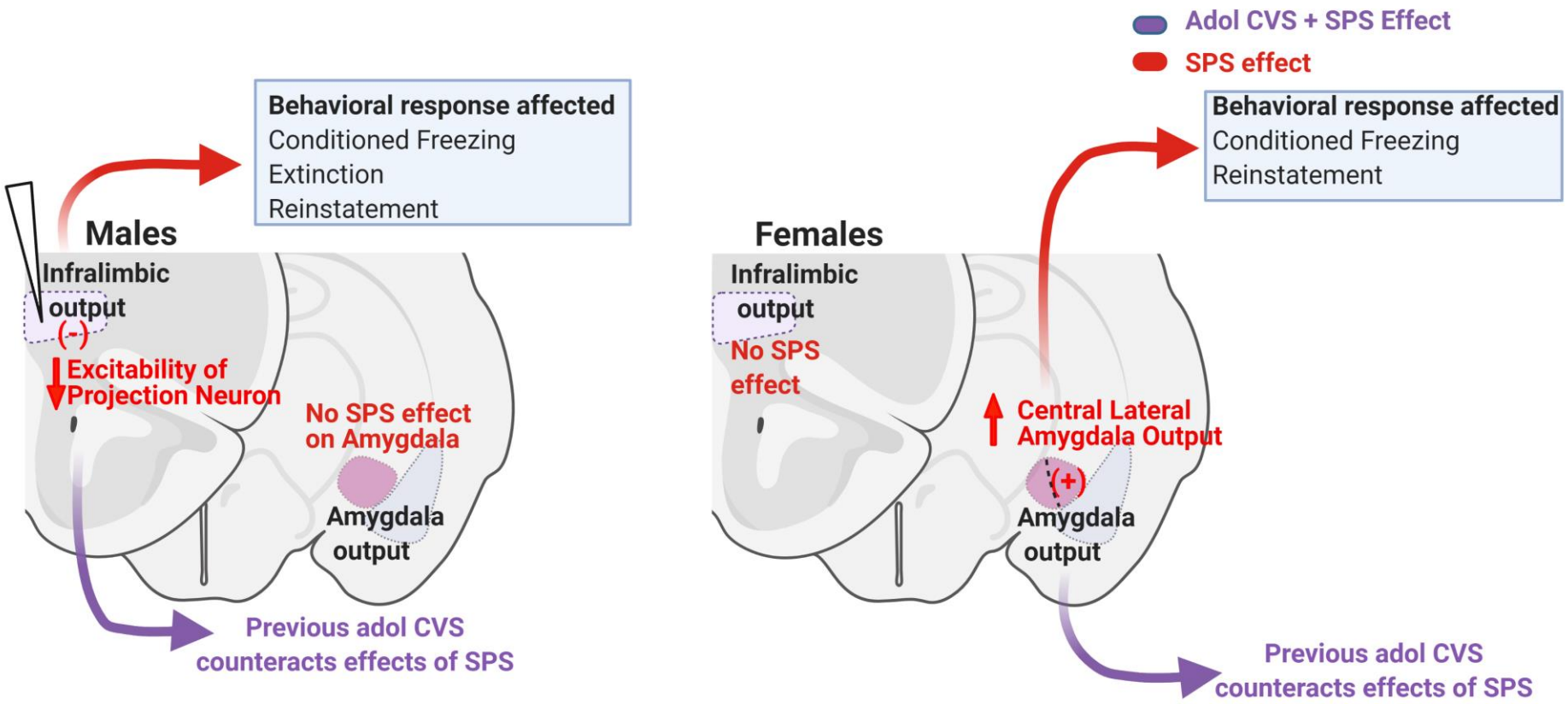

A
Adol CVS (PND 45 +/- 2) 2 weeks
4 weeks
SPS
1 week
Patch Clamp Electrophysiology
B
Bregma
3.2
2.2
C
Control
Adol CVS
SPS
Adol CVS + SPS
Control
Adolescent CVS
D
Rheobase
Rheobase (pA)
No SPS
SPS
E
Membrane Resistance
Membrane Resistance (MΩ)
F
Action Potential Threshold
Action Potential Threshold (mV)
G
Action Potential Amplitude
Action Potential Amplitude (mV)
H
AP50
AP50 (ms)
I
Resting Membrane Potential
Resting Membrane Potential (mV)
J
Capacitance
Cm (pF)
K
Peak Firing Frequency
Peak Firing Frequency (Hz)
L
Control
SPS
Adolescent CVS
Adolescent CVS + SPS

Figure 5


Adol CVS + SPS Effect
SPS effect
Behavioral response affected
Conditioned Freezing
Extinction
Reinstatement
Males
Infralimbic
output
(-)
Excitability of
Projection Neuron
No SPS effect
on Amygdala
Amygdala
output
Previous adol CVS
counteracts effects of SPS
Behavioral response affected
Conditioned Freezing
Reinstatement
Females
Infralimbic
output
No SPS
effect
Central Lateral
Amygdala Output
(+)
Amygdala
output
Previous adol CVS
counteracts effects of SPS