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Adolescent social defeat alters N-methyl-D-aspartic acid receptor expression and impairs fear learning in adulthood

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Abstract

Repeated social defeat of adolescent male rats results in adult mesocortical dopamine hypofunction, impaired working memory, and increased contextual anxiety-like behavior. Given the role of glutamate in dopamine regulation, cognition, and fear and anxiety, we investigated potential changes to N-methyl-D-aspartic acid (NMDA) receptors following adolescent social defeat. As both NMDA receptors and mesocortical dopamine are implicated in the expression and extinction of conditioned fear, a separate cohort of rats was challenged with a classical fear conditioning paradigm to investigate whether fear learning is altered by adolescent defeat. Quantitative autoradiography was used to measure 3H-MK-801 binding to NMDA receptors in regions of the medial prefrontal cortex, caudate putamen, nucleus accumbens, amygdala and hippocampus. Assessment of fear learning was achieved using an auditory fear conditioning paradigm, with freezing towards the auditory tone used as a measure of conditioned fear. Compared to controls, adolescent social defeat decreased adult NMDA receptor expression in the infralimbic region of the prefrontal cortex and central amygdala, while increasing expression in the CA3 region of the hippocampus. Previously defeated rats also displayed decreased conditioned freezing during the recall and first extinction periods, which may be related to the observed decreases and increases in NMDA receptors within the central amygdala and CA3, respectively. The alteration in NMDA receptors seen following adolescent social defeat suggests that dysfunction of glutamatergic systems, combined with mesocortical dopamine deficits, likely plays a role in the some of the long-term behavioral consequences of social stressors in adolescence seen in both preclinical and clinical studies.

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Keywords

NMDA; adolescence; stress; social defeat; conditioned fear

1. Introduction

Glutamate, as one of the primary excitatory amino acids, plays a ubiquitous role in brain function. Importantly, glutamate activity at N-methyl-D-aspartic acid (NMDA) receptors has been linked to changes in synaptic plasticity, learning and memory, and modulation of other neurotransmitter systems [1–3]. Given its influence on brain structure and function, alterations in NMDA receptor activity as a result of stressful experience have been linked to a variety of psychiatric disorders [3, 4].

Psychiatric disorders are particularly common in individuals who have experienced severe social stressors during adolescence [5], but the role of NMDA receptors in outcomes of adolescent social stress has yet to be fully delineated. It is known that NMDA receptor expression undergoes changes throughout the brain during adolescence [6], potentially enhancing the vulnerability of adolescents to stress-induced NMDA receptor dysfunction. In support of this, rats exposed to social instability stress during adolescence have been found to demonstrate deficits in spatial memory as well altered hippocampal synaptic plasticity when tested in adulthood [7], both processes linked to NMDA receptor function [8]. Strikingly, these changes in hippocampal plasticity and behavior were specific to stress being experienced in adolescence [7]. Furthermore, social isolation stress in adolescence causes synaptic loss in the adult prefrontal cortex, which can be reversed by treatment with the NMDA receptor antagonist MK-801 [9]. While changes to NMDA receptor expression, as measured by 3H-MK-801 receptor binding to NMDA receptors, have been found following physical stressors in both adults and adolescents [10, 11], the effects of social stress experienced in adolescence on 3H-MK-801 binding have yet to be evaluated.

Adolescent social defeat represents an ethologically relevant model of social stress that results in long term behavioral changes known to be mediated by NMDA receptors, such as decreased spatial working memory [12], increased contextual anxiety-like behavior [13], and enhanced drug seeking behavior [14]. In addition, male rats defeated in adolescence show deficits in adult mesocortical dopamine (DA) function [13–18], a system at least partially regulated by NMDA receptor activation [3, 19, 20]. Thus, dysfunction of NMDA receptors may represent a potential mechanism by which adolescent social defeat leads to long term changes in behavior and neurochemistry. Furthermore, recent research suggests that symptoms associated with NMDA dysfunction may benefit from the use of both experimental and clinically-available drugs that target the glutamatergic system [21–23]. Therefore, in order to further investigate the mechanisms involved in adolescent social stress as well as inform future research on treatment strategies, the present study looked at adult changes in NMDA receptor expression following adolescent social defeat in discrete cortical, striatal and limbic brain regions, as measured by quantitative autoradiography with 3H-MK-801.

In addition to measurement of NMDA receptors, we also investigated whether adolescent defeat alters conditioned fear learning and extinction in adulthood, processes that are heavily reliant on NMDA receptor activation in several brain regions [24, 25]. Exposure to severe social stressors in adolescence are known to be associated with an increased incidence of anxiety- and fear-related disorders later in life, such as generalized anxiety, agoraphobia and social phobia [26, 27]. One mechanism driving increased fear and anxiety following social stressors may be deficits in fear extinction [28, 29], which allows the individual to relearn that cues previously associated with danger are no longer threatening and hence promotes contextually-appropriate behavioral expression. Studies have demonstrated that DA activity in the medial prefrontal cortex (mPFC) is necessary for the extinction of conditioned fear, with DA lesions resulting in prolonged retention of fearful responses [30, 31]. Given that adolescent social defeat results in DA hypofunction within the mPFC [17], we hypothesized that previously defeated rats would demonstrate impaired fear extinction following auditory fear conditioning, providing a potential model to research the fear and anxiety symptoms observed following adolescent exposure to social stressors in humans.

2. Materials and Methods

2.1. Animals

Male juvenile post-weanling Sprague-Dawley rats (postnatal day [P]21), obtained from the University of South Dakota Animal Resource Center, were pair-housed such that cage-mates were in the same treatment group (social defeat or control) and kept at 22°C on a reverse 12-hr light-dark cycle (lights off 10.00). Food and water were available *ad libitum*. Behavioral experiments were conducted between 11:00 and 15:00 under red lighting. All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and received approval from the Institutional Animal Care and Use Committee of the University of South Dakota. Every effort was made to minimize the number of animals used and their suffering.

2.2. Adolescent Social Defeat

The adolescent social defeat procedure was conducted using a modified resident-intruder paradigm as described previously [13, 17, 18]. Briefly, at P35 (mid-adolescence [32]), male rats were placed in the cage of a larger, aggressive adult (>P90) Sprague Dawley resident male rat, which had been housed in isolation for 3 weeks and pre-selected for aggressive behavior against practice male adolescent intruders (practice intruders were not included in any subsequent experiments). Social defeat was defined *a priori* as the adolescent intruder adopting a minimum of 3 submissive supine postures in response to consecutive resident attacks, which typically occurs within 5 min. The adolescent and resident were allowed 10 minutes of social interaction, during which attacks by the resident male usually resulted in approximately 7–10 adolescent submissions. The adolescent was also inevitably subject to additional attacks that did not elicit a submissive posture. Immediately after the 10 minute period, the rats were separated by a wire mesh barrier for an additional 25 minutes to prevent further physical attack while still allowing exposure to visual, auditory, and olfactory intimidation from the resident. The adolescent rat was then returned with its cage mate to the home cage. Adolescent subjects ($N = 10$) were exposed to social defeat once a

day for 5 days (P35 – 39), and were confronted with a different resident male each day to control for variance in individual defeat intensity. Aged-matched controls ($N = 10$) did not undergo social defeat but were instead placed into a novel empty cage for the duration of the defeat procedure to control for handling and novel environment stress. After the final defeat trial, all rats were left in their original pairs in their home cages and allowed to mature undisturbed until early adulthood (P56).

2.3. [³H]MK-801 Binding

At P56, animals were sacrificed by decapitation and the brains were removed immediately and stored at -80°C till use. Brain tissue sections ($16\text{ }\mu\text{m}$) were cut at -18°C using a cryostat microtome according to the Brain Atlas of Paxinos and Watson [33] and mounted on gelatin-coated microscope slides (two sections per slide). For each animal, quadruplicate sections from plates 8, 12, 30 and 42 from [33] were used in this study, encompassing the mPFC (cingulate, prelimbic and infralimbic cortices), the striatum (caudate putamen, CPu), the nucleus accumbens (NAc) core and shell, the basolateral amygdala (BLA) and the central nucleus of the amygdala (CeA), and the cornu ammonis areas (CA1, CA2, CA3) of the hippocampus. Brain section availability allowed for analysis of $N = 9$ and $N = 8$ in the social defeat and control groups, respectively, for regions within the mPFC, striatum, and NAc. For regions within the amygdala and hippocampus, $N = 6$ were available for analysis for both control and defeat groups. NMDA receptors were labeled with [³H] MK-801 (35 Ci/mmol, Perkin Elmer) as described previously [34] and based on the method of Sakurai et al. [35] with minor modifications. Specifically, brain sections were preincubated for 30 min at 4°C in 50 mM Tris-HCl buffer (pH 7.4). The sections were then incubated in 50 mM Tris-HCl buffer containing 15 nM [³H] MK-801 and 30 μM glutamate/10 μM glycine for 120 min at room temperature, rinsed for 30 min with cold 50 mM Tris-HCl buffer, dipped once in ice-cold distilled water, and immediately dried in a stream of cool air. Non-specific binding was determined with the addition of 50 μM non-radioactive MK-801. The slides were then dried at room temperature, transferred into cassettes, and exposed to Kodak BioMax MS film along with [³H] standards. Following a 4-week exposure period at 4°C , the films were developed in Kodak GBX developer at room temperature.

2.4 Fear Learning Paradigm

Upon reaching early adulthood (P56), a separate group of previously defeated and control rats ($N = 12/\text{group}$) underwent auditory fear conditioning and extinction testing. As adolescent defeat is known to reduce DA activity in the adult mPFC [17], we used a fear learning paradigm modified from Morrow et al. [30], which demonstrated impairments in conditioned fear expression following mPFC DA depletion. This involved a two-day habituation period where the subject rat was placed into a foot-shock chamber each day for a total of 30 min. The chamber contained both an overhead camera for behavioral observation and a speaker for delivery of an auditory stimulus, and was fitted with a metal grid floor (Noldus Information Technology, Leesburg, VA). The entire foot-shock chamber was housed within a light and sound-attenuating enclosure (Med Associates, Inc., St Albans, VT). On day three, the subject rat underwent fear conditioning, where it learned to associate a benign conditioned stimulus (auditory tone) with an aversive unconditioned stimulus (foot-shock). During conditioning, the rat was first placed in the chamber for 10 min with no

presentation of either tone or foot-shock (pre-tone period). In the second 10 min period (11–20 min; tone period), the subject experienced the tone-shock pairing treatment, with an auditory tone (56 dB, 5 s duration) being presented once every minute (10 tone presentations total). Each tone was paired with a mild foot-shock (0.7 mA, 0.5 s duration), with the foot-shock overlapping the last 0.5 s of the 5 s tone. Tone-shock pairings were controlled by Ethovision 3.1 (Noldus Information Technology, [36]). The rat then remained in the chamber for 10 more minutes following the tone-shock pairing treatment (post-tone period). Exposing the subject to the tone-shock pairing only in the second 10 min period (tone) ensured that fear learning would be associated solely with the auditory tone, and not to the context in general. On day four, the rat was once again placed into the chamber for 30 min and tested for recall of conditioned fear behavior by being exposed to 10 tone presentations (56 dB, 5 s duration) given every minute over the middle 10 min block in the absence of foot-shock. Days five through eight consisted of fear extinction tests, in which the rat was again placed into the chamber for 30 min with the tone presented 10 times (once every minute over the middle 10 min) sans the foot-shock. Duration of freezing behavior was measured over each 10-minute period (pre-tone, tone, post-tone) within each test session for days 3–8 (Conditioning, Recall, Extinction sessions 1 through 4) and was scored from video by an observer blind to treatment using Observer XT 7 (Noldus Information Technology).

2.5. Quantification and Statistics

2.5.1. Analysis of [3H]MK-801 Binding—Autoradiographic films were analyzed using the computer software program, ImageJ [37]. The average grayscale intensity within a region of interest was measured, providing a measurement of optical density that was independent of a region's size. The data were then applied to a calibration curve of optical densities generated from a series of tritiated standards of known concentrations (3H fmol/mg protein) that were exposed along with the brain sections [15, 34]. Nonspecific binding was subtracted from the total binding to provide the specific binding in the regions of interest. Data were thus expressed as mean $\pm$ S.E.M. specific binding (fmol/mg brain protein). Levels of NMDA receptor binding in each region of interest were compared between previously defeated and control rats using separate Student's t-tests. Receptor binding was not compared across different regions. Statistical analysis was performed using SigmaPlot 11.0 for Windows, with the level of significance set *a priori* at $p < 0.05$.

2.5.2 Analysis of Conditioned Fear—Data from two control rats for Extinction session 3 were not available owing to failure of the video recording software partway through their testing. Thus, subject numbers for Extinction 3 were Controls N = 10, defeat N = 12. For all other sessions, N = 12/group. Grubbs' tests were applied separately to each behavioral data set to identify statistical outliers, which were then excluded from subsequent analyses. This resulted in the removal of 35 data points from a total of 1,846 observations.

To examine for possible differences between control and defeated rats in unconditioned fear responses to delivery of the foot-shock on the Conditioning day (day 3), the duration of freezing within each 10 min time block (pre-tone, tone, post-tone) of the Conditioning session was compared using two way ANOVA with a repeated measure of time block, with subsequent one way repeated measures ANOVA with Student-Newman-Keuls (SNK)

multiple comparisons of means tests performed for each treatment group when there was a main effect of time block. A main effect of treatment or an interaction was followed by one way ANOVA with SNK tests. To confirm that subsequent conditioned freezing responses were specific to tone presentation, repeated one way ANOVA plus SNK tests within each stress treatment group were used to compare freezing duration across the 10 min time blocks (pre-tone, tone, post-tone) separately within each succeeding testing session (Recall, Extinction 1 through 4).

Conditioned freezing behavior was then compared across the five daily testing sessions in which only the conditioned tone was presented (Recall, Extinctions 1–4). Duration of freezing during the middle 10 min time block when the tone was presented was calculated for each session, then compared between control and defeated rats using a two way ANOVA with a repeated measure of session. Significant main effects of treatment within each session, or interactions between treatment and session, were followed by separate one way ANOVA with SNK tests, while one way repeated measures ANOVA with SNK tests were used to compare freezing within each treatment group across sessions.

During exposure to a conditioned fearful stimulus (such as a tone) freezing responses typically decline with successive tone presentation within a session, indicating acquisition of fear extinction learning [38]. Therefore, when differences in the total amount of freezing between control and defeated rats were found within a session, the pattern of conditioned freezing expression across tone presentation was compared using separate two way ANOVA with the repeated measure of tone, followed by SNK tests and one way within-treatment ANOVA where appropriate. This allowed examination of whether within-session extinction differed between stress treatment groups. All analyses were performed using SigmaPlot 11, with the alpha level set to 0.05 throughout.

3. Results

3.1 NMDA receptor expression as measured by 3H-MK801

3.1.1 Medial prefrontal cortex (mPFC)—Adolescent social defeat resulted in decreased NMDA receptor binding within the adult infralimbic cortex ($t(15) = 2.696$, $p = 0.017$; Fig. 1A). No significant differences between defeated rats and controls were found within the prelimbic ($t(15) = 1.689$, $p = 0.112$; Fig. 1A) or cingulate cortices ($t(15) = 1.907$, $p = 0.076$; Fig. 1A).

3.1.2 Striatal and accumbal regions—Adolescent social defeat did not result in any significant differences in adult NMDA receptor binding in the CPu ($t(15) = -0.448$, $p = 0.661$), NAc shell ($t(15) = -0.220$, $p = 0.829$), or NAc core ($t(15) = -0.0403$, $p = 0.968$) (Fig. 1B).

3.1.3 Hippocampal CA regions—No significant differences in NMDA receptor binding were found in either the CA1 ($t(10) = -1.072$, $p = 0.309$) or CA2 ($t(10) = -1.788$, $p = 0.104$) region of the hippocampus between rats defeated in adolescence and their controls (Fig. 1C). However, adolescent social defeat resulted in a significant increase in adult NMDA receptor binding in the CA3 region ($t(10) = -4.013$, $p = 0.002$; Fig. 1C).

3.1.4 Amygdala—Rats defeated in adolescence had significantly decreased NMDA receptor binding in the CeA in early adulthood ($t(10) = 2.674, p = 0.023$; Fig. 1D). No significant differences were found within the BLA ($t(10) = 0.811, p = 0.436$; Fig. 1D).

3.2. Fear Learning

3.2.1 Freezing Behavior to Unconditioned and Conditioned Stimuli—The duration of freezing for control and defeated rats in each 10 min time block on each testing day (Conditioning, Recall, Extinctions 1 through 4) is presented in Table 1. There was no difference in unconditioned fear responses between treatment groups during the Conditioning day. Two way repeated measures ANOVA revealed no main effect of treatment ($F(1, 43) = 0.005, p = 0.95$) or an interaction between treatment and time block ($F(2, 43) = 0.008, p = 0.99$). However, there was a main effect of time ($F(2, 43) = 128.56, p < 0.001$), such that control and defeated rats exhibited equivalent increases in freezing during the middle time block when foot shock was delivered, which was maintained in the third 10 min time block following foot shock (Table 1). For both control and defeated rats, freezing responses within each of the post-Conditioning sessions (Recall, Extinctions 1 – 4) were specific to tone presentation, being highest in the middle 10 min time block when the tone was played (Table 1).

3.2.2 Freezing Behavior across Conditioned Stimulus Sessions—Comparison of the amount of freezing during tone presentation across all sessions where the conditioned tone was presented (Recall, Extinction 1 through 4) showed significant effects of treatment ($F(1, 83) = 7.78, p = 0.011$), session ($F(4, 83) = 108.76; p < 0.001$), and an interaction between the two factors ($F(4, 83) = 2.53, p = 0.046$). One way repeated measures ANOVA within each treatment group revealed that both control and previously defeated rats displayed the greatest freezing during Recall (Control: $F(4, 39) = 58.92, p < 0.001$; Defeat: $F(4, 44) = 49.53, p < 0.001$), with responses for both groups decreasing in each subsequent session (Fig. 2, Holm-Sidak $p < 0.05$). However, defeated rats showed significantly less freezing than controls during both Recall (Fig. 2, SNK $p = 0.002$) and Extinction 1 (Fig. 2, SNK $p = 0.002$). Freezing was equivalent between control and defeated rats in all subsequent Extinction sessions (Fig. 2, SNK $p > 0.09$).

3.2.3 Freezing Behavior to Repeated Conditioned Stimulus Presentation—Control and defeated rats also showed differences in the expression of freezing to successive tone presentation within the Recall and Extinction 1 sessions. Two way repeated measures ANOVA showed that for the Recall session, there was a main effect of treatment ($F(1, 195) = 7.61, p = 0.011$) and of tone ($F(9, 195) = 10.23, p < 0.001$), but no interaction ($F(9, 195) = 0.69, p = 0.71$). Within control rats, one way repeated measures ANOVA ($F(9, 97) = 5.63, p < 0.001$) revealed that freezing to tones 2 through 7 was significantly higher than to tone 1 (Fig. 3A, Holm-Sidak $p < 0.05$). In defeated rats (one way repeated ANOVA $F(9, 98) = 5.29, p < 0.001$), increases in freezing relative to tone 1 were restricted to tones 2 through 5 (Fig. 3A, Holm-Sidak $p < 0.05$). A similar pattern was observed for freezing to each tone in the Extinction 1 session, with two way repeated ANOVA showing main effects of treatment ($F(1, 196) = 4.97, p = 0.036$) and of tone ($F(9, 196) = 8.57, p < 0.001$), but no interaction ($F(9, 196) = 1.49, p = 0.15$). Control rats (one way repeated ANOVA $F(9, 99) = 6.99, p <$

0.001) displayed increased freezing relative to tone 1 during presentation of tones 2 through 4 (Fig. 3B, Holm-Sidak $p < 0.05$). In contrast, despite a significant main effect being revealed by a one way repeated measures ANOVA ($F(9, 97) = 2.63, p = 0.009$), defeated rats showed no significant change in freezing across tone presentation (Fig. 3B, Holm-Sidak $p > 0.1$). The lack of statistical interaction in the initial two-way ANOVA for either the Recall or the Extinction 1 sessions precluded any direct comparison of freezing to each tone between stress groups. However, while both groups increased freezing with successive tone presentation, control rats appeared to return to baseline levels more slowly than defeated subjects. This pattern was particularly apparent during Extinction session 1.

4. Discussion

In the present study, adult rats exposed to adolescent social defeat demonstrated significant differences in adult NMDA receptor expression compared to controls. Specifically, adolescent social defeat resulted in decreased levels of NMDA receptors in both the central nucleus of the amygdala (CeA) and the infralimbic region of the mPFC, while increased NMDA receptor expression was noted in the CA3 region of the hippocampus. Several other studies have looked at NMDA receptor expression in response to stress, albeit utilizing different quantification methods, developmental time points, and stress procedures [10, 11, 39–45]. While the majority of these studies looked at expression of specific NMDA receptor subunits, one study measuring 3H-MK-801 binding immediately following repeated immobilization stress in adolescent mice found an increase in NMDA expression in the lateral septum and dentate gyrus, but no other regions [10]. Although unique methodologies might partially account for the differences in binding pattern from the present study, the long term effects of a stressor such as social defeat are likely different from changes measured immediately following a physical stressor like immobilization.

Adolescent defeat also had a marked effect on fear learning in adulthood, with previously defeated rats displaying reduced conditioned freezing responses towards an auditory tone previously paired with foot shock. While we did not directly assess nociception, it is unlikely that adolescent defeat would increase pain tolerance in adulthood to explain the decrease in conditioned freezing. In support of this, defeated rats showed equivalent levels of unconditioned freezing to controls during both foot shock + tone presentation and the subsequent 10 min period. Although recent studies have shown that experience of adolescent defeat increases resilience to stress in adulthood, this was specific to subsequent social defeat, with responses to heterotypic stressors not assessed [46]. In addition, early life social isolation encompassing adolescence (P21 to 42) alters conditioned fear learning in a paradigm equivalent to that used here, without any corresponding change to nociception [36]. Adolescent defeat also causes impairments in adult spatial learning tasks that do not involve painful stimuli [12]. Collectively, this implies that the reduced conditioned fear response shown here by previously defeated rats is a result of impaired fear learning rather than blunted pain perception.

Reduced NMDA receptor expression in the infralimbic cortex adds to previous findings that the mPFC represents one of the major brain regions sensitive to the effects of adolescent social defeat [9, 13, 47]. Presuming that the reduced expression reflects decreased

glutamatergic tone at NMDA receptors, it may represent an additional mechanism underlying changes to cognition and psychostimulant responses induced by adolescent defeat, which were previously attributed to decreased mPFC DA activity [17]. Specifically, rats defeated in adolescence show deficits in adult working memory tasks [12] along with enhanced conditioned place preference and locomotion responses to amphetamine [14, 16], similar to effects of pharmacologically reducing mPFC DA function [48–51]. However, these behaviors are also modulated by NMDA receptors in the mPFC. For instance, NMDA receptor activation in the primate dorsolateral PFC (equivalent of rat mPFC [52]) appears to be necessary for maintaining cognitive representations of information across a temporal delay [53], and NMDA antagonists have been found to disrupt working memory performance when administered either systemically or into the mPFC [54–56]. With regards to drugs of abuse, mutant mice deficient in NMDA receptor function show blunted amphetamine-evoked neuronal activation in the infralimbic cortex in comparison to wild-type controls [57], and blockade of NR2B-containing NMDA receptors in infralimbic pyramidal neurons prevents extinction of cocaine conditioned place preference [58]. Cue-induced reinstatement of alcohol seeking is also increased following ablation of infralimbic neurons that are specifically responsive to drug-associated cues [59]. Combined, this suggests that increased amphetamine conditioned place preference following adolescent defeat may be potentiated and maintained by reduced NMDA receptor-mediated excitation of the infralimbic cortex. Thus, reductions in NMDA-mediated glutamatergic activity in the adult infralimbic cortex may combine with decreased DA activity in this region to promote the impaired working memory and enhanced behavioral responses to amphetamine caused by adolescent social defeat.

Increased c-Fos expression in the infralimbic cortex is associated with expression of freezing responses to foot shock-associated stimuli [60, 61], implying that a reduction in neural activity in this region would be associated with decreased conditioned freezing. Thus, it would seem that decreased NMDA receptor-mediated excitation of the infralimbic cortex following adolescent defeat could account for the reduced conditioned fear seen in adulthood. However, pharmacological inactivation of the infralimbic cortex has no effect on conditioned fear expression [62], and single-unit recordings from infralimbic neurons reveal no relationship between neuronal firing and freezing during presentation of a conditioned tone [63]. Instead, firing only increases after exposure to a session of extinction training, and lower firing is correlated with poorer extinction, i.e., higher freezing [63]. Moreover, microstimulation of infralimbic neurons potentiates the extinction-induced reduction in freezing [63]. Conversely, stimulation of the prelimbic cortex causes increases in conditioned fear and prevents extinction [64], and deficits in extinction recall are seen following antagonism of infralimbic NMDA receptors [65]. Combined, this makes it unlikely that decreased NMDA receptor expression in the infralimbic cortex following adolescent defeat would have contributed both to the decrease in conditioned fear and to the seemingly greater extinction seen in adulthood.

The decreased conditioned fear following adolescent defeat may instead be related to the reduced adult expression of NMDA receptors within the amygdala. This was specific to the CeA, with no difference in expression seen in the basolateral amygdala (BLA). The amygdala has been widely studied for its role in fear and anxiety, with the acquisition and

expression of conditioned fear responses being attributed to the BLA and CeA, respectively [24, 66]. However, blockade of NMDA receptors in either the BLA or CeA prior to training prevents auditory conditioned fear acquisition [67, 68]. While demonstrating direct link between changes in amygdalar NMDA expression and fear conditioning behavior following adolescent social defeat would require additional experimental manipulation, the co-occurrence of changes in the present study along with the known role of amygdalar NMDA activity does suggest potential relationship for further investigation. For example, if the decreased NMDA receptor expression in the CeA found in the current study indeed translates to reduced NMDA receptor function, it may have contributed to the corresponding decrease in conditioned fear by hindering acquisition of associative learning. The fact that previously defeated rats did demonstrate some level of fear conditioning may reflect retention of acquisition capability through preservation of NMDA receptor expression/function in the BLA. Alternatively, the lowered freezing response shown by defeated rats may have nothing to do with impairments in conditioned fear acquisition, and could instead simply reflect dampened NMDA receptor-mediated activation of the CeA to result in reduced fear expression [69, 70] upon subsequent tone exposure.

Given adolescent defeat causes reductions in adult mPFC DA activity [17], we had initially hypothesized that adolescent defeat would impair fear extinction, similar to effects of either mPFC DA depletion or DA receptor blockade [30, 31, 71, 72]. Instead, previously defeated rats showed reduced freezing compared to controls during the first extinction trial (2 days after conditioning). However, conditioned freezing by defeated rats was also lower during the initial recall session, the day immediately after conditioning, suggesting that the seemingly potentiated extinction response was actually a function of deficits in conditioned fear expression. Fearful responses to conditioned stimuli are reduced by decreasing mPFC DA activity [73, 74] or by local blockade of DA D4 receptors [75]. Thus, decreased adult mPFC DA activity following adolescent defeat may have dampened conditioned fear expression, which then obscured any effect on subsequent fear extinction. In addition, various studies have reported similar results when testing fear learning in adulthood following experience of stress in adolescence, with adolescent rats exposed to either social instability stress [76] or traumatic underwater stress [77] demonstrating decreased fear expression following conditioning. Such decreased conditioned fear expression following early life stress has been attributed to deficits in learning and memory [78], possibly related to insults on the developing hippocampus [76]. Our results add to this by suggesting that decreased conditioned fear expression following adolescent social defeat may partly be due to a combination of reduced adult mPFC DA activity and decreased NMDA receptor expression in the CeA, with the latter potentially resulting in decreased output to brain regions controlling fear-related behavior [69, 70].

Adolescent social defeat was also found to result in increased levels of NMDA receptor expression within the CA3 region of the adult hippocampus, similar to the effects of acute social defeat in adult rats [40]. The adult CA3 region is known to be particularly sensitive to the effects of stressors, including social stress, demonstrating neuronal atrophy and dendritic retraction up to 3 weeks following termination of the stressor [1, 8, 79]. Chronic restraint stress also reduces dendrite length and branching in the adolescent CA3 [80], and chronic variable physical stress throughout adolescence arrests CA3 growth well into adulthood

[81]. While it is unknown whether the adolescent defeat procedure in the current study leads to any type of hippocampal atrophy, the previously reported long-term (up to 3 weeks) morphological changes are in line with the time course of NMDA receptor changes seen here. Excitotoxicity as mediated by NMDA receptors contributes to dendritic atrophy following stress [1, 8, 9]. This excitotoxic effect may be enhanced by serotonin, which can increase glutamate binding to NMDA receptors [39]. Interestingly, adolescent social defeat was found to result in increased levels of serotonin in the ventral hippocampus in adulthood [13], but further study would be necessary to determine the influence this might have on NMDA receptor binding as well as potential morphological changes.

Alternatively, the increased NMDA receptor expression in the CA3 of previously defeated rats may represent enhanced glutamatergic signaling to have functional effects on behavior, without causing excitotoxicity. In the current study, defeated rats appeared to return to baseline levels of freezing more quickly than controls upon successive presentation of conditioned tones during the Recall and first Extinction sessions, suggesting faster acquisition of within-session extinction learning [38]. The hippocampus, particularly the ventral hippocampus, is known to play a role in the acquisition of fear conditioning as well as memory for extinction [62, 82]. While the present analysis did not dissociate ventral from dorsal hippocampus, the decreased conditioned fear expression shown by defeated rats possibly relates to the observed increase in CA3 NMDA receptors. The ability to distinguish fearful contexts is dependent on NMDA receptors in the CA3 [83], and reduced CA3 firing upon transfer between different mildly anxiogenic environments is associated with impairments in rapid (within 3 min) discrimination of contexts associated with fearful experience [84]. In addition, the ventral CA3 is also required for retrieval of auditory-cued fear memory [62, 85], and the acquisition and recall of fear extinction to a conditioned auditory tone is enhanced by infusions of D-cycloserine, a partial NMDA receptor agonist, into the CA regions of the hippocampus [86]. Rats defeated in adolescence also display heightened fearful reactions as adults when re-exposed to the defeat context in the absence of an aggressive resident [13]. Thus, increased NMDA receptor function in the CA3 following adolescent defeat may promote either short or long term formation and recall of fearful memories depending on the nature of the fearful stimulus and at what developmental stage it was experienced. Specifically, previously defeated rats appear to acquire extinction learning rapidly (within minutes) to cues associated with aversive non-social stimuli in adulthood, but retain long term memories for fearful contexts associated with the adolescent defeat experience. However, direct investigations of effects on intra-CA3 NMDA receptor antagonism prior to re-exposure either to conditioned auditory stimuli predicting footshock, or to the adolescent defeat context, would be required to support this hypothesis definitively.

In conclusion, adolescent social defeat results in regionally-specific differences in adult NMDA receptor expression that may underlie subsequent behavioral alterations. For instance, decreased NMDA receptor expression in the infralimbic cortex of the mPFC may contribute to previously noted deficits in adult working memory and increased responses to psychostimulants, while reduced expression in the CeA and increased expression in the CA3 may be related to the decreases in the expression of conditioned fear observed here. This reduction in conditioned fear is seemingly at odds with the higher adult incidence of anxiety disorders shown by victims of adolescent social stress [26, 27]. However, victims also

exhibit a range of other mental health disorders in later life, including attention-deficit hyperactivity, bipolar disorder, depression, schizotypy, substance abuse, and suicidality [27, 87–90]. Such disorders, along with anxiety, are promoted in part by impairments in prefrontal cortex-mediated executive function [91–95], which is in turn regulated by glutamatergic and monoaminergic signaling [96, 97]. Our adolescent defeat model may therefore represent the consequences of broad-based impairments in executive control, rather than disruption limited to specific domains of emotive behavior such as fear learning. Interestingly, while chronic treatment with NMDA antagonists such as PCP and MK-801 decrease mesocortical DA and impair executive function [20, 98], treatment with the clinically available NMDA antagonist memantine appears to have the opposite effect [99]. Extinction of cocaine seeking is also enhanced by strengthening NMDA receptor-mediated currents in cortical pyramidal neurons [58]. The pharmacological modulation of NMDA receptor activity to treat negative outcomes of adolescent social stress thus presents a potentially beneficial area of future research.

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Abbreviations

NMDA	N-methyl-D-aspartic acid
mPFC	medial prefrontal cortex
CPu	Caudate Putamen
NAc	Nucleus Accumbens
CeA	Central Amygdala
BLA	Basolateral Amygdala
CA	Cornu ammonis

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Highlights

- Adolescent defeat alters NMDA receptor expression in discrete brain areas
- Adult fear conditioning and extinction are decreased following adolescent defeat
- Altered glutamate signaling may underlie behavioral outcomes of adolescent stress

Figure 1.

Specific binding of ^3H -MK801 to NMDA receptors in brain regions of adult rats that underwent adolescent social defeat versus controls. (A) medial prefrontal cortex (mPFC), (B) caudate putamen (CPu) and nucleus accumbens (NAc), (C) hippocampal CA, and (D) basolateral amygdala (BLA) and central nucleus of the amygdala (CeA). Data are expressed as the mean $\pm$ S.E.M. determinations made in quadruplicate sections from each brain. $N = 6-9/\text{group}$. *Significant difference between treatment groups ($p < 0.05$).

Fig. 2.

All rats displayed greatest freezing responses to the conditioned tone during the Recall session (24 hr after foot shock conditioning), with conditioned freezing declining thereafter. However, previously defeated rats showed reduced conditioned freezing compared to controls on both the Recall and Extinction 1 sessions. Values represent mean + SE.

#Different from Recall session, *Difference between control and defeat ($N = 10-12/\text{group}$, $p < 0.05$).

Fig. 3.

Freezing responses to successive presentations of the conditioned tone during the Recall (A) and Extinction 1 (B) sessions. Values represent mean + SE. *Different from response to tone 1 ($N = 12/\text{group}$, $p < 0.05$)

Table 1

Freezing duration (s) across each 10 min time block within each testing session. Values represent mean $\pm$ S.E.M.

Session	0 – 10 min	11 – 20 min (tone)	21 – 30 min	ANOVA (one way repeated measures within stress treatment)
<i>Conditioning</i>				
Control	3.86 $\pm$ 0.73	357.43 $\pm$ 25.85 [#]	367.11 $\pm$ 41.19 [#]	$F(2,22) = 68.03, p < 0.001$
Defeat	2.85 $\pm$ 0.71	360.41 $\pm$ 24.25 [#]	372.10 $\pm$ 46.89 [#]	$F(2,21) = 60.8, p < 0.001$
<i>Recall</i>				
Control	71.44 $\pm$ 34.34	522.61 $\pm$ 15.89 [#]	67.18 $\pm$ 15.83	$F(2,20) = 114.6, p < 0.001$
Defeat	17.76 $\pm$ 7.67	387.82 $\pm$ 38.78 ^{#*}	73.61 $\pm$ 26.74	$F(2,21) = 73.9, p < 0.001$
<i>Extinction 1</i>				
Control	12.63 $\pm$ 2.37	300.68 $\pm$ 45.08 [#]	29.64 $\pm$ 6.67	$F(2,22) = 40.3, p < 0.001$
Defeat	19.73 $\pm$ 7.09	172.78 $\pm$ 39.27 ^{#*}	17.15 $\pm$ 7.53	$F(2,21) = 14.8, p < 0.001$
<i>Extinction 2</i>				
Control	5.51 $\pm$ 0.53	170.96 $\pm$ 29.80 [#]	16.52 $\pm$ 2.75	$F(2,21) = 29.7, p < 0.001$
Defeat	3.12 $\pm$ 0.66	104.89 $\pm$ 26.48 [#]	11.02 $\pm$ 2.51	$F(2,20) = 10.5, p < 0.001$
<i>Extinction 3</i>				
Control	8.44 $\pm$ 1.19	90.29 $\pm$ 10.87 [#]	25.98 $\pm$ 4.99	$F(2,17) = 38.6, p < 0.001$
Defeat	5.61 $\pm$ 0.83	56.46 $\pm$ 11.57 [#]	18.98 $\pm$ 3.51	$F(2,21) = 11.9, p < 0.001$
<i>Extinction 4</i>				
Control	5.38 $\pm$ 0.91	44.32 $\pm$ 4.31 [#]	12.22 $\pm$ 3.19	$F(2,21) = 36.5, p < 0.001$
Defeat	3.47 $\pm$ 0.76	33.05 $\pm$ 7.58 [#]	12.77 $\pm$ 3.68	$F(2,21) = 13.6, p < 0.001$

[#] Difference among time blocks (one way repeated ANOVA within stress treatment, SNK $p < 0.005$),

^{*} Difference between control and defeat within the 11 – 20 min (tone) time block (two way repeated ANOVA stress x session, SNK $p < 0.002$). $N = 12$ /group.