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Behavioral and accumbens synaptic plasticity induced by cues associated with restraint stress

Constanza Garcia-Keller¹ [✉], Jordan S. Carter¹, Anna Kruyer¹, Angela M. Kearns¹, Jordan L. Hopkins¹, Ritchy Hodebourg¹, Peter W. Kalivas^{1,2} [✉] and Carmela M. Reichel^{1,2} [✉]

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Exposure to acute stress can increase vulnerability to develop or express many psychiatric disorders, including post-traumatic stress disorder. We hypothesized that stress-induced psychiatric vulnerability is associated with enduring neuroplasticity in the nucleus accumbens core because stress exposure can alter drug addiction-related behaviors that are associated with accumbens synaptic plasticity. We used a single 2-h stress session and 3 weeks later exposed male and female rats to stress-conditioned odors in a modified defensive burying task, and quantified both active and avoidant coping strategies. We measured corticosterone, dendritic spine and astrocyte morphology in accumbens, and examined reward sensitivity using a sucrose two-bottle choice and operant sucrose self-administration. Exposure to stress odor increased burying (active coping) and immobility (avoidant coping) in the defensive burying task in female and male rats. Systemic corticosterone was transiently increased by both ongoing acute restraint stress and stress-conditioned odors. Three weeks after administering acute restraint stress, we observed increased dendritic spine density and head diameter, and decreased synaptic association with astroglia and the astroglial glutamate transporter, GLT-1. Exposure to conditioned stress further increased head diameter without affecting spine density or astroglial morphology, and this increase by conditioned stress was correlated with burying behavior. Finally, we found that stress-exposed females have a preference for sweet solutions and higher motivation to seek sucrose than stressed male rats. We conclude that acute stress produced enduring plasticity in accumbens postsynapses and associated astroglia. Moreover, conditioned stress odors induced active behavioral coping strategies that were correlated with dendritic spine morphology.

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INTRODUCTION

Exposure to acute life-threatening stress, such as military combat or sexual assault, increases the incidence of many psychiatric disorders, including post-traumatic stress disorder (PTSD). PTSD is a debilitating disorder that can evolve from a single traumatic event, defined as an event where an individual experienced, witnessed, or was confronted by death threat, serious injury, threat to physical integrity of oneself or another, and can be responded to with intense fear, helplessness, or horror (Diagnostic and Statistics Manual of Mental Health Disorders-V). A diagnosis of PTSD often occurs months or years after the stress exposure [1], implying enduring neurobiological vulnerabilities are instilled by the initial stressful experience [2]. Three consistently identified PTSD behavioral symptom clusters are the intrusive, avoidant, and hyperarousal clusters [3]. Importantly, intrusive memories are often initiated by external cues and contexts that resemble aspects of the stressful experience, and can trigger the avoidant and hyperarousal (active) behavioral clusters of PTSD [1]. Based on previous studies [4], we recently recapitulated the pathological behavioral sequence of a stress-associated cue triggering avoidant and active behavioral clusters in rats, by using a defensive burying task [15]. Thus, 8 weeks after acute restraint stress the presence of the stress-conditioned odor (stress-conditioned

stimulus, stress-CS) increased both burying (active coping) and immobility (avoidant coping).

The enduring nature of vulnerability to a stress-CS inducing avoidant and active coping behaviors indicates enduring changes in synaptic architecture that may be coding for stress-CS learning and retrieval. Consistent with this possibility, stress induces synaptic plasticity in classic fear circuitry, including the prefrontal cortex and amygdala [2]. Also, 3 weeks after acute restraint stress AMPA/NMDA currents were potentiated in the nucleus accumbens core (NAcore) [5]. This was accompanied by a decrease in the glutamate transporter, GLT-1, and preventing the stress-induced decrease in GLT-1 by ceftriaxone treatment blocked the stress-induced potentiation of cocaine-induced locomotor sensitization and self-administration. Importantly, the enduring synaptic changes induced by acute stress in NAcore resemble the changes produced by repeated use of addictive cocaine [6]. This study complemented a large literature showing that stress exposure promotes drug-induced reward seeking and sensitization, and points to an overlap in stress and drug-induced synaptic plasticity in the NAcore that may contribute to the high level of comorbidity between PTSD and substance use disorder [2, 7–15].

In addition to enduring effects on synaptic plasticity by stress and addictive drug use, drug-conditioned cues produce transient

¹Department of Neuroscience, Medical University of South Carolina, Charleston, SC, USA. ²These authors contributed equally: Peter W. Kalivas, Carmela M. Reichel. ✉email: garciak@muscc.edu; reichel@muscc.edu

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synaptic potentiation in NAcore [6]. Given all the potential links between stress, reward seeking, and NAcore plasticity, we hypothesized that exposure to an odor stress-CS induces plasticity in NAcore synaptic and astroglial morphology, akin to that produced by drug-conditioned cues. We used a single 2-h restraint session to establish an odor stress-CS and 3 weeks later used a defensive burying task to quantify coping strategies and synaptic plasticity in rats exposed to the stress-CS. We included measures of systemic corticosterone and tested rats for the effect of stress on sucrose reward seeking.

METHODS

See Supplemental Methods and Tables (S1, S2, and S3) for complete statistical and methodological information.

Animal housing

Male and female Sprague–Dawley rats (~250 g; Charles River Laboratories, Raleigh, NC, USA) were double-housed according to sex with a 12:12 h dark/light cycle. Animals were 2 months old (± 1 week) and experiments were conducted during the dark cycle. Rats received food and water ad libitum, and were allowed to acclimate for 1 week before treatment. Experimental procedures were approved by the Animal Care and Use Committee of the Medical University of South Carolina and performed in accordance with National Institutes of Health guidelines.

Acute restraint stress and scent exposure

Rats were randomly assigned into sham or stress groups. The stress rats were inserted into acrylic flat bottom restrainers (PLAS Labs, Thomas Scientific, Swedensboro, NJ, USA) that restricted the movement for 2 h at the beginning of the dark cycle. During restraint, three drops of an odor fluid in a Petri dish container ("stress-CS") were placed in a cage similar to the home cage to elicit an association between the odor and the physiological discomfort of restraint. The scents used were lemon (LM, dōTERRA Intl., West Pleasant Grove, UT, USA) and sandalwood (SW, Wyndmere Naturals, Minneapolis, MN, USA), using a counter balanced design. Sham rats received exposure to the odor without the stress experience; thus, exposure to this odor is termed a "neutral stimulus" (NS) throughout. Each pair of rats were exposed to the same scent and stress/sham condition.

Defensive burying

Defensive burying quantifies approach/avoidance behaviors through exploiting an innate response in rodents to bury threatening objects [16]. We used odor CS or NS to test cue reactivity during a defensive burying task [17]. Bedding was placed in one half of the cage opposite to a dish containing the odor. The cage was divided in half and labeled to define the bedding versus odor cue zone. Each rat was placed facing away from the odor on the bedding side of the cage. Behavior was digitally recorded for 15 min.

Defensive burying index

To determine the burying index, the last frame of the recorded video was cropped to visualize the odor cue zone (Fig. S1A-1). Using ImageJ (Fiji), the picture was converted to 8-bit greyscale (Fig. S1A-2) and the threshold was adjusted (Fig. S1A-3). Bedding on the cage floor was quantified as area and integrated density, and normalized to the size of the picture in pixels (Fig. S1A-4). Because the measures of integrated density and area were highly correlated (Fig. S1B; $r^2 = 0.85$, $p < 0.001$), the results were presented as the percentage of the total area covered by bedding.

Plasma corticosterone levels

Rats were surgically implanted with intravenous catheters for sampling blood at 1 week before restraint stress, during the stress session, and in response to the stress-CS. Other samples were taken 1 week after the stress and 1 week after CS exposure. All blood draws were made 7 days apart between 9 and 11 a.m. Whole blood (1.2 ml) was collected using 1000 U heparin. Plasma was isolated by centrifugation (10,000 r.p.m. at 4 °C for 20 min) and stored at -80 °C until assayed. Plasma corticosterone was determined using Eliza (Arbor Assays Corticosterone Enzyme Immunoassay; Ann Arbor, MI USA). Given the qualitative sex difference, the group

mean was calculated separately for males and females. Each data point reported in Fig. 2D was divided by the group mean, resulting in an adjusted group mean of 100% [(individual data point/group mean) \times 100].

Quantification of dendritic spine morphology

NAcore tissue was sliced, cell labeled using the fluorescent lipophilic indocarbocyanine dye, DiIC₁₈(3), and spine dendrite morphology quantified [18]. Confocal Z-series data sets of spine morphology were acquired using a Leica SP5 laser-scanning confocal microscope at 63 \times (1024 \times 256 frame; 0.21 μ m/Z step). Images were deconvolved using Autoquant (Media Cybernetics, Bethesda, MD), and a 3-D perspective rendered using the Surface module of Imaris software package V9 (Bitplane; Saint Paul, MN).

Quantification of astrocyte morphology

NAcore astrocyte labeling and morphology was quantified, as described previously [19]. Briefly, z-stacks were acquired using a Leica SP5 at 63 \times with a 1024 \times 1024 frame size, 12-bit image resolution, four-frame averaging, and 1-mm step size. Images were deconvolved using Autoquant and a 3-D perspective was rendered using the surface module of Imaris software (V9).

Sucrose self-administration, progressive ratio, and reinstatement

Sucrose self-administration, progressive ratio (PR), extinction, and cued-reinstatement tests were conducted, as described previously [20, 21]. The first 5 days rats were on a fixed ratio (FR) 1 schedule of reinforcement, followed by an FR3 for 3 days, and then placed on an FR5 for 12 more days. PR testing occurred when responding stabilized. Break point was defined as the highest number of lever presses required by the rat to receive a single pellet. The session terminated if a rat failed to receive a sucrose pellet for 1 h or after a total of 5 h. PR test was also evaluated in the presence of CS or NS in the self-administration chamber with 2 days of sucrose self-administration on an FR5 between tests using crossover randomized design. The CS or NS was placed on the floor of the self-administration chamber for the entire extent of the test (see ref. [22]). Following the PR tests, rats underwent 2 h daily extinction sessions for a minimum of 9 days, where responses on both levers were recorded, but had no scheduled consequences. Upon reaching extinction criteria (<25 active lever presses for 2 days), rats underwent cue reinstatement. During the cue test, active lever pressing resulted in the presentation of the light + tone sucrose-associated cue, but no sucrose was available. Animals were not food deprived at any time point.

Data analysis and statistics

All statistics were done using GraphPad Prism V9. The overall analysis included between subjects' variables of sex (male and female) and group (sham and stress). Behavioral data were analyzed using ANOVA's followed by Holm–Sidak post hoc tests for multiple comparisons. Corticosterone data were analyzed with Student's *t* test for baseline and mixed effects for multiple time points. Spine density and dh data were analyzed with nested ANOVA (between factors variation among group condition, and within-subject factors variation within subject across different segments), and post hoc comparisons conducted using a Holm–Sidak post hoc tests. Astrocyte morphology was statistically analyzed by Kruskal–Wallis because groups were not normally distributed, and Dunn's test for post hoc comparisons. Sucrose preference and sucrose self-administration, PR and reinstatement ANOVAs were used to determine behavior and positive interactions were followed by Holm–Sidak's post hoc comparisons. All data except sucrose self-administration and two-bottle choice were obtained and quantified by persons unaware of animal treatment.

RESULTS

Stress-CS increases corticosterone and avoidant and active coping behaviors

One hundred twenty-six female and male Sprague–Dawley rats entered a restraint stress or sham protocol and 3 weeks later performed a defensive burying task. Following the task, rats were immediately assigned to either spine morphology, sucrose self-administration, or sucrose two-bottle choice (Fig. 1A). During defensive burying, rats were exposed to either an odor stress-CS,

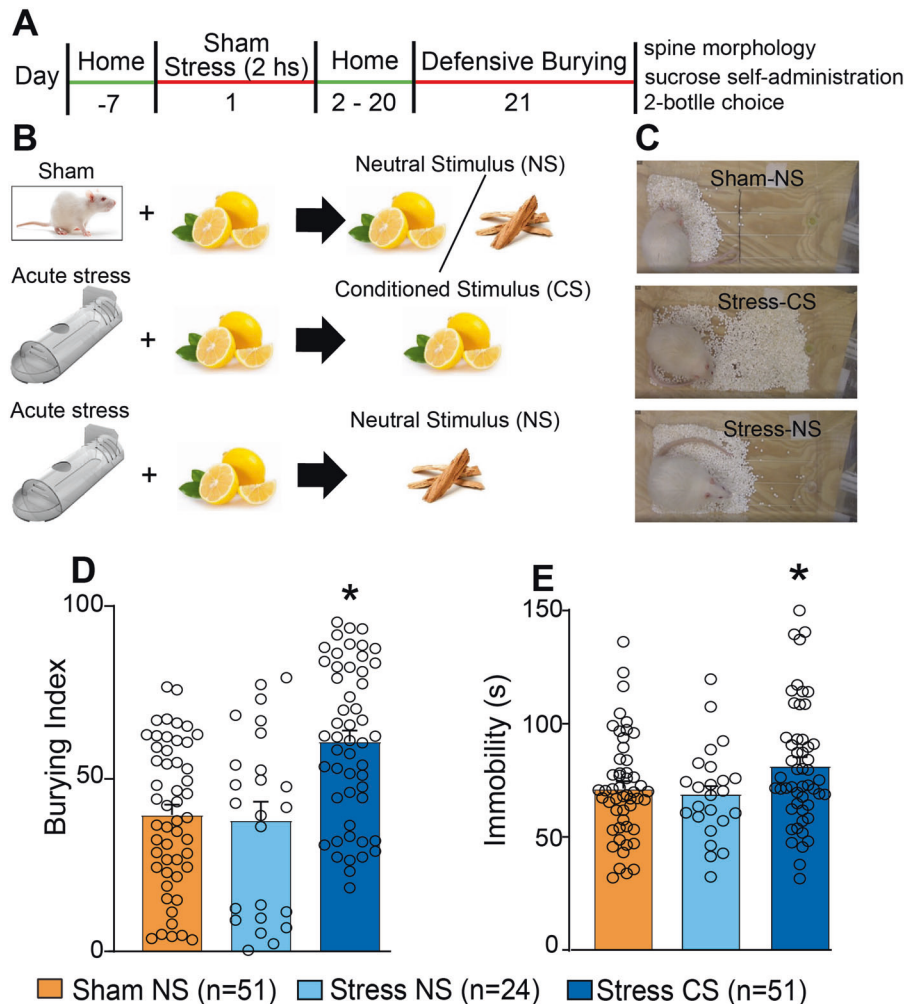


Fig. 1 The stress-conditioned stimulus increased stress responses in a defensive burying task. **A** Timeline: female and male rats were either restrained or left undisturbed in the home cage for 2 h, 3 weeks after all the animals were tested on a defensive burying task. **B** Experimental design: sham animals were exposed in the home cage to an odor (lemon or sandalwood); thus, this odor is termed neutral stimulus (top panel). During acute restraint stress animals were exposed to an odor (lemon or sandalwood) that became a conditioned stimulus to elicit an association between the odor and the stress of restraint (middle panel), while the opposite odor become a neutral stimulus (bottom panel). Three experimental groups were used: sham-NS, stress-CS, and stress-NS. **C** Representative pictures of the three groups conditions in the defensive burying chamber. **D** Increased burying index, calculated as displaced bedding toward the cue odor, in stress-CS compared to sham-NS and stress-NS groups. Note that the higher *N* in the stress-CS group is because this group includes behavior from rats examined for corticosterone levels (see Fig. 2). **E** Increased immobility time in stress-CS compared to sham-NS and stress-NS groups. Data are shown as mean \pm SEM. Each dot in bar represents an animal. * $p < 0.05$ comparing stress-CS to stress-NS and sham-NS, using Holm–Sidak’s post hoc; since stress-NS did not pass normality test we used Kruskal–Wallis test and revealed identical statistical conclusion.

previously associated with the stressful experience, or a neutral odor stimulus (NS; Fig. 1B, C). For sham animals, the NS was an odor added to the home cage during sham conditioning, while for stress animals the NS was a different odor from the one associated with stress.

The burying index and immobility time were first statistically evaluated for sex differences, and finding no main effect of sex or interaction with sex and stress group, the data were collapsed across sex (Fig. S2A). Stress-CS-exposed rats had an increased burying index compared to sham-NS and stress-NS (Fig. 1D; one-way ANOVA $F_{(2,123)} = 13.86$, $p < 0.001$). Similarly, stress-CS rats spent more time immobile (Fig. 1E; one-way ANOVA $F_{(2,123)} = 3.11$, $p = 0.048$) relative to the sham-NS and stress-NS. We also examined the relationship between the burying index and other behavioral measures in the defensive burying task (Fig. S1C–E), and there was a positive correlation between total time spent burying and burying index ($r^2 = 0.134$, $p < 0.001$). Conversely, there was a negative correlation between the latency to begin burying and the burying index ($r^2 = 0.119$, $p < 0.001$).

Corticosterone is released during stressful events in rodents and is often used as a biomarker for stress [23]. We sampled blood from 13 rats at different time points during the stress protocol (Fig. 2A, B), including prior to restraint stress, 15-min into restraint stress, 1-week post-stress exposure, immediately after defensive burying, and 1-week post-defensive burying. Since, male and female rats had significantly different baseline levels of corticosterone (Fig. 2C; unpaired t test $t_{(11)} = 2.49$, $p = 0.029$), data were converted to a percentage of baseline to evaluate the time course of changes in corticosterone (Fig. 2D). Exposure to 15 min restraint stress and re-exposure to stress-CS during the defensive burying task increased blood corticosterone levels in male and female rats compared to baseline level (Fig. 2D; one-way within subjects ANOVA, $F_{(5,50)} = 16.91$, $p < 0.001$ and Holm–Sidak’s, $p < 0.05$). Note that some catheters lost patency during the multiple extractions, resulting in reduced sample number over time (a total of four rats lost catheter patency, two after obtaining the 15 min after stress samples, and two more after obtaining the 1 week after stress samples).

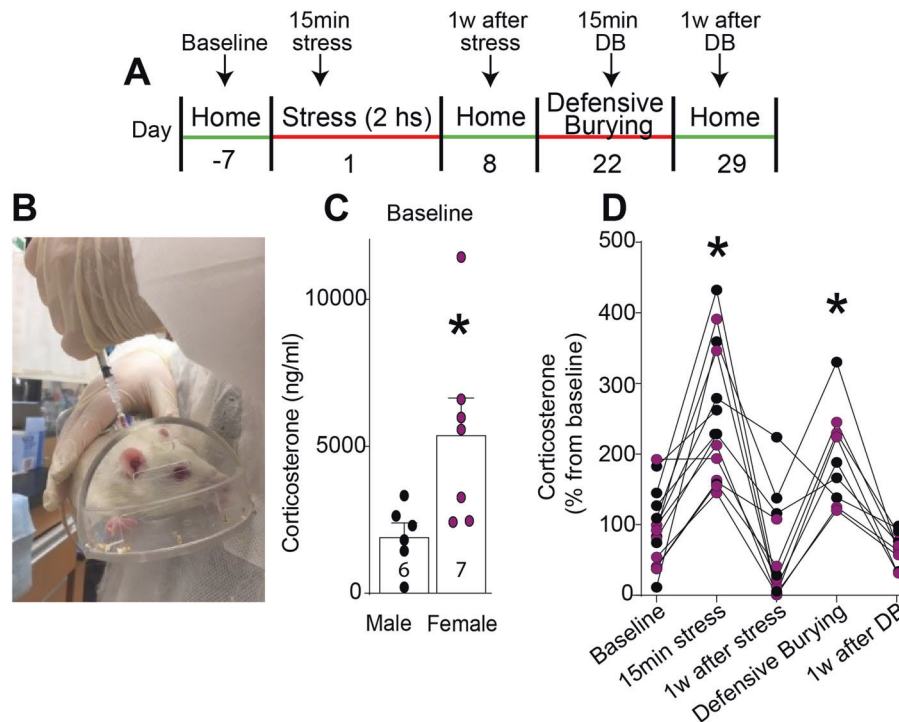


Fig. 2 Restraint stress and stress-CS increased plasma corticosterone levels. **A** Timeline with arrows indicates the different time points when the blood was drawn. **B** Image showing blood withdrawal inside the restrainer after 15 min of the stress session. **C** Female and male rats had different baseline corticosterone levels. Data are shown as mean \pm SEM and individual rat values. Number of rats shown in bars * $p < 0.05$ comparing female to male. **D** Plasma corticosterone increased after 15 min of restraint stress and upon re-exposure to the CS during defensive burying task. * $p < 0.05$ indicates difference from baseline sample using Holm–Sidak's post hoc.

Acute stress and stress-CS exposure alter dendritic spine morphology at accumbens synapses

To determine if acute restraint stress and subsequent stress-CS exposure induced synaptic plasticity, 35 female and male rats were perfused immediately after the defensive burying task and Z-series confocal images were made of diolistically labeled neurons in the NAcCore (Fig. 3A, B). Data were collapsed across sex, and spine density was increased in NAcCore 3 weeks after acute restraint stress, regardless of whether the rats were in the stress-CS or stress-NS groups (Fig. 3C; nested one-way ANOVA $F_{(2,32)} = 11.37$, $p < 0.001$). Similarly, stress increased dh (dh) in both stress groups compared to sham-NS, but the stress-CS rats showed a further increase in head diameter compared to stress-NS rats (Fig. 3D; nested one-way ANOVA $F_{(2,32)} = 13.88$, $p < 0.001$). Group differences also occurred in the cumulative frequency distribution of both density and dh. The number of spines per μm in stress-NS and stress-CS rats was increased relative to sham-NS rats (Fig. 3E; female: KS test = 0.603, $p < 0.001$; male: KS test = 0.599, $p < 0.001$). The distribution of head diameters showed increases in stress-NS relative to sham-NS, and a further increase in stress-CS rats compared to stress-NS (Fig. 3F; female: KS test = 0.559, $p = 0.001$; male: KS test = 0.403, $p = 0.014$). Supporting a relationship between a stress-CS induced increase in spine density and burying behavior, spine density was positively correlated with the latency to start burying the CS (Fig. 3G; $r^2 = 0.635$ and $p = 0.006$) and negatively correlated with the total time the animal spent burying in stress-CS rats (Fig. 3H; $r^2 = 0.401$ and $p = 0.048$). No other correlations between immobility and spine morphology were statistically significant (Table S2).

Stress and sex interacted with spine density (Fig. S2B left; $F_{(2,173)} = 5.06$, $p = 0.007$), and there were main effects of sex ($F_{(1,173)} = 7.07$, $p < 0.001$) and stress ($F_{(2,173)} = 26.22$, $p < 0.001$). The only sex difference that emerged in post hoc analysis showed that male stress-NS rats had greater spine density than female stress-NS rats.

Regarding dh (Fig. S2B right), there was a main effect of sex ($F_{(1,173)} = 6.11$, $p = 0.014$) and stress ($F_{(2,173)} = 45.21$, $p < 0.001$), but post hoc comparisons did not reveal any specific sex differences.

Stress reduced synaptic proximity of astroglia and stress-CS decreased synapse-associated GLT-1

Synaptic adjacency of astroglial processes and astrocyte expression of the glutamate transporter, GLT-1, are important factors influencing synaptic glutamate spillover [24], and astroglial proximity to synapses in the NAcCore is changed after cocaine or heroin withdrawal [25]. In order to examine the impact of conditioned stress on astroglia, we labeled astroglia with a membrane-bound fluorescent reporter (AAV5-GFAP-hM3dqmCherry) for subsequent confocal imaging, digitization, and quantification of astrocyte morphology (Fig. 4A). Three weeks after acute stress or sham, male rats were assigned to an experimental group (sham-NS, stress-NS, or stress-CS) and brains removed 15-min after exposure to the NS or CS. Levels of GLT-1 and co-registration of the astroglial membrane or astroglial GLT-1 with the synaptic marker Synapsin I were quantified [19]. The majority of quantified parameters were not normally distributed in tissue from sham animals (Table S3). Stressed animals showed no change in astroglial surface area or volume between treatment groups (Fig. S3A, B). Tissue was immunolabeled for Synapsin I to quantify synaptic adjacency of the astroglial surface (Fig. 4A–2). Near adjacency was defined as co-registration of Synapsin I puncta with the astroglial membrane reporter mCherry indicating proximity < 250 nm, according to the limit of confocal resolution. Synaptic co-registration of NAcCore astroglia was constitutively reduced after acute stress (stress-NS), and partially recovered during CS exposure (Fig. 4B, Kruskal–Wallis = 8.53, $p = 0.014$). The reduction in synaptic co-registration was due to reduced number of Synapsin I puncta co-registered with the astroglial surface (Fig. 4B; Kruskal–Wallis = 14.21, $p < 0.001$), not from group

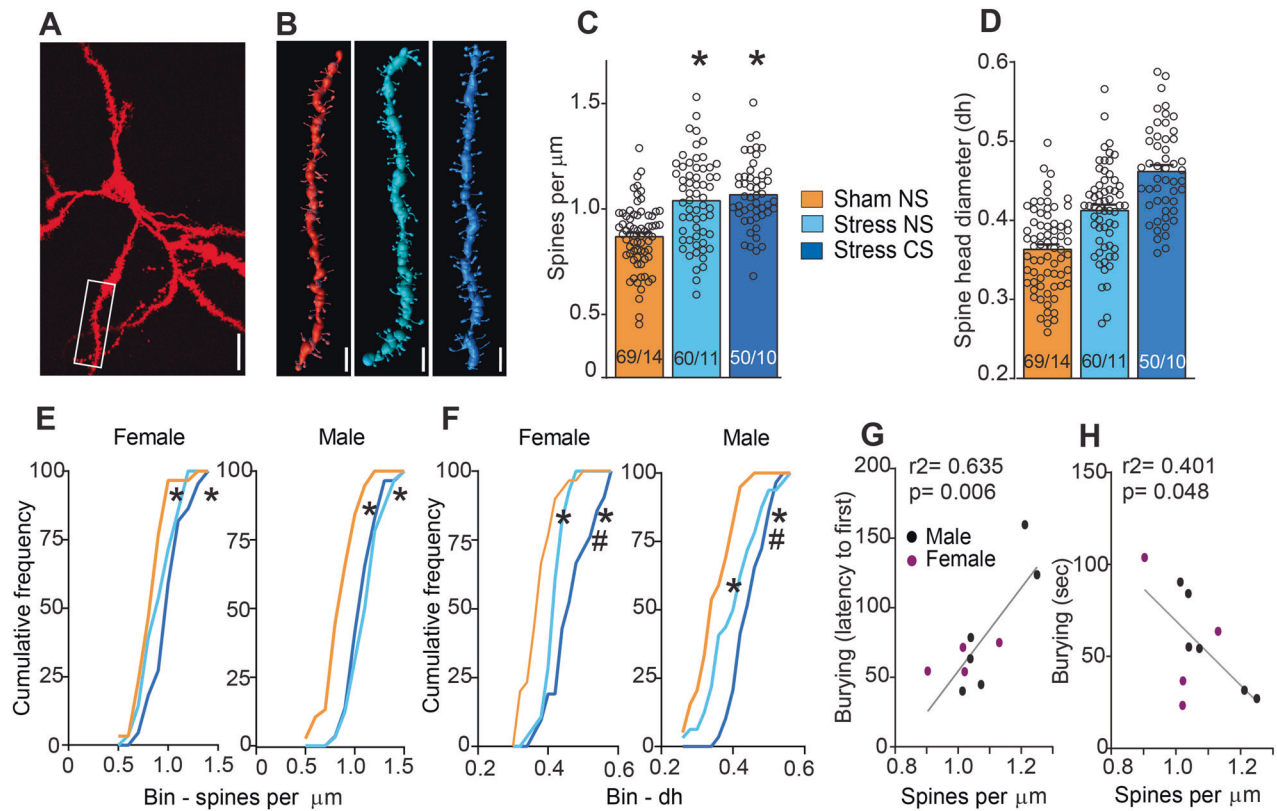


Fig. 3 Acute stress induced an increase in spine density and head diameter (dh) was potentiated by stress-CS exposure. **A** Representative Dil filled MSN. Box shows area sampled from this neuron for spine analysis. Scale bar = 30 μm . **B** Representative dendritic segments from each treatment group. Scale bar = 2 μm . **C** Acute stress increased spine density in stress-NS and stress-CS rats. **D** Stress-CS induced an increase in dh after 15 min of exposure. **E** Differential cumulative frequency distribution of spine density of stress vs sham-treated animals in females and males. **F** Differential cumulative frequency distribution of dh across the three different groups in females and males. **G** Positive correlation between spine density and latency to start burying during 15 min of defensive burying task. **H** Negative correlation between spine density and the amount of time spent burying during 15 min of defensive burying task. Data are shown as mean \pm SEM. Each dot in bar represents an analyzed segment. *N* is shown as number of neurons quantified over number of animals in each condition. * $p < 0.05$ difference from sham group, # $p < 0.05$ difference from stress-NS using Holm–Sidak’s post hoc or Kolmogorov–Smirnov test (for frequency plots).

differences in the volume of overlapping regions (Fig. S3C), or the total synapsin density (Fig. S3D).

Astroglia were also assessed for GLT-1 expression (Fig. 4A–4). Stress-NS rats showed a trend toward reduced GLT-1 immunoreactivity that was statistically significant after exposure to the stress-CS (Fig. 4D; Kruskal–Wallis = 7.523, $p = 0.023$). To determine the impact of GLT-1 downregulation and synaptic retraction by astroglia on synaptic adjacency of GLT-1, tissue was immunolabeled for Synapsin I and GLT-1, and triple co-registration of these markers with astroglia surface mCherry was quantified and normalized to the astroglial volume. The stress-CS reduced synaptic co-registration of GLT-1 (Fig. 4E; Kruskal–Wallis = 8.657, $p = 0.013$). The reduction in astroglia synaptic proximity and downregulation of GLT-1 is consistent with the stress-CS producing conditions facilitating glutamate spillover [26].

Acute restraint stress increased the motivation for sucrose

Following the restraint stress/defensive burying protocol, 32 female and male, stress and sham rats were trained to self-administer sucrose by increasing the FR (FR1, FR3, and FR5; Fig. 5A). Rats were then tested on a PR schedule of reinforcement. This schedule is a measure of the motivational value of foods, fluids, and drugs in operant lever-pressing tasks [27]. All rats acquired sucrose self-administration (Fig. 5B; lever \times day interaction, $F_{(19,1104)} = 65.7$, $p = 0.0001$). On the PR tests, active lever pressing (Fig. 5C; $t_{(30)} = 1.95$, $p = 0.041$) and break point (Fig. 5C; $t_{(30)} = 1.798$, $p = 0.041$) were higher in stress relative to sham

rats indicating greater motivation to work for sucrose reward, following acute restraint stress. There were no differences in inactive lever (Fig. 5A), between stress-CS and stress-NS (Fig. 5A, B, C), or between sex on any measures (Fig. 5S).

Following PR testing, rats were placed into extinction. There were no sex or group differences during extinction in active (Fig. 5D) or inactive lever presses (Fig. 5A, D). Reinstatement of lever pressing was precipitated by presenting light/tone cues associated with sucrose delivery during self-administration. Sham and stress rats (Fig. 5E) reinstated equally in response to sucrose cues ($F_{(1,28)} = 70.85$, $p < 0.001$). There was no difference in inactive levers (Fig. 5A, E) or sex differences on these measures (Fig. 5S, D).

Acute restraint stress did not induce anhedonia

We also tested whether acute stress produced anhedonic affects akin to chronic models of stress-induced depression [28–30]. The lack of sucrose preference is interpreted as anhedonia [31]. Following the defensive burying, rats 24-h sucrose and water consumption were measured daily (Figs. S6–S8). Animals were first exposed to 3 days 1% sucrose intake, then two-bottle choice (1% sucrose or water), followed by ascending and randomized sucrose concentrations (0.1, 0.3, 1.0, 3.0 and 10%; Fig. S6A). Main effects were observed with ascending sucrose concentrations (0.1, 0.3, 1.0, 3.0, and 10%) in one bottle or water in the other bottle for 5 days at each concentration. The amount of sucrose consumed (w/v) interacted across sucrose concentration and stress (Fig. S6D;

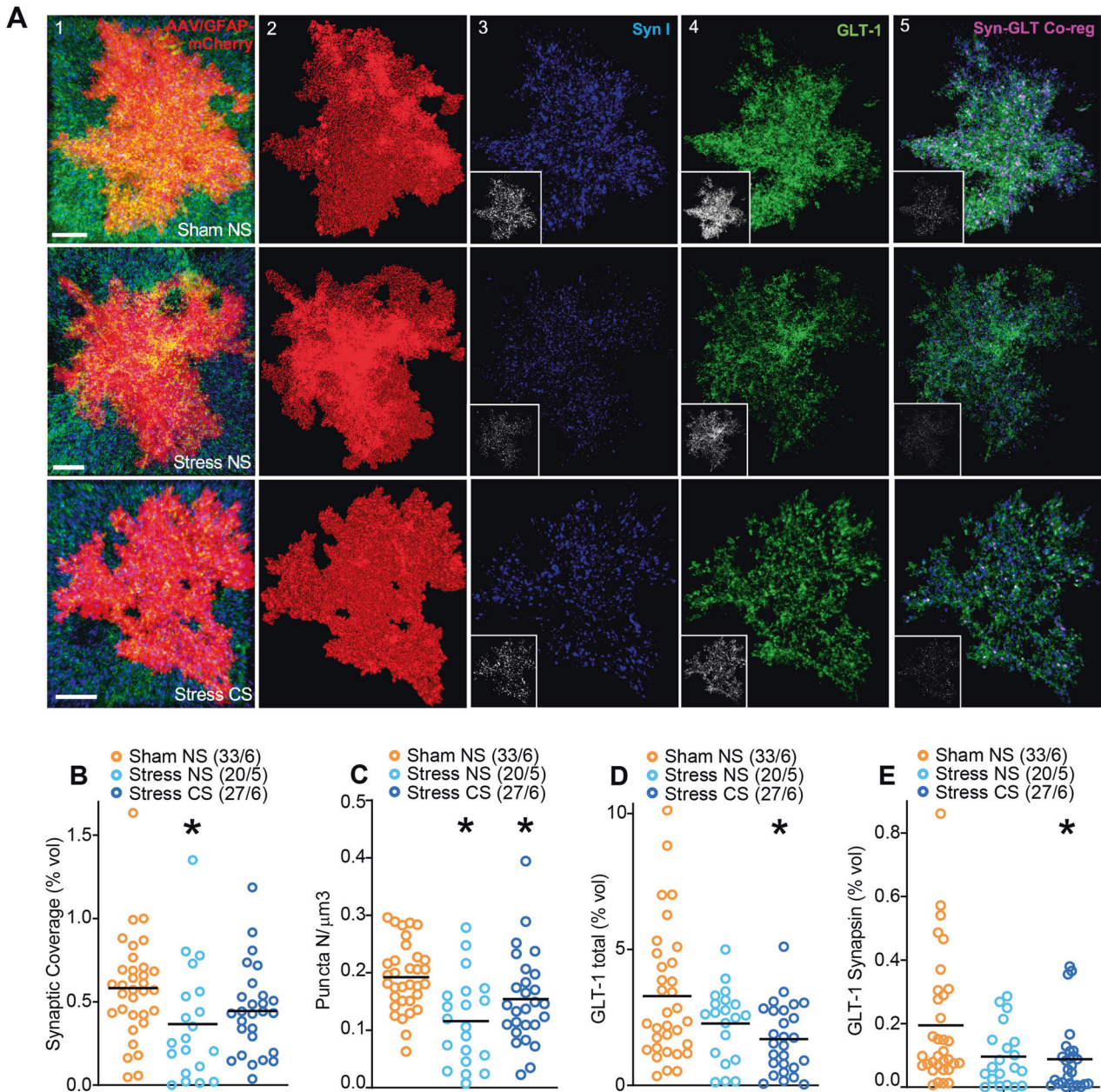


Fig. 4 Acute stress induced long-lasting retraction of astroglia from NAc core synapses and stress-CS reduced synaptic co-registration of GLT-1. **A** Z-series depicting an NAc core astrocyte from the three experimental groups. (1) Astrocyte transfected with AAV5/GFAP-hM3d-mCherry (red) with immunolabeled GLT-1 (green) and Synapsin I (blue). Bars = 10 μm . (2) Digitized model of astrocyte in panel 1. (3) Digitally isolated Synapsin I (blue) puncta that co-registered with the astroglial volume. (4) Digitally isolated GLT-1 (green) signal that co-registered with the astroglial volume. (5) GLT-1 and Synapsin I co-registration (pink) within the territory occupied by the astroglia in panel 1. Insets show quantified signal in white for illustrative contrast. **B** Synaptic proximity by the astroglial membrane was constitutively reduced after restraint stress (stress-NS group), and partially increased toward sham-NS- in stress-CS-exposed animals. **C** The number of puncta where astrocytes co-registered with Synapsin I was reduced after restraint stress (stress-NS group) and in stress-CS-exposed animals. **D** GLT-1 expression was decreased in rats exposed to the stress-CS. **E** The triple co-registration between the astroglial membrane, Synapsin I, and GLT-1 was reduced in stress-CS exposed animals, consistent with downregulated GLT-1 and synaptic retraction by astroglia. Data are shown as median. * $p < 0.05$, compared to sham using a Dunn's post hoc test.

$F_{(4,172)} = 3.52$, $p = 0.009$) and sucrose concentration and sex (Fig. S7C; $F_{(1,172)} = 6.34$, $p < 0.001$). Further evaluation of a stress by sucrose concentration interaction, showed that stress rats consumed greater amounts of 3.0% sucrose relative to sham (Fig. S6D; Holm-Sidak $p = 0.04$). The sex by sucrose concentration interaction revealed that females consumed more 1.0% (Holm-Sidak $p = 0.02$), 3.0% (Holm-Sidak $p = 0.006$), and 10.0% (Holm-Sidak $p = 0.006$) sucrose relative to males (Fig. S7C). Surprisingly, rather than anhedonia-like effects, acute stress

modestly increased sucrose preference. Although there were overall sex differences, these appeared unrelated to stress.

DISCUSSION

Using a modified defensive burying task, we found that presenting a stress-conditioned odor initiated a stress response that was characterized by increases in both active (burying) and avoidant (immobility) coping, as well as an increase in blood

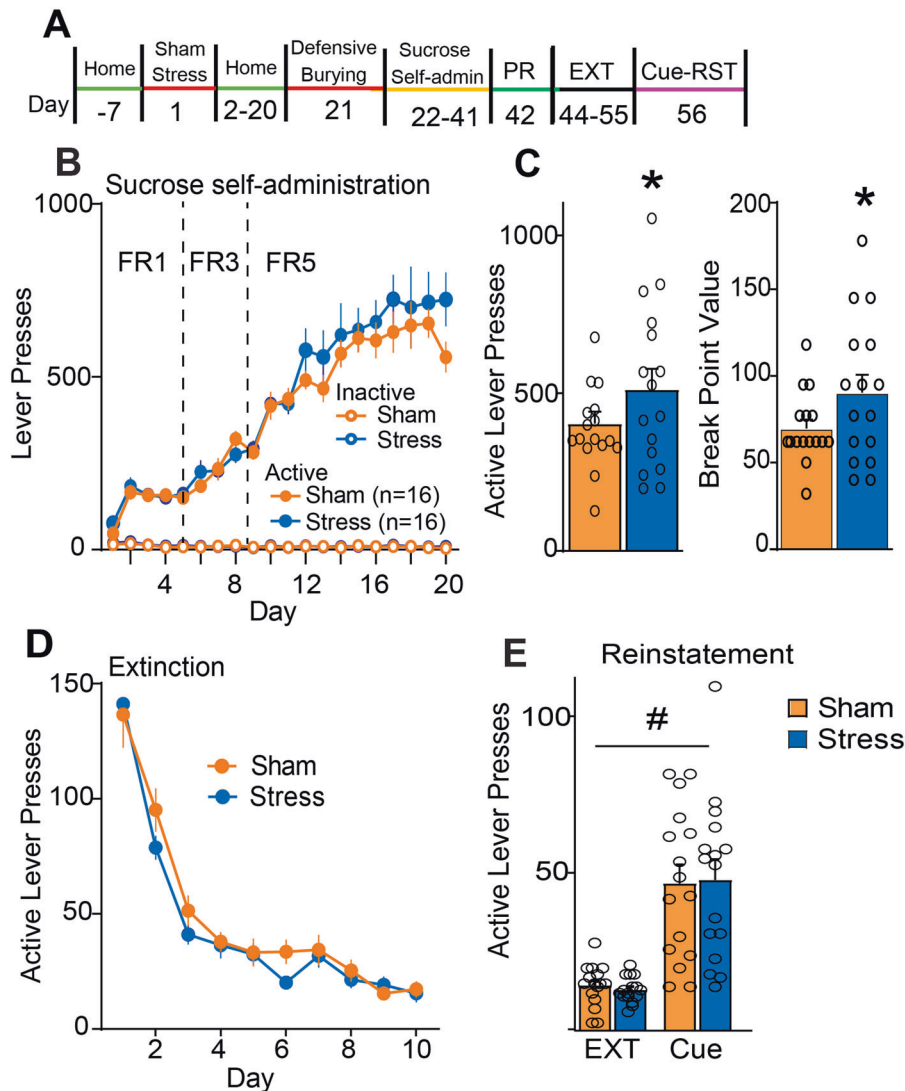


Fig. 5 Acute stress did not increase the motivation to work for sucrose pellets and did not affect stress-, cue-, or the combination of both induced reinstatement. **A** Experimental timeline used for sucrose self-administration, progressive ratio (PR), extinction (EXT), and reinstatement (RST). **B** There were no group or sex differences in the number of active or inactive lever presses on any of the three fixed ratio (FR1, FR3, and FR5) schedules during sucrose self-administration. **C** Increased number of active lever presses and break point value during progressive ratio test in stressed animals compared to sham. **D** No change in active lever presses during extinction training between stress and sham animals. **E** No difference in the number of active lever presses between sham and stress animals; however, both groups reinstate to cues associated with sucrose and cue + CS. Data are shown as mean \pm SEM. Each dot in bar represents an animal. Number of animals is expressed in brackets. * $p < 0.05$ difference from sham group, # $p < 0.05$ difference from extinction baseline using Holm-Sidak's post hoc.

corticosterone levels. In parallel with the stress odor-induced coping behaviors, there was an increase in *dh* in NAc_{core} and indices of active coping (latency to begin burying and total time burying) were correlated with the odor-induced change in spine morphology. In parallel with increased *dh*, the stress-CS reduced the amount of astroglial glutamate transporter (GLT-1) adjacent to the synapse. In addition to the stress-CS, acute restraint stress itself caused enduring morphological changes in both dendritic spine and astroglial morphology. Thus, in the stress-NS group NAc_{core} spine density and *dh* were increased, and the association between astroglia surface and synapses was decreased. Together these data show that acute restraint stress can condition a stimulus to elicit a complex stress coping behavioral response, and simultaneously produce morphological changes in NAc_{core} synapses. It is possible that the NAc_{core} adaptations may underlie the capacity of acute restraint stress to potentiate rewarding stimuli, as shown before by stress inducing increase cocaine, alcohol, and heroin intake [5, 17, 22], and here we observed

increase in PR responding for sucrose. Furthermore, by finding that the morphological synaptic plasticity in NAc_{core} produced by stress-CS resembles the plasticity elicited in other studies by a drug-conditioned cue [6].

Stress-induced constitutive and transient changes in neuronal synapse morphology

Quantification of structural plasticity most consistently reveals stress-induced dendrite and spine atrophy in allocortical and cortical brain regions, such as hippocampus and medial prefrontal cortex [2], although increased spine density has been reported in amygdala [32]. The accumbens integrates excitatory input from several stress-sensitive cortical and allocortical regions [33–35], and we previously found that acute restraint stress produces enduring potentiation of glutamatergic synapses in the NAc_{core} [5]. Similarly, stress-induced depression-like behaviors have also been associated with increased dendritic spine density specifically on accumbens D2-dopamine receptor expressing neurons [36–38]. In

addition to these enduring effects, a stress-conditioned odor increased dh above the enduring constitutive elevations induced by acute stress. The correlation between the constitutive increase in spine density and burying latency or total burying time only in stress-CS rats indicates that spine density may influence active coping to a stress-CS. The fact that the more the original stress exposure constitutively increased spine density, the more slowly the rats responded to a stress-CS and the shorter duration the overall burying response supports an hypothesis that the greater the stress-induced increase in spine density the more resistant the rats were to the stress-CS inducing an active coping response (burying). The resistance to stress-CS induced active coping may be an indication of poor ability to cope with stress or that the stress-CS may be less stressful, when spine density is higher in the NAcCore.

Stress-induced constitutive and transient changes in astrocyte morphology near neuronal synapses

Many studies show a role for astroglia in regulating glutamate transmission, which has led to conceptualizing a dynamic tripartite relationship between the pre- and postsynapse and astroglia that actively regulates both physiological and pathological synaptic plasticity [39, 40]. Three weeks after acute restraint stress, we showed there is an increase in synaptic glutamate spillover in NAcCore that is mediated by decreased expression and function of the astroglial glutamate transporter GLT-1 [5]. We found that following stress-CS total astrocytic GLT-1 and the synaptic proximity of GLT-1 was reduced. Moreover, reduced GLT-1 was associated with a reduction in overall synaptic proximity of astroglia. Together, this stress-associated astroglial plasticity would be expected to promote synaptic glutamate spillover by reducing synaptic proximity of GLT-1 [41].

Stress and reward seeking

Anhedonia, the inability to experience pleasure from rewarding activities, and negative changes in affect comprise part of the current diagnostic criteria for PTSD (DSM 5) [42]. Correspondingly, many studies show that repeated stress in rodents induces anhedonia-like behaviors, including decreased preference for natural rewards, such as sucrose [28–30, 43]. The deficits in these behaviors could reflect “motivational anhedonia,” diminished drive to obtain the reward, and/or “consummatory anhedonia,” the capacity to experience reward [44]. We conducted two experiments distinguishing between these anhedonic processes: (1) operant sucrose self-administration with PR testing and, (2) sucrose preference with two-bottle choice task. In the first experiment, restraint stress increased PR operant responding for sucrose. However, the extent to which restraint stress impacts motivational processes needs to be further elucidated, because social defeat stress results in contrasting patterns of natural and drug reward-seeking behavior depending on methodology. For example, acute social defeat decreased motivational drive for saccharin-reinforced responding [45], and chronic social defeat decreased PR responding for cocaine and cumulative cocaine intake [45]. However, in alignment with our findings, other reports indicate that chronic and intermittent social defeat-induced stress increase PR responding for alcohol [46] and cocaine [47, 48].

As a direct measure of “consummatory anhedonia” males and females underwent a two-bottle sucrose preference task. In our study, stress rats showed sucrose preference for the 3% sucrose concentration, an effect driven by females. In a prior study, acute restraint stress decreased saccharin preference 24 h after stress [49]. In response to foot shock stress, male rats decreased saccharin intake, but increased sucrose intake indicating differences in the qualitative value of the reward [50]. Combined, the enhancement for sweet preferences and motivation for sucrose reward suggest acute restraint stress increases both motivational and consummatory hedonic processes.

CONCLUSIONS

Stress-related disorders, such as PTSD, affect millions of individuals world-wide. While modeling a diagnosis of PTSD in rats is not possible, here we recapitulated four key traits of PTSD, hyperarousal (burying), avoidant symptoms (immobility), hyperarousal, and avoidant behavior initiated by a stress-associated cue and enduring vulnerability to cue-induced initiation of behavioral response (i.e., 3 weeks after stress exposure). One major obstacle in developing new treatments for stress-related disorders is an incomplete understanding of the basic neurobiology of stress and stress-associated cues. Currently, the only pharmacotherapy approved by the US Food and Drug Administration for treating PTSD are selective serotonin reuptake inhibitors [51], which are only marginally effective at treating PTSD and comorbid PTSD and SUDs [52]. Similarly, most preclinical studies have focused on stress-induced adaptations in monoaminergic circuits and how corticotrophin-releasing factor (CRF) modulates monoamines [53, 54]. It has been proposed that pathological substrates may predispose individuals suffering from stress disorders to develop comorbid SUDs [2, 5]. This possibility is supported by a single stress event and exposure to a stress-associated cue, causing alterations at glutamatergic synapses in the NAcCore (spine morphology changes and astrocyte-GLT-1 regulation) that also occur during cue-induced drug seeking [6]. Future studies will examine corticosterone and CRF contributions to these glutamatergic adaptations. Moreover, the similarity in glutamatergic neuroadaptations in NAcCore between acute stress and stress-CS to those produced by the self-administration and cue-induced drug seeking poses common points of pharmacological intervention that may be particularly useful in treating PTSD and SUDs comorbidity. Along these lines, normalizing GLT-1 with *N*-acetylcysteine has been generally successful at reducing drug craving and other forms of intrusive thinking associated with other neuropsychiatric diseases, such as obsessive-compulsive disorder, PTSD, and major depression [52, 55].

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AUTHOR CONTRIBUTIONS

CG-K, PWK, and CMR designed research; CG-K, JSC, AK, AMK, and JLH performed research; CG-K, JSC, AK, RH, PWK, and CMR discussed data; CG-K, PWK, and CMR analyzed data; and CG-K, PWK, and CMR wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to C.G.-K. or C.M.R.

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