Published in final edited form as:

Horm Behav. 2017 March ; 89: 137–144. doi:10.1016/j.yhbeh.2017.01.002.

Allopregnanolone induces state-dependent fear via the bed nucleus of the stria terminalis

Gillian M. Acca^a, Abel S. Mathew^{b,1}, Jingji Jin^a, Stephen Maren^{a,b}, and Naomi Nagaya^{a,b,*}

^aInstitute for Neuroscience, Texas A&M University, College Station, TX, USA

^bDepartment of Psychology, Texas A&M University, College Station, TX, USA

Abstract

Gonadal steroids and their metabolites have been shown to be important modulators of emotional behavior. Allopregnanolone (ALLO), for example, is a metabolite of progesterone that has been linked to anxiety-related disorders such as posttraumatic stress disorder. In rodents, it has been shown to reduce anxiety in a number of behavioral paradigms including Pavlovian fear conditioning. We have recently found that expression of conditioned contextual (but not auditory) freezing in rats can be suppressed by infusion of ALLO into the bed nucleus of the stria terminalis (BNST). To further explore the nature of this effect, we infused ALLO into the BNST of male rats prior to both conditioning and testing. We found that suppression of contextual fear occurred when the hormone was present during either conditioning or testing but not during both procedures, suggesting that ALLO acts in a state-dependent manner within the BNST. A shift in interoceptive context during testing for animals conditioned under ALLO provided further support for this mechanism of hormonal action on contextual fear. Interestingly, infusions of ALLO into the basolateral amygdala produced a state-independent suppression of both conditioned contextual and auditory freezing. Altogether, these results suggest that ALLO can influence the acquisition and expression of fear memories by both state-dependent and state-independent mechanisms.

Keywords

Neurosteroid; Fear conditioning; Contextual fear; Basolateral amygdala

1. Introduction

The importance of sex steroid hormones in anxiety-related disorders has gained recent support from both preclinical and clinical studies (Glover et al., 2015; Maeng and Milad, 2015). Estrogen has been shown to affect different aspects of fear learning including acquisition of contextual fear (Barha et al., 2010), facilitation of fear extinction in female rats (Chang et al., 2009; Gupta et al., 2001; Milad et al., 2009) and humans (Milad et al., 2009; Milad et al., 2010; Zeidan et al., 2011) as well as modulation of fear generalization in male and female rats (Lynch et al., 2014; Lynch et al., 2016). Progesterone has mixed effects on fear extinction; it is facilitatory in some instances (rats; Milad et al., 2009) and impairing

*Corresponding author at: Texas A&M University, 301 Old Main Drive, College Station, TX 77843-3474, USA.

¹Current address: Department of Psychology, University of Wisconsin-Milwaukee, Milwaukee, WI, USA.

in others (humans; Graham and Daher, 2016; Pineles et al., 2016). Support for a role of androgens in conditioned fear has also varied across studies. Castration with or without testosterone replacement had no effect on the acquisition, expression, or extinction of conditioned contextual fear in male rats (Anagnostaras et al., 1998). In contrast, when light-enhanced startle (similar to contextual fear) and fear-enhanced startle (similar to cued fear) were examined in male rats, androgens were found to suppress the former but not the latter (Toufexis et al., 2005).

Modulation of conditioned fear processes may involve steroid metabolites as well. Allopregnanolone (ALLO), a major metabolite of progesterone, has been associated with decreased levels of context-related fear (Pibiri et al., 2008; Toufexis et al., 2004) and its synthetic analogue, ganaxolone, has been shown to facilitate extinction in mice (Pinna and Rasmusson, 2014). We have recently shown that local ALLO activity in the bed nucleus of the stria terminalis (BNST) modulates the expression of conditioned contextual fear (Nagaya et al., 2015). In male rats, increased ALLO activity via local infusion suppressed context-dependent freezing whereas in female rats, decreased ALLO activity via pharmacological means resulted in enhanced freezing. Given that ALLO has been shown to be a potent allosteric potentiator of GABA_A receptor (GABAR) currents (Majewska et al., 1986), one possible mechanism of ALLO action on contextual fear may involve GABARs within the BNST.

The importance of GABARs in regulating contextual fear has been recently demonstrated by studies of fear acquisition and expression following intra-hippocampal infusion of gaboxadol (Jovasevic et al., 2015). Interestingly, this work revealed that activation of extrasynaptic GABARs within the hippocampus produces state-dependent contextual fear. That is, intra-hippocampal gaboxadol infusions before either fear conditioning or retention testing blunted the expression of contextual fear in mice. However, gaboxadol infusions before both conditioning and testing resulted in high levels of conditioned freezing—indicating a state-dependent effect of GABAR modulation. ALLO shares similarities with gaboxadol in that it promotes GABAergic tone especially at extrasynaptic GABARs (Brown et al., 2002; Mortensen et al., 2012). As such, we explored the mechanism by which ALLO affects conditioned fear by administering it into the BNST or the BLA prior to acquisition, expression, or both.

2. Materials and methods

2.1. Subjects

Adult male Long-Evans rats (200–224 g at arrival; Blue Spruce) were purchased from a commercial supplier (Envigo, Indianapolis, IN, USA). Rats were individually housed in clear plastic cages and maintained on a 14:10 h light:dark cycle (lights on at 7:00 AM) in a temperature- and humidity-controlled vivarium with unrestricted access to food and water. All experiments occurred during the light phase. Upon arrival, rats were handled for five consecutive days for approximately 20 s each day to acclimate to the experimenter. All procedures were performed in accordance with the guidelines approved by the Texas A&M University Institutional Animal Care and Use Committees.

2.2. Behavioral apparatus

All behavioral sessions occurred in eight identical observation chambers (30 × 24 × 21 cm; Med Associates, St. Albans, VT, USA) in a single testing room. Each chamber consisted of two aluminum sidewalls, a Plexiglas ceiling, rear wall, and hinged front door. A speaker for delivery of the auditory conditioned stimulus (CS) was mounted on one sidewall and an incandescent house light (15 W) was mounted on the other. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center to center) for delivery of the footshock unconditioned stimulus (US). Rods were connected to a shock source and solid-state grid scrambler (Med Associates). A removable stainless steel tray underneath the rods was used to manipulate contexts with odors. Each chamber was situated inside a sound-attenuating cabinet equipped with a ventilation fan to provide background noise (65 dB).

Behavioral procedures were conducted using two distinct contexts. For “Context A”, white fluorescent room lights, cabinet fans, and house lights were all on and cabinet doors left open. Each chamber was cleaned with 1% acetic acid; a small amount of liquid remained in the tray beneath the floor rods. Subjects were transported to and from the vivarium in white plastic boxes and the computer monitor in the room was left on. For “Context B”, red fluorescent room lights and cabinet fans were on whereas house lights were off and cabinet doors kept closed. Each chamber was cleaned with 1% ammonium hydroxide; a small amount of liquid remained in the tray beneath the floor rods. Subjects were transported to and from the vivarium in black plastic boxes and the computer monitor in the room was turned off.

Each chamber rested on a load cell to measure locomotor activity via Threshold Activity Software (Med Associates). Before the experiment, each load cell was calibrated to a specific displacement with the output of each amplifier set to a specific gain. The results from this output were digitized at 5 Hz, such that one observation every 200 ms was recorded. Freezing was only detected if the rat was immobile for, at minimum, 1 s.

2.3. Surgery

Rats were anesthetized with isoflurane (5% induction, 2% maintenance). The top of the head was shaved and the rat was secured in the stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and given a subcutaneous injection of lidocaine before incision. After incision and retraction of the scalp, the head was adjusted such that lambda and bregma were aligned in the same horizontal plane. Three small holes were drilled for placement of anchoring screws and two small holes were drilled for bilateral implantation of guide cannulae (stainless steel, 26-gauge, 9 mm for the BNST, 11 mm for the BLA; Plastics One, Roanoke, VA, USA) attached to internal cannulae (33-gauge, 9 or 11 mm with 1-mm projection; Plastics One) directed at the BNST (0.0 mm AP, ± 2.7 mm ML, −6.9 mm DV from dura at a 10° angle towards the midline) and BLA (−2.9 mm AP, ± 4.9 mm ML, −7.4 mm DV from dura). Dental acrylic was applied around the anchoring screws and guide cannulae. After surgery, internal cannulae were removed and replaced with dummy cannulae, projecting 1 mm beyond the guide cannulae (33-gauge; Plastics One). Rats received one week of recovery prior to the start of behavioral procedures. To habituate the

animals to the infusion procedure, rats were transported in white, 5-gal buckets lined with bedding to the infusion room. Rats were gently restrained and dummy cannulae were changed twice prior to the start of behavioral experiments.

2.4. Drugs

Allopregnanolone or (3 α ,5 α)-3-hydroxy-pregnan-20-one (ALLO; R&D Systems, Minneapolis, MN, USA) was solubilized (8 mg/ml) in 30% (w/v) hydroxypropyl- β -cyclodextrin in purified water (VEH; Sigma-Aldrich, St. Louis, MO, USA).

2.5. Infusions

All rats were individually transported from their home cages to the infusion room in 5-gal white buckets and dummy cannulae were removed. Infusions were made with internal cannulae secured to either polyethylene tubing (PE-20, Braintree Scientific, Braintree, MA, USA) for VEH or polytetrafluoroethylene tubing (PTFE; 28-gauge, SAI Infusion Technologies, Lake Villa, IL, USA) for ALLO. Tubing was connected to 10- μ l Hamilton syringes mounted on an infusion pump (KD Scientific, Holliston, MA, USA). Bilateral infusions of VEH and ALLO were made at a rate 0.25 μ l/min for a total volume of 0.25 μ l in the BNST and 0.3 μ l in the BLA. Internal cannulae were left in place for a 2 min post-infusion period to allow for drug diffusion. Fresh dummy cannulae were then inserted and rats remained in buckets until 10 min post-infusion after which they were transported to the testing rooms in plastic boxes for behavior. Previous work in our laboratory has found reliable effects of ALLO at this dosage without producing sedation (Nagaya et al., 2015).

2.6. Experiment 1: Effects of intra-BNST ALLO on the acquisition and expression of contextual and cued fear

Rats were housed and implanted with cannulae targeting the BNST as described above. The behavioral experiment followed a 2 $\times$ 2 factorial design consisting of four different groups: V/V, V/A, A/A, and A/V (V = VEH, A = ALLO; the first letter signifies drug state on conditioning day and the second letter signifies drug state on testing days). On Day 1, rats were transported to the infusion room and received bilateral infusions of VEH or ALLO (2 μ g/side) targeting the BNST. Ten minutes following the start of the infusion, rats were transported in white plastic boxes to the observation chambers for conditioning (Context A). Conditioning consisted of a 3-min baseline period, followed by five tone (CS; 10 s, 80 dB, 2 kHz)-shock (US; 2 s, 1 mA) pairings in which the tone immediately followed the shock. A 1-min inter-trial interval (ITI) separated each tone-shock pairing. Following the last conditioning trial, there was a 1-min waiting period after which rats were returned to their home cages. Contextual and cued fear testing occurred over Days 2 and 3. Half of the animals were counterbalanced for order of context and cued tests. As previously shown (Nagaya et al., 2015), the suppressive effect of intra-BNST ALLO on freezing was limited to context exposure. Therefore, the remaining half of the animals only received context tests. On Day 2, rats were infused in the same manner as on Day 1 with either the same drug or a different drug. Ten minutes after infusion, rats were transported to either the conditioning context (Context A) for context testing or a novel context (Context B) for cued fear testing. Context testing consisted of a 10-min test with no tones or shocks given. Cued fear testing consisted of a 3-min baseline period followed by four CS presentations with a 1-min ITI and

a 1-min period after the final tone. On Day 3, rats were infused in the same manner and with the same drug as on Day 2. After 10 min, rats were transported to the testing room for either context or cued fear testing.

2.7. Experiment 2: Effects of intra-BLA ALLO on the acquisition and expression of contextual and cued fear

Rats were housed and implanted with cannulae targeting the BLA as described above. Similar to Experiment 1, this experiment followed a 2×2 factorial design yielding groups conditioned under VEH or ALLO (2.4 $\mu\text{g}/\text{side}$) and tested in either the same or different drug state. All subjects were tested for both context and cued fear with the order of testing counterbalanced across groups. On Day 1, rats were infused with either VEH or ALLO and then conditioned in Context A. On Days 2 and 3, rats were given either context (Context A) or cued fear (Context B) testing. On Day 2, rats were infused with either the same or different drug as on conditioning day. On Day 3, rats received the same drug as on Day 2.

2.8. Experiment 3: Contribution of intra-BNST ALLO to interoceptive and exteroceptive states

Subjects were housed and cannulated as described above. Unlike Experiments 1 and 2, this experiment involved animals that were all conditioned under the same context (Context A) and drug state (ALLO) and then tested in context and drug states that were either the same as those at conditioning or different, yielding the following groups with different shift conditions for context testing: None (no shift), Context (context shift only), Drug (drug shift only), and Context & drug (context and drug shift). On Day 1, rats were transported to the infusion room and received bilateral infusions of ALLO (2 $\mu\text{g}/\text{side}$) into the BNST in the same manner as previously described. Ten minutes after the infusion, rats were transported to the conditioning room (Context A) and trained under the same conditions as in Experiment 1. On Day 2, rats were infused with either VEH or ALLO. After 10 min, rats were placed in either the conditioning context (Context A) or a novel context (Context B). As in the previous experiments, context testing consisted of a 10-min test with no tone or shock presentations. Subjects were not tested for cued fear.

2.9. Histology

After all behavioral testing, animals were overdosed with sodium pentobarbital (100 mg/kg) and perfused transcardially with 0.9% saline and 10% formalin. Brains were rapidly extracted and post-fixed for 24 h in 10% formalin at 4 °C before being transferred to a 30% sucrose-formalin solution. Using a cryostat set at -20 °C, brains were sectioned at 40 μm and mounted onto gelatin-subbed slides with 70% ethanol. Sections were stained with 0.25% thionin and imaged at 10 $\times$ (Leica Microsystems, Buffalo Grove, IL) to ensure proper cannula placement for each experiment.

2.10. Data analysis

The percentage of freezing behavior during conditioning was averaged across the 3-min pre-CS baseline period, the 1-min ITI for each trial, and the final 1-min post-trial period. For context tests, mean of percentage freezing was averaged across the entire 10-min period. For

cued tests, freezing was computed as the mean of percentage freezing during the 1-min ITI for each trial and the final 1-min post-trial period. All data were analyzed with repeated or factorial analysis of variance (ANOVA) and represented as means $\pm$ SEMs ($\alpha = 0.05$). In the case of significant F ratios, Fisher's protected least significant difference (PLSD) post hoc comparisons were performed. Effect size estimates were calculated using η^2 for ANOVAs (ratio of effect variance to total variance) and Cohen's d for pairwise comparisons (<http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php>).

3. Results

3.1. Effects of intra-BNST ALLO on the acquisition and expression of contextual and cued fear

3.1.1. Histology—A photomicrograph of a thionin-stained brain section shows the representative bilateral placement of cannulae targeted to the anterior BNST (Fig. 1A). Fig. 1B depicts the cannula placements for all subjects included in Experiments 1 and 3. For Experiment 1, 64 rats were implanted; nine were excluded due to improper cannula placement. Cannula damage during experimental procedures resulted in the exclusion of two subjects and an additional four did not survive the study. This yielded the following group sizes: V/V ($n = 12$), A/A ($n = 11$), A/V ($n = 14$), and V/A ($n = 12$). All subjects included had tip placements localized to the anterior BNST, both dorsal and ventral to the anterior commissure. Cannula placements for Experiment 1 were distributed from 0.00 to -0.46 mm relative to bregma, with the majority occurring between -0.11 and -0.26 mm.

3.1.2. Behavior—Groups receiving VEH or ALLO prior to acquisition started with low baseline levels of freezing which then increased during conditioning trials (Fig. 1C). Throughout conditioning, ALLO-treated subjects exhibited increased levels of freezing relative to VEH-treated animals. A repeated measures ANOVA showed a significant main effect of drug state ($F_{(1,47)} = 8.1$, $p < 0.01$, $\eta^2 = 0.147$) and trial number ($F_{(5,235)} = 48$, $p < 0.0001$, $\eta^2 = 0.496$) on freezing. There was no significant interaction between drug state and trial number ($F_{(5,235)} = 1.3$, $p = 0.26$, $\eta^2 = 0.014$). These data indicate that pre-training infusions of ALLO into the BNST facilitate acquisition of fear conditioning.

Context testing (Fig. 1D) revealed elevated levels of freezing in subjects trained and tested in the same drug state (V/V and A/A) and reduced levels of freezing in subjects trained and tested in different drug states (V/A and A/V). A factorial ANOVA showed no significant main effect of training state ($F_{(1,45)} = 0.60$, $p = 0.44$, $\eta^2 = 0.001$) or testing state ($F_{(1,45)} = 0.16$, $p = 0.69$, $\eta^2 = 0.003$) on context-specific freezing. There was, however, a significant interaction between training and testing states ($F_{(1,45)} = 15$, $p < 0.001$, $\eta^2 = 0.247$). Post hoc comparisons revealed significant differences in percentage freezing between the A/A group and the A/V ($p < 0.01$, $d = 1.16$) and V/A ($p < 0.05$, $d = 1.03$) groups. Percentage freezing for the V/V group was also found to be significantly different from the A/V ($p < 0.01$, $d = 1.21$) and V/A ($p < 0.05$, $d = 1.06$) groups. These data indicate that training and testing under the same drug state results in the expression of higher levels of contextual fear.

In contrast, differences in drug state between training and testing did not affect freezing in response to cued fear (Fig. 1E). A factorial ANOVA with training state and testing state as

between-subjects variables and trial number as the within-subject variable revealed no significant main effect of training state ($F_{(1,19)} = 1.9$, $p = 0.18$, $\eta^2 = 0.089$) or testing state ($F_{(1,19)} = 0.032$, $p = 0.86$, $\eta^2 = 0.001$) and no significant interaction between training state and testing state ($F_{(1,19)} = 0.86$, $p = 0.37$, $\eta^2 = 0.039$). These data indicate that drug state during training or testing does not influence the expression of cued fear.

3.2. Effects of intra-BLA ALLO on the acquisition and expression of contextual and cued fear

3.2.1. Histology—A photomicrograph of a thionin-stained brain section shows the representative bilateral placement of cannulae targeted to the BLA (Fig. 2A). Fig. 2B depicts cannula placements for all subjects used in the analysis for Experiment 2. Of the 64 rats implanted, 12 were excluded due to improper cannula placement. Additional subjects were excluded due to loss of head caps ($n = 2$) and blocked cannulae ($n = 2$). This yielded the following group sizes: V/V ($n = 14$), A/A ($n = 11$), A/V ($n = 10$), and V/A ($n = 13$). All subjects included had tip placements localized to the BLA. Cannula placements for Experiment 2 were distributed from -2.45 to -3.70 mm relative to bregma, with the majority between -2.85 and -3.25 mm.

3.2.2. Behavior—During conditioning, initial freezing for VEH- and ALLO-treated animals was negligible and then steadily rose across tone-shock presentations (Fig. 2C). A repeated measures ANOVA with a between-subjects variable of drug group revealed a significant main effect of trial ($F_{(5,230)} = 53$, $p < 0.0001$, $\eta^2 = 0.527$) but neither an effect of drug state prior to training ($F_{(1,46)} = 1.1$, $p = 0.3$, $\eta^2 = 0.023$) nor an interaction between the two ($F_{(5,230)} = 1.6$, $p = 0.17$, $\eta^2 = 0.016$). Therefore, regardless of drug manipulation prior to training, all subjects acquired conditioned fear at similar rates.

Context testing showed that subjects receiving ALLO prior to training (A/V), testing (V/A), or both (A/A) had reduced freezing compared to those receiving VEH prior to both training and testing (V/V; Fig. 2D). A factorial ANOVA with between-subjects variables of training state and testing state revealed a significant main effect of testing state ($F_{(1,44)} = 12$, $p < 0.01$, $\eta^2 = 0.187$), but not training state ($F_{(1,44)} = 0.64$, $p = 0.43$, $\eta^2 = 0.01$). There was, however, a significant interaction between training state and testing state ($F_{(1,44)} = 8.8$, $p < 0.01$, $\eta^2 = 0.134$). Post hoc analysis revealed significant differences between the V/V group and all other groups ($p < 0.05$, $d = 1.04$ for A/V; $p < 0.01$, $d = 1.24$ for A/A; $p < 0.0001$, $d = 2.24$ for V/A).

Similar results were found with drug infusions prior to cued fear testing; animals treated with VEH prior to both training and testing had elevated levels of freezing compared to other groups (Fig. 2E). A factorial ANOVA revealed a significant main effect of testing state ($F_{(1,44)} = 7.4$, $p < 0.01$, $\eta^2 = 0.117$), but not training state ($F_{(1,44)} = 2.7$, $p = 0.11$, $\eta^2 = 0.042$). A significant interaction between training state and testing state was also found ($F_{(1,44)} = 9.0$, $p < 0.01$, $\eta^2 = 0.143$). Post hoc analysis revealed significant differences between the V/V group and all other groups ($p < 0.01$, $d = 1.19$ for A/A; $p < 0.01$, $d = 1.26$ for A/V; $p < 0.0001$, $d = 1.56$ for V/A).

3.3. Contribution of intra-BNST ALLO to interoceptive and exteroceptive states

3.3.1. Histology—Cannula placements for all subjects in Experiment 3 are depicted in Fig. 1B. Of the 64 rats implanted, eight were excluded due to improper cannula placement. This yielded the following group sizes: None ($n = 12$), Context ($n = 16$), Drug ($n = 12$), and Context & drug ($n = 16$). All subjects included had tip placements localized to the anterior BNST. Cannula placements for Experiment 3 were distributed from 0.00 to -0.46 mm from bregma, with the majority between -0.11 and -0.26 mm.

3.3.2. Behavior—In order to explore the possible contribution of intra-BNST ALLO to interoceptive and exteroceptive aspects of conditioned context, the design illustrated in Fig. 3A was devised for Experiment 3. All animals were initially infused with ALLO and then conditioned in Context A. The following day, subjects were tested under one of four assigned context shift test conditions: 1) the same drug state and context (None), 2) the same drug state but different context (Context), 3) the same context but different drug state (Drug), or 4) different context and drug state (Context & drug). Acquisition of fear conditioning did not differ between subjects assigned to these groups (data not shown). Baseline freezing was low across all groups and as seen in ALLO-treated animals from Experiment 1, rats exhibited robust freezing after the onset of tone-shock trials. A repeated measures ANOVA with a between-subjects variable of assigned context shift test condition (None, Context, Drug, or Context & drug) and a within-subject variable of trial number revealed a significant main effect of trial number ($F_{(5,260)} = 53$, $p < 0.0001$, $\eta^2 = 0.493$) but no significant main effect ($F_{(3,52)} = 1.4$, $p = 0.26$, $\eta^2 = 0.074$) or interaction of context test assignment ($F_{(15,260)} = 0.99$, $p > 0.05$, $\eta^2 = 0.027$). Therefore, subjects in all assigned test groups acquired conditioned fear similarly across the five trials.

During context testing, changes in both context and drug state as well as a change in drug state alone had suppressive effects on freezing behavior (Fig. 3B). There was a significant main effect of both test context ($F_{(1,52)} = 4.1$, $p < 0.05$, $\eta^2 = 0.065$) and test drug state ($F_{(1,52)} = 6.5$, $p < 0.05$, $\eta^2 = 0.103$), but no interaction between the two factors ($F_{(1,52)} = 0.47$, $p = 0.50$, $\eta^2 = 0.007$). Post hoc analysis revealed a significant difference between None and Drug groups ($p < 0.05$, $d = 0.99$) and None and Context & drug groups ($p < 0.01$, $d = 1.46$). In addition, a trend towards a significant difference between percentage freezing expressed by None and Context groups ($p = 0.06$, $d = 0.78$) was obtained. These data indicate that a change in the interoceptive drug state of the BNST at test can impair recall of a fear-conditioned exteroceptive context.

4. Discussion

The present study demonstrates that intra-BNST administration of ALLO results in state-dependent contextual fear in male rats. Thus, our previous finding that intra-BNST ALLO suppresses contextual fear (Nagaya et al., 2015) may not be due to a simple pharmacological impairment of context-specific fear expression. Rather, our current data suggest that intra-BNST ALLO may induce an interoceptive state that becomes conditioned to exteroceptive aversive stimuli. This effect of ALLO was unique to the BNST, insofar as ALLO infusions into the BLA produced a state-independent impairment in both the acquisition and

expression of conditional freezing to context and cue. The state-dependent modulation of contextual fear by intra-BNST ALLO suggests a novel mechanism by which gonadal steroids and their metabolites might influence the acquisition and expression of fear memories.

In addition to producing state-dependent contextual fear, we found, unexpectedly, that ALLO infused into the BNST prior to conditioning facilitated the acquisition of post-shock freezing. The increase in post-shock freezing after ALLO infusion was unique to the BNST and, did not necessarily result in increased contextual fear—in fact, the opposite was true when VEH rather than ALLO was administered prior to testing. Infusion of ALLO or other GABAR modulators into the BNST prior to fear conditioning have not been reported, although chemical lesions of the BNST have been found to either impair fear to context-like, long-duration stimuli (Waddell et al., 2006) or have no effect on contextual fear (LeDoux et al., 1988). The increased level of freezing we observed in ALLO-treated animals during acquisition could arise from enhanced synaptic inhibition within the BNST resulting in 1) increased activity in anxiogenic circuits and/or, 2) facilitated acquisition of a US association with the drug-induced interoceptive state. ALLO infusion into the BNST may shift GABAergic inhibition such that anxiogenesis is promoted during fear conditioning. Optogenetic and in vitro slice recording studies support the presence of heterogeneous subnuclei within the BNST that have opposing functions in regulating anxiety (Gungor et al., 2015; Jennings et al., 2013; Kim et al., 2013). GABAergic inputs from the lateral portion of the central amygdala (CeL) have been proposed to inhibit neurons in the anterolateral portion of the BNST (BNST-AL), which in turn, inhibit neurons in the anteromedial portion of the BNST (BNST-AM) and modulate fear (Gungor and Paré, 2014; Haufler et al., 2013). Infused ALLO may facilitate CeL inhibition and thereby increase disinhibition of BNST-AM neurons that generate fear. Alternatively, fear acquisition in ALLO-treated animals may be enhanced by the 10 min pre-conditioning exposure to ALLO, which could promote greater salience between the US (footshock) and interoceptive state rather than the tone CS or context. In other words, ALLO within the BNST may serve as a ‘cue’ that becomes associated with footshock (Fanselow, 1980), thereby facilitating acquisition in ALLO-treated but not VEH-treated animals. Indeed, the greater level of freezing induced by ALLO infusion is apparent after the first conditioning trial (Fig. 1C).

Of course, it is of considerable interest that despite increasing post-shock freezing, ALLO in the BNST produced marked deficits in contextual freezing during retention testing when infused either before conditioning or before testing. While this might suggest a general inhibitory effect of GABAR modulation on the acquisition and expression of fear, animals for which ALLO was infused prior to both fear conditioning and retention testing exhibited high levels of conditioned freezing. This suggests that impairments in contextual freezing in animals experiencing differing drug states were due to state-dependent generalization deficits. In particular, we suggest that a shift in the interoceptive context between conditioning ‘on drug’ and testing ‘off drug’ (and vice versa) reduced contextual fear. To our knowledge, this is the first time state-dependent drug effects have been reported for the BNST. Not surprisingly, given that the BNST has been predominantly implicated in contextual fear, state dependence was not observed for cued fear (Hammack et al., 2004; Resstel et al., 2008; Sullivan et al., 2004; Zimmerman and Maren, 2011). State-dependent

learning occurs when the learned event is more strongly recalled when training and testing occur in the same “state” (Overton, 1991). The state may refer to endogenous cues that create an interoceptive context resulting from drug consumption or alteration of brain excitation through receptor modulation as opposed to external contextual attributes such as lighting or odor.

The present finding that the BNST is involved in a state-dependent form of contextual fear is somewhat surprising and casts previous work on its role in fear learning and expression in a different light. For example, in Experiment 3, matching of the interoceptive and exteroceptive contexts resulted in the greatest degree of expressed fear, whereas testing under different interoceptive and exteroceptive contexts resulted in the lowest degree of expressed fear. Not surprisingly, animals trained and tested with either the same interoceptive or the same exteroceptive context showed intermediate levels of fear. Differences in freezing of animals tested in the same drug state but different contexts approached significance ($p = 0.06$; Fig. 3B). These results suggest that the interoceptive (e.g., hormonal) contextual representation generated by the BNST may carry as much weight as the exteroceptive representation of spatial context generated by the hippocampus. In other words, the level of activation within the BNST may serve as a critical component to the overall “context” encoded during fear acquisition.

The ability of ALLO to induce state dependence in contextual fear learning is perhaps not surprising when the precedence of GABAR modulators in state-dependent learning is considered. Studies involving active avoidance in the T-maze have shown that barbiturates, such as pentobarbital, and steroids, such as hydroxydione and progesterone, can induce state-dependent learning upon systemic administration (Overton, 1964; Stewart et al., 1967). Passive avoidance has also been shown to be state-dependent in ovariectomized females treated with progesterone (Ebner et al., 1981), which can be metabolized to ALLO, and in intact males treated with diazepam or muscimol (Nakagawa et al., 1993). More recently, Jovasevic et al. (2015) have demonstrated that state-dependent contextual fear learning can be induced by altering interoceptive state with gaboxadol infusion into the hippocampus prior to conditioning, suggesting that activation of extrasynaptic GABARs can “gate” access to contextual fear memory (Holmes and Chen, 2015).

Although the effects we observed of intra-BLA ALLO infusion on acquisition and expression of contextual and cued fear are distinctly different from those observed for the BNST, they are in concordance with previous studies involving pharmacological blockade of synaptic activity within the BLA (Fanselow and Kim, 1994; Harris and Westbrook, 1998; Helmstetter and Bellgowan, 1994; Maren et al., 1996; Maren and Holt, 2004; Muller et al., 1997). They are also consistent with ALLO acting to increase GABAergic tone and inhibit activity within the BLA. Helmstetter and Bellgowan (1994) found that infusions of muscimol (prior to conditioning or testing) produce deficits in the expression of conditioned contextual fear (cued fear was not examined), suggesting that essential neural activity within the BLA is required for both acquisition and expression of conditioned contextual fear. Similarly, Maren and Holt (2004) reported deficits in both conditioned contextual and cued fear with pre-conditioning and pre-testing infusion of muscimol into the BLA. Such effects of pharmacological suppression of synaptic transmission are not limited to GABAR agonists

but have also been observed with GABAR potentiators such as midazolam, a benzodiazepine (Harris and Westbrook, 1998). The effects of ALLO infusion into the BLA are also very similar to the effects of APV infusion into the BLA, except that ALLO did not attenuate freezing during acquisition of contextual fear whereas APV significantly decreased freezing during conditioning (Maren et al., 1996) and during testing (Fanselow and Kim, 1994; Maren et al., 1996). The effects of APV were not due to state-dependent decrements in generalization, were evident when infusions were given before conditioning, testing, or both, and did not affect consolidation. Thus, the Maren et al. (1996) study supports the role of NMDA receptors in the acquisition and expression of conditioned contextual fear. Interestingly, the effects we observed of ALLO infusions prior to conditioning and testing on contextual and cued fear look very similar to those seen by Muller et al. (1997). They found that muscimol infusions into the lateral and basal amygdala suppress both context- and cue-dependent freezing when administered before conditioning, testing, or both. When considered along with previous reports, our present findings on the suppressive effects of intra-BLA ALLO on contextual and cued fear suggest that ALLO, like muscimol and APV, is blocking synaptic transmission essential to acquisition and expression of conditioned fear. Given that ALLO was not administered post-training, we cannot directly address whether or not consolidation was affected.

We acknowledge that the effects of ALLO we observed could involve molecular mechanisms other than GABAR potentiation. ALLO may modulate other ionotropic neurotransmitter receptors (5-HT₃ and NMDA; Frye et al., 2014) or act at nonclassical steroid receptors (membrane progesterone and pregnane xenobiotic; Porcu et al., 2016). In addition, local availability of metabolic enzymes could convert ALLO into its precursor, dihydroprogesterone, which acts at intracellular progesterone receptors (Rupprecht et al., 1993), or its 3 β epimer, isoallopregnanolone, which can antagonize ALLO-mediated potentiation of GABARs (Johansson et al., 2016).

5. Conclusions

We have presented evidence that local infusion of ALLO into the BNST generates state-dependent contextual fear in male rats. This finding strongly suggests that the effects of intra-BNST ALLO we recently observed (Nagaya et al., 2015) are due to impaired retrieval of conditioned contextual fear due to ALLO's role as an interoceptive component of the conditioned context. It also suggests that previously reported deficits in contextual freezing following pre-test administration of ALLO and other GABAergic modulators are due to the pharmacologically induced state-dependent nature of contextual fear learning. Additionally, pre-training administration of GABAergic modulators may similarly produce deficits in performance if they are absent during testing. The implications of our work with regards to anxiety-related disorders are that levels of endogenous gonadal steroids and their metabolites may shape CS-US associations during acquisition and serve as an important component to retrieval of conditioned context-specific fear. Consideration of hormonally induced interoceptive state may contribute to more effective treatment of disorders such as PTSD.

Acknowledgments

We thank Christina Fu and Barbara Tsao for their expert assistance in running the experiments. This work was supported by the National Institutes of Health R01 MH065961 (SM), the Heep Graduate Fellowship (GMA), the McKnight Foundation Memory and Cognitive Disorders Award (SM), and the College of Liberal Arts, Texas A&M University (SM, NN).

References

- Anagnostaras SG, Maren S, DeCola JP, Lane NI, Gale GD, Schlinger BA, Fanselow MS. Testicular hormones do not regulate sexually dimorphic Pavlovian fear conditioning or perforant-path long-term potentiation in adult male rats. *Behav Brain Res.* 1998; 92:1–9. [PubMed: 9588680]
- Barha CK, Dalton GL, Galea LM. Low doses of 17 α -estradiol and 17 β -estradiol facilitate, whereas higher doses of estrone and 17 α - and 17 β -estradiol impair, contextual fear conditioning in adult female rats. *Neuropsychopharmacology.* 2010; 35:547–559. [PubMed: 19847162]
- Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA. Pharmacological characterization of a novel cell line expressing human $\alpha 4\beta 3\delta$ GABA_A receptors. *Br J Pharmacol.* 2002; 136:965–974. [PubMed: 12145096]
- Chang YJ, Yang CH, Liang YC, Yeh CM, Huang CC, Hsu KS. Estrogen modulates sexually dimorphic contextual fear extinction in rats through estrogen receptor β . *Hippocampus.* 2009; 19:1142–1150. [PubMed: 19338017]
- Ebner DL, Richardson R, Riccio DC. Ovarian hormones and retention of learned fear in rats. *Behav Neural Biol.* 1981; 33:45–58. [PubMed: 7198905]
- Fanselow MS. Conditional and unconditional components of post-shock freezing. *Pavlov J Biol Sci.* 1980; 15:177–182. [PubMed: 7208128]
- Fanselow MS, Kim JJ. Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist d,l-2-amino-5-phosphonovaleric acid to the basolateral amygdala. *Behav Neurosci.* 1994; 108:210–212. [PubMed: 7910746]
- Frye CA, Koonce CJ, Walf AA. Novel receptor targets for production and action of allopregnanolone in the central nervous system: a focus on pregnane xenobiotic receptor. *Front Cell Neurosci.* 2014; 8:106. [PubMed: 24782710]
- Glover EM, Jovanovic T, Norrholm SD. Estrogen and extinction of fear memories: implications for posttraumatic stress disorder treatment. *Biol Psychiatry.* 2015; 78:178–185. [PubMed: 25796471]
- Graham BM, Daher M. Estradiol and progesterone have opposing roles in the regulation of fear extinction in female rats. *Neuropsychopharmacology.* 2016; 41:774–780. [PubMed: 26156559]
- Gungor NZ, Paré D. CGRP inhibits neurons of the bed nucleus of the stria terminalis: implications for the regulation of fear and anxiety. *J Neurosci.* 2014; 34:60–65. [PubMed: 24381268]
- Gungor NZ, Yamamoto R, Paré D. Optogenetic study of the projections from the bed nucleus of the stria terminalis to the central amygdala. *J Neurophysiol.* 2015; 114:2903–2911. [PubMed: 26400259]
- Gupta RR, Sen S, Diepenhorst LL, Rudick CN, Maren S. Estrogen modulates sexually dimorphic contextual fear conditioning and hippocampal long-term potentiation (LTP) in rats. *Brain Res.* 2001; 888:356–365. [PubMed: 11150498]
- Hammack SE, Richey KJ, Watkins LR, Maier SF. Chemical lesion of the bed nucleus of the stria terminalis blocks the behavioral consequences of uncontrollable stress. *Behav Neurosci.* 2004; 118:443–448. [PubMed: 15113272]
- Harris JA, Westbrook RF. Benzodiazepine-induced amnesia in rats: reinstatement of conditioned performance by noxious stimulation on test. *Behav Neurosci.* 1998; 112:183–192. [PubMed: 9517826]
- Haufler D, Nagy FZ, Paré D. Neuronal correlates of fear conditioning in the bed nucleus of the stria terminalis. *Learn Mem.* 2013; 20:633–641. [PubMed: 24131794]
- Helmstetter FJ, Bellgowan PS. Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. *Behav Neurosci.* 1994; 108:1005–1009. [PubMed: 7826507]

- Holmes A, Chen A. GABA receptors in a state of fear. *Nat Neurosci.* 2015; 18:1194–1196. [PubMed: 26308977]
- Jennings JH, Sparta DR, Stamatakis AM, Ung RL, Pleil KE, Kash TL, Stuber GD. Distinct extended amygdala circuits for divergent motivational states. *Nature.* 2013; 496:224–228. [PubMed: 23515155]
- Johansson M, Strömberg J, Ragagnin R, Doverskog M, Bäckström T. GABA_A receptor modulating steroid antagonists (GAMSA) are functional in vivo. *J Steroid Biochem Mol Biol.* 2016; 160:98–105. [PubMed: 26523675]
- Jovasevic V, Corcoran KA, Leaderbrand K, Yamawaki N, Guedea AL, Chen HJ, Shepherd GMG, Radulovic J. GABAergic mechanisms regulated by miR-33 encode state-dependent fear. *Nat Neurosci.* 2015; 18:1265–1271. [PubMed: 26280760]
- Kim SY, Adhikari A, Lee SY, Marshel JH, Kim CK, Mallory CS, Lo M, Pak S, Mattis J, Lim BK, Malenka RC, Warden MR, Neve R, Tye KM, Deisseroth K. Diverging neural pathways assemble a behavioural state from separable features in anxiety. *Nature.* 2013; 496:219–223. [PubMed: 23515158]
- LeDoux JE, Iwata J, Cicchetti P, Reis DJ. Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *J Neurosci.* 1988; 8:2517–2529. [PubMed: 2854842]
- Lynch JF III, Dejanovic D, Winiecki P, Mulvany J, Ortiz S, Riccio DC, Jasnow AM. Activation of ER β modulates fear generalization through an effect on memory retrieval. *Horm Behav.* 2014; 66:421–429. [PubMed: 25007980]
- Lynch JF III, Vanderhoof T, Winiecki P, Latsko MS, Riccio DC, Jasnow AM. Aromatized testosterone attenuates contextual generalization of fear in male rats. *Horm Behav.* 2016; 84:127–135. [PubMed: 27368147]
- Maeng LY, Milad MR. Sex differences in anxiety disorders: interactions between fear, stress, and gonadal hormones. *Horm Behav.* 2015; 76:106–117. [PubMed: 25888456]
- Majewska MD, Harrison NL, Schwartz RD, Barker JL, Paul SM. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science.* 1986; 232:1004–1007. [PubMed: 2422758]
- Maren S, Holt WG. Hippocampus and Pavlovian fear conditioning in rats: muscimol infusions into the ventral, but not dorsal, hippocampus impair the acquisition of conditional freezing to an auditory conditional stimulus. *Behav Neurosci.* 2004; 118:97–110. [PubMed: 14979786]
- Maren S, Aharonov G, Stote DL, Fanselow MS. *N*-methyl-D-aspartate receptors in the basolateral amygdala are required for both acquisition and expression of conditional fear in rats. *Behav Neurosci.* 1996; 110:1365–1374. [PubMed: 8986338]
- Milad MR, Iggo SA, Lebron-Milad K, Novales JE. Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience.* 2009; 164:887–895. [PubMed: 19761818]
- Milad MR, Zeidan MA, Contero A, Pitman RK, Klibanski A, Rauch SL, Goldstein JM. The influence of gonadal hormones on conditioned fear extinction in healthy humans. *Neuroscience.* 2010; 168:652–658. [PubMed: 20412837]
- Mortensen M, Patel B, Smart TG. GABA potency at GABA_A receptors found in synaptic and extrasynaptic zones. *Front Cell Neurosci.* 2012; 6:1. [PubMed: 22319471]
- Muller J, Corodimas KP, Fridel Z, LeDoux JE. Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. *Behav Neurosci.* 1997; 111:683–691. [PubMed: 9267646]
- Nagaya N, Acca GM, Maren S. Allopregnanolone in the bed nucleus of the stria terminalis modulates contextual fear in rats. *Front Behav Neurosci.* 2015; 9:205. [PubMed: 26300750]
- Nakagawa Y, Iwasaki T, Ishima T, Kimura K. Interaction between benzodiazepine and GABA-A receptors in state-dependent learning. *Life Sci.* 1993; 52:1935–1945. [PubMed: 8389411]
- Overton DA. State-dependent or “dissociated” learning produced with pentobarbital. *J Comp Physiol Psychol.* 1964; 57:3–12. [PubMed: 14125086]
- Overton DA. Historical context of state dependent learning and discriminative drug effects. *Behav Pharmacol.* 1991; 2:253–264. [PubMed: 11224069]

- Pibiri F, Nelson M, Guidotti A, Costa E, Pinna G. Decreased corticolimbic allopregnanolone expression during social isolation enhances contextual fear: a model relevant for posttraumatic stress disorder. *Proc Natl Acad Sci U S A*. 2008; 105:5567–5572. [PubMed: 18391192]
- Pineles SL, Nilni YI, King MW, Patton SC, Bauer MR, Mostoufi SM, Gerber MR, Hauger R, Resick PA, Rasmusson AM, Orr SP. Extinction retention and the menstrual cycle: different associations for women with posttraumatic stress disorder. *J Abnorm Psychol*. 2016; 125:349–355. [PubMed: 26866677]
- Pinna G, Rasmusson AM. Ganaxolone improves behavioral deficits in a mouse model of posttraumatic stress disorder. *Front Cell Neurosci*. 2014; 8:256. [PubMed: 25309317]
- Porcu P, Barron AM, Frye CA, Walf AA, Yang SY, He XY, Morrow AL, Panzica GC, Melcangi RC. Neurosteroidogenesis today: novel targets for neuroactive steroid synthesis and action and their relevance for translational research. *J Neuroendocrinol*. 2016; 28:12351. [PubMed: 26681259]
- Resstel LBM, Alves FHF, Reis DG, Crestani CC, Corrêa FMA, Guimarães FS. Anxiolytic-like effects induced by acute reversible inactivation of the bed nucleus of stria terminalis. *Neuroscience*. 2008; 154:869–876. [PubMed: 18479825]
- Rupprecht R, Reul JMHM, Trapp T, van Steensel B, Wetzel C, Damm K, Ziegglänsburger W, Holsboer F. Progesterone-receptor mediated effects of neuroactive steroids. *Neuron*. 1993; 11:523–530. [PubMed: 8398145]
- Stewart J, Krebs WH, Kaczender E. State-dependent learning produced with steroids. *Nature*. 1967; 216:1223–1224. [PubMed: 4383824]
- Sullivan GM, Apergis J, Bush DEA, Johnson LR, Hou M, LeDoux JE. Lesions in the bed nucleus of the stria terminalis disrupt corticosterone and freezing responses elicited by a contextual but not by a specific cue-conditioned fear stimulus. *Neuroscience*. 2004; 128:7–14. [PubMed: 15450349]
- Swanson, LW. *Brain Maps: Structure of the Rat Brain*. third. Academic Press; San Diego: 2003.
- Toufexis DJ, Davis C, Hammond A, Davis M. Progesterone attenuates corticotropin-releasing factor-enhanced but not fear-potentiated startle via the activity of its neuroactive metabolite, allopregnanolone. *J Neurosci*. 2004; 24:10280–10287. [PubMed: 15537900]
- Toufexis D, Davis C, Hammond A, Davis M. Sex differences in hormonal modulation of anxiety measured with light-enhanced startle: possible role for arginine vasopressin in the male. *J Neurosci*. 2005; 25:9010–9016. [PubMed: 16192392]
- Waddell J, Morris RW, Bouton ME. Effects of bed nucleus of the stria terminalis lesions on conditioned anxiety: aversive conditioning with long-duration conditional stimuli and reinstatement of extinguished fear. *Behav Neurosci*. 2006; 120:324–336. [PubMed: 16719697]
- Zeidan MA, Igoe SA, Linnman C, Vitalo A, Levine JB, Klibanski A, Goldstein JM, Milad MR. Estradiol modulates medial prefrontal cortex and amygdala activity during fear extinction in women and female rats. *Biol Psychiatry*. 2011; 70:920–927. [PubMed: 21762880]
- Zimmerman JM, Maren S. The bed nucleus of the stria terminalis is required for the expression of contextual but not auditory freezing in rats with basolateral amygdala lesions. *Neurobiol Learn Mem*. 2011; 95:199–205. [PubMed: 21073972]

Fig. 1. Effects of intra-BNST ALLO infusions on the acquisition and expression of contextual and cued fear. A) Representative thionin-stained coronal section showing cannula placements. B) Schematic coronal sections showing infusion sites for vehicle (VEH) or allopregnanolone (ALLO). Experiment 1 sites are indicated by circles and Experiment 3 sites are indicated by triangles. Coronal brain section images are adapted from Swanson (2003). C) Mean percentage of freezing ($\pm$ SEM) during the conditioning session (data are shown for a 3-min pre-trial period followed by five tone-shock pairings). Freezing was quantified before the first trial (baseline, BL) and during the 1-min period after each trial. D) Mean percentage of freezing ($\pm$ SEM) averaged across the 10-min context test. The drug states for each group are indicated by a label on each bar indicating the drug infused before conditioning followed by the drug infused before testing (V for VEH, A for ALLO). The V/V group differed from the V/A ($p < 0.01$) and A/V ($p < 0.05$) groups. The A/A group differed from the V/A ($p < 0.05$) and A/V ($p < 0.01$) groups. E) Mean percentage of freezing ($\pm$ SEM) averaged across each 1-min period after four tone presentations.

Fig. 2. Effects of intra-BLA ALLO infusions on the acquisition and expression of contextual and cued fear. A) Representative thionin-stained coronal section showing cannula placements. B) Schematic coronal sections showing infusion sites for vehicle (VEH) or allopregnanolone (ALLO). Experiment 2 sites are indicated by circles. Coronal brain section images are adapted from Swanson (2003). C) Mean percentage of freezing ($\pm$ SEM) during the conditioning session (data are shown for a 3-min pre-trial period followed by five tone-shock pairings). Freezing was quantified before the first trial (baseline, BL) and during the 1-min period after each trial. D) Mean percentage of freezing ($\pm$ SEM) averaged across the 10-min context test. The drug states for each group are indicated by a label on each bar indicating the drug infused before conditioning followed by the drug infused before testing (V for VEH, A for ALLO). The V/V group differed from the A/A ($p < 0.01$), V/A ($p < 0.0001$), and A/V ($p < 0.05$) groups. E) Mean percentage of freezing ($\pm$ SEM) averaged across each 1-min period after four tone presentations. The V/V group differed from the A/A ($p < 0.01$), V/A ($p < 0.0001$), and A/V ($p < 0.01$) groups.

Fig. 3. Contribution of intra-BNST ALLO to interoceptive and exteroceptive states. A) Schematic diagram showing the design for Experiment 3. For conditioning, all animals were in the same interoceptive (infused with allopregnanolone; ALLO) and exteroceptive (Context A) states. For context testing, animals were assigned to one of four groups with varying degrees of shift in interoceptive and exteroceptive states: 1) the None group had no shifts, 2) the Context group had a shift in exteroceptive state (infused with ALLO, exposed to Context B), 3) the Drug group had a shift in interoceptive state (infused with vehicle, exposed to Context A), and 4) the Context & drug group had shifts in both states (infused with vehicle, exposed to Context B). B) Mean percentage of freezing ($\pm$ SEM) averaged across the 10-min context test. The None group differed from the Drug ($p < 0.05$) and Context & drug groups ($p < 0.01$).