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Noradrenergic Regulation of central Amygdala in Aversive Pavlovian-to-Instrumental Transfer

Noradrenergic regulation of aversive motivation

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1 **Abstract**

2 The neural mechanisms through which a Pavlovian conditioned stimulus (CS) elicits
3 innate defense responses are well understood. But a Pavlovian CS can also invigorate
4 ongoing instrumental responding, as shown by studies of aversive Pavlovian-to-
5 instrumental transfer (PIT). While the neural circuitry of appetitive PIT has been studied
6 extensively, little is known about the brain mechanisms of aversive PIT. We recently
7 showed the central amygdala (CeA) is essential for aversive PIT. In the current studies,
8 using pharmacology and designer receptors in rodents, we demonstrate that
9 noradrenergic activity negatively regulates PIT via brainstem locus coeruleus (LC)
10 activity and LC projections to CeA. Our results provide evidence for a novel pathway
11 through which response modulation occurs between brainstem neuromodulatory
12 systems and CeA to invigorate adaptive behavior in the face of threat.

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1 **Significance Statement**

2 The results reported herein use Pavlovian-to-instrumental transfer, a test of the
3 motivational value of a CS, to study noradrenergic contributions to aversive motivation.
4 During transfer tests, a shock-paired cue elevates separately trained shock-avoidance
5 responding. Designer receptor excitation of locus coeruleus and its projections to central
6 amygdala prior to transfer testing eliminated the effect of the cue on shock-avoidance
7 behavior. These findings provide the first evidence that noradrenaline negatively
8 regulates this phenomenon in aversive motivation.

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1 **Introduction**

2 Much has been learned about the neural basis of threat processing using Pavlovian
3 threat conditioning (PTC; see LeDoux 2015). In this procedure a neutral conditioned
4 stimulus (CS) paired with a noxious unconditioned stimulus (US) such as footshock
5 comes to elicit conditioned responses (CRs), such as freezing behavior (Blanchard &
6 Blanchard, 1972; Bolles & Fanselow, 1980). In addition to eliciting simple CRs, Pavlovian
7 cues can also motivate or enhance other actions that are associated with the US through
8 prior experience. For example, in Pavlovian-to-instrumental transfer (PIT) a previously
9 shock-paired CS, rather than generating passive freezing CRs, increases the rate of
10 ongoing active footshock avoidance behavior (e.g., two-way shuttling; Campese et al.,
11 2013; also see Rescorla & Lolordo, 1965; Overmier et al., 1971; Weisman & Litner,
12 1969; Overmier & Brackbill, 1977; Patterson & Overmier, 1981). In recent work we have
13 begun to explore the neural basis of aversive PIT. Using brain lesions to disrupt neural
14 activity, we found that the facilitative effect of the aversive CS on avoidance is impaired
15 by damage to specific nuclei of the amygdala (Campese et al., 2014; 2015; see McCue
16 et al., 2014). Thus, damage to the lateral, central or medial nucleus, but not to the basal
17 nucleus, disrupted aversive PIT.

18 In appetitive PIT, dopamine release in the striatum modulates instrumental
19 performance (Corbit & Balleine, 2011; Laurent et al., 2014; Wassum et al., 2011; Ostlund
20 et al., 2011; Shifflet & Balleine, 2011). In contrast, although it is well established that
21 noradrenergic (NE) neuromodulation in the amygdala regulates aversive learning
22 (Johansen, Cain, Ostroff & LeDoux, 2011; Tully & Bolshakov 2010; Murchison et al.,

1 2004; Ferry & Quirarte, 2012; Gertner & Thomas, 2006; Bush et al., 2010; Sears et al.,
2 2014; Schiff et al., 2016), a role for NE in aversive PIT is not known.

3 In order to express aversive PIT, CS-elicited freezing responses must be suppressed
4 and avoidance must be increased. The central nucleus of the amygdala (CeA) has been
5 shown to be involved in both of these processes (Moscarello & LeDoux, 2013; Campese
6 et al., 2013). CeA also receives NE inputs from brainstem locus coeruleus (LC) and
7 expresses NE receptors (Kravets, Reyes, Unterwald, & Van Bockstaele, 2015).
8 Therefore, in the present study we explored the contribution of NE to aversive PIT. We
9 first used systemic pharmacology to show a role for NE in PIT. We then examined the
10 effect of increasing NE release from the LC in the central amygdala on PIT. Terminal
11 manipulations of NE in the CeA were accomplished using designer receptors exclusively
12 activated by designer drugs (DREADDs). These studies suggest that NE release in CeA
13 plays a critical role in CS-elicited behavior and that this neuromodulatory system
14 negatively regulates aversive PIT.

15

16 **Materials and methods**

17 *Subjects.* Forty-eight male Sprague-Dawley rats, purchased from Hilltop Lab Animals
18 (Scottsdale, PA) were used for Experiment 1 and eighteen for Experiment 2. Subjects
19 weighed approximately 275g at the start of experimentation. Rats were housed in
20 standard Plexiglas cages on a 12:12 hour light:dark cycle. Subjects had free access to
21 food and water while in their home cages, which were lined with paper bedding. Animal
22 care and housing met the current standards of the International Association for
23 Assessment and Accreditation of Laboratory Animal Care (AAALAC). The University

1 Animal Welfare Committee (UAWC) at New York University approved all procedures
2 reported herein.

3

4 *Apparatus.* Pavlovian conditioning took place in Context A, a set of standard training
5 chambers manufactured by Coulbourn Instruments (26 X 28 X 20 cm, length X width X
6 height; Whitehall, PA model no H10-11R- TC) with stainless steel grid floors to deliver
7 the footshock US and an 8ohm speaker for the 30-second 5kHz tone CS presentations.
8 Avoidance training and PIT testing took place in Context B, which was a two-way shuttle
9 chamber (50.8 X 25.4 X 30.5 cm, length X width X height; model no H10- 11R-SC) also
10 manufactured by Coulbourn. Stainless steel grid floors similar to those in context A
11 presented the footshock US and had an 8ohm speaker on each side of the chamber to
12 present the tone CS. All chambers were housed in light and sound attenuating shells.
13 Follow up tests for CS-elicited freezing were conducted in Context C. Context C was
14 another set of standard training chambers also manufactured Coulbourn in a different
15 room and made different from Context A by insertion of striped patterns on the plastic
16 walls and peppermint scent (Dr. Bronner's Magic Soaps) in the waste pan. Additionally,
17 the floor was made from thin mesh-wiring.

18

19 *Procedure.* Subjects were trained using the procedure developed in our lab to study
20 aversive PIT (see Campese et al., 2013). The studies included three main phases - 1.
21 Pavlovian threat (fear) conditioning (PTC) 2. Unsignaled Sidman active avoidance
22 training (USAA) and 3. Pavlovian-to-instrumental transfer testing (PIT). An additional test
23 was included where the CS was presented outside of the avoidance context (Context B).

1 For Experiment 1, subjects were given intraperitoneal (IP) treatment with propranolol,
2 procaterol or the saline vehicle prior to PIT tests. These pharmacological treatments
3 were also administered prior to the test for CS-elicited freezing in Context C. For
4 Experiment 2, following recovery from surgical treatments for viral injections subjects
5 underwent training and testing for PIT as described above. Prior to tests subject were
6 treated with vehicle or clozapine-N-oxide (CNO) IP in a counterbalanced fashion.
7 Subjects were then retrained and implanted with guide cannula targeting the CeA and
8 given further retraining and testing. These final transfer tests were preceded by
9 intracranial drug treatments but otherwise identical to the previous tests. Finally, subjects
10 were tested for CS-elicited freezing in a non-avoidance context (Context C) following IP
11 drug treatments.

12

13 *Pavlovian threat conditioning.* Subjects first received Pavlovian threat conditioning (PTC)
14 in context A where, following a 5 minute baseline, the 30 second tone CS co-terminated
15 with a 1 second duration footshock US (0.7mA). There were three trials separated by a
16 180 second intertrial interval.

17

18 *Unsignaled Sidman active avoidance.* Starting on day two of the study, subjects
19 underwent 15-sessions of unsignaled Sidman active avoidance (USAA) in context B.
20 There was one session per day with four or five sessions per week and each session
21 was 25 minutes in duration. During these sessions, shuttle responses were reinforced by
22 extension of shock free periods. One-second 0.7mA footshocks were delivered every 5
23 seconds unless a shuttle response was performed. Shuttling during the shock terminated

1 the event (i.e., *escape*), while shuttling between shocks (i.e., *avoidance*) produced a
2 shock free interval of 30 seconds. All shuttle responses were accompanied by a 0.3
3 second blinking house light as a feedback cue (for more information see Lazaro-Munoz
4 et al., 2010). Due to a computer error during experiment 1, training data from day 11 of
5 USAA training were not recorded. Therefore, block 4 in the analysis only includes two
6 sessions. Video recording issues resulted in the loss of two subjects' footage for USAA
7 freezing. One of these was from the propranolol group, the other from the procaterol
8 group, though subjects had not yet been treated. Subjects that failed to meet the training
9 criteria were eliminated from the study following day 10 of USAA. For inclusion, a subject
10 was required to reach at least twenty avoidance responses in two consecutive training
11 sessions within the first ten days of this phase (for more information see Lazaro-Munoz
12 et al., 2010).

13

14 *Pavlovian-to-instrumental transfer testing I.* Pavlovian-to-instrumental transfer (PIT)
15 testing took place over two consecutive days in two sessions in context B following
16 completion of USAA training. These tests used parameters established in prior work that
17 produce strong and reliable PIT effects while still maintaining uniform response
18 requirements during transfer (see Campese et al., 2013). In each test, following a fifteen-
19 minute baseline shuttling period the CS was evaluated for its capacity to augment
20 instrumental responding (i.e., shuttling) in a single trial. There were no shocks delivered
21 and shuttle responses still produced the blinking house light feedback cue throughout
22 the test phase, including during the CS trials. The tone was presented to each subject
23 when shuttle response rates fell below two responses per minute for a full two minutes

1 following the mandatory 15-minute baseline. The tone then remained on until ten shuttle
2 responses were performed, at which point the CS terminated and the houselights turned
3 off. The following day this was repeated. For Experiment 1 PIT testing was carried out as
4 described above except that each of these tests were preceded 15-minutes earlier by
5 systemic injections of propranolol, procaterol or the saline vehicle. For Experiment 2, PIT
6 tests were conducted in the same way except for the pre-test drug treatments. Instead of
7 receiving beta-receptor drugs 15-minutes prior to tests, subjects received IP treatment
8 with CNO (2mg/kg; Sigma Aldrich) or the saline vehicle only for the first two tests.
9 Additionally, for Experiment 2, following one week, an additional two tests were
10 conducted with reversed drug assignments. Subjects were matched on USAA
11 performance for assignment into these conditions.

12

13 Retraining (Experiment 2): *Pre/Post-cannulation retraining*. The day following the fourth
14 PIT test for Experiment 2, subjects received a session of PTC retraining identical to the
15 session described above for day one of the study. Over the next two days, subjects then
16 received an additional two USAA training sessions run identically to those described
17 above. These retraining sessions were done 1) to avoid a floor effect for later testing
18 since pilot data we've collected suggests PIT can extinguish over multiple tests and 2) to
19 provide a performance measure against which to compare USAA following cannula
20 implants. Pilot data we've collected suggest that cannula implants in CeA can interfere
21 with USAA acquisition and PIT. In the current study, we compared a third USAA
22 retraining session conducted following recovery from cannula implants to the pre-surgical

1 sessions to confirm that post training implants did not interfere with USAA behavior.

2 Aside from the timing in relation to surgery, these sessions did not differ.

3

4 *Infusions and PIT testing II.* Prior to the tests following the retraining phase, infusions into
5 the CeA were made using a Harvard Apparatus pump (PHD 22/2000). Each hemisphere
6 was infused simultaneously with 0.3uL of CNO (1mg/ml) or the saline vehicle at a rate of
7 0.15uL/min. Each round of testing included two individual test sessions as described
8 above and the two rounds were separated by 1-week to encourage response recovery.
9 Infusions were counterbalanced over these tests such that half of the animals received
10 vehicle and the other half CNO for the first round of these postoperative tests, and the
11 assignments were reversed the following week for the second round. Infusion
12 assignments were made orthogonal to IP drug assignments from the first round of tests.
13 Half of the CNO-vehicle treated subjects over the first round of IP tests received CNO-
14 vehicle treatment during the intracranial infusion tests while the other half received
15 vehicle-CNO treatment. The same was true of animals treated vehicle-CNO during the
16 first round of tests. Otherwise, these tests were as described above.

17

18 *CS-elicited freezing test.* One week after PIT testing in Experiment 1, subjects underwent
19 an additional test session in context C, also preceded by systemic drug treatments using
20 the same assigned groups, where three trials of the CS were presented under extinction
21 conditions and freezing was evaluated in the absence of the shuttle response. These
22 sessions were identical to the PTC sessions, but did not include any footshock. For
23 Experiment 2 this was done later, following intracranial testing and used a slightly

1 different approach. Subjects received two identical test sessions in the standard non-
2 avoidance context C preceded 20-minutes earlier by IP CNO (2mg/kg) or vehicle
3 treatment after both rounds of PIT testing concluded. These assignments were
4 counterbalanced in order to produce within-subjects measure of the effects of CNO and
5 further balanced with regard to earlier assignments across PIT testing.

6
7 *Surgery.* Adeno-Associated Virus (AAV) injections in LC were achieved using
8 procedures previously described (Sears et al., 2013). The viral vector
9 (AAV9.PRSx8.hM3Dq-mCherry.WPRE.rBG) was subcloned and packaged by the
10 University of Pennsylvania Viral Vector Core in AAV2/9. The PRSx8 promoter was used
11 to restrict expression of the HA-tagged hM3Dq DREADD gene to noradrenergic neurons
12 in the LC area (Vazey & Aston-Jones, 2014). Animals were anesthetized using a mixture
13 of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), with supplementation as
14 needed, along with buprenorphine–HCl (0.02 mg/kg, s.c.) for analgesia, and placed in a
15 stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skull was
16 exposed and the LC targeted using the following coordinates (Interaural: - 0.72 AP, ±
17 1.35 ML, - 7.5 DV from skull). AAV (2 uL/side) was delivered via a Hamilton Neuros
18 syringe (5 uL) at a rate of 0.05 uL/min. Following infusion, the syringe was left in place
19 for a minimum of 5 minutes for diffusion of the virus in the tissue. Animals were sutured
20 with dissolvable sutures, and returned to the vivarium where they recovered for 3 weeks
21 before training in PTC. Approximately six weeks separated the surgical phase from the
22 time PIT testing began. For CeA cannula implants subjects were anesthetized as
23 described above and had stainless steel guide cannula implanted above the CeA

1 (Bregma: - 2.8 AP, \pm 4.3 ML, - 7.0 DV), which were fixed to the skull using dental cement
2 and jeweler's screws. Following implantation, subjects recovered for one week prior to
3 retraining and the second rounds of tests. Perfusions were done within two weeks of the
4 end of testing.

5
6 *Perfusions and immunohistochemistry.* Upon completion of the behavioral component of
7 the study, subjects from experiment 2 were perfused for immunohistochemical analysis
8 as described previously (Sears et al., 2013; Ramirez et al., 2015). Briefly, animals were
9 transcardially perfused with room temperature (RT) 4% paraformaldehyde (PFA) in 0.2
10 M phosphate buffer (PB). Tissue was post-fixed 24-72 hours in 4% PFA/0.2 M PB and
11 cut into 40 μ M sections using a vibrating blade microtome (Leica Biosystems, Nussloch,
12 Germany). For assessment of LC infection, CeA cannula placement and axonal
13 DREADD expression in CeA, every 5th section of each structure was processed using a
14 floating immunohistochemistry procedure. Sections were washed with PBS (0.01 M, pH
15 7.4) at room temperature 3 times for 5 minutes between all steps. Sections were
16 incubated for 30 min in 1% bovine serum albumin (Sigma) to block nonspecific binding
17 and then incubated overnight (18 h) in primary antibody. Primary and secondary
18 antibody incubations were made in 1% bovine serum albumin (BSA)/PBS containing
19 0.2% Triton-X 100.

20 For LC sections, tissue was incubated in rabbit polyclonal antibody directed at the
21 hemagglutinin (HA) tag fused to hM4Di (1:250 or 1:500; HA-Tag (C29F4) Rabbit mAb)
22 and mouse anti-dopamine beta-hydroxylase (DBH; (1:2000; clone 4F10.2; EMD
23 Millipore, MA) for 24 hours at RT. Tissue was then incubated for 30 minutes-1 hour at RT

1 in secondary antibodies (1:200; goat anti-mouse Alexa Fluor 488 and 1:200 goat anti-
2 rabbit Alexa Fluor 594; Life Technologies, CA). Sections were mounted on gelatin-
3 subbed slides, and dried briefly (10-20 minutes) in a dark place. Tissue was briefly
4 rehydrated with a few drops of 0.2 M PB saline and coverslipped with 3-4 drops of
5 aqueous mounting media (ProLong Gold Antifade Mountant, Life Technologies, CA).
6 Slides were cured overnight at room temperature and then kept at 4°C before
7 fluorescence imaging and image capture.

8 For assessment of cannula targeting and axon DREADD expression, sections were
9 incubated in rabbit anti-HA tag (1:500; HA-Tag (C29F4) Rabbit mAb, Cell Signaling, MA)
10 at RT overnight. Tissue was then incubated for 30 minutes-1 hour at room temperature in
11 secondary antibodies (1:200 goat anti-rabbit Alexa Fluor 594; Life Technologies, CA).
12 Sections were then mounted, coverslipped and imaged using fluorescent microscopy as
13 described above.

14

15 **Results**

16 **Beta-adrenergic processes constrain aversive PIT**

17 To determine if NE contributes to aversive PIT, subjects were treated systemically with
18 the NE beta-receptor antagonist propranolol, the agonist procaterol, or the saline vehicle
19 prior to transfer tests where the effect of the CS on shuttling was measured. These drugs
20 were also tested (via systemic administration) for effects on CS-elicited freezing in a non-
21 avoidance context after PIT testing had concluded. Based on published findings where
22 NE antagonism impaired memory retrieval in the Morris water-maze task (Murchison et

1 al, 2004) we anticipated that propranolol treatment would also impair PIT and that
2 procaterol would enhance the effect.

3

4 *Pavlovian conditioning and USAA training*

5 Data from the PTC and USAA phases are presented in Figure 1. A repeated-
6 measures analysis of variance (ANOVA) on freezing scores during Pavlovian
7 conditioning confirmed that subjects acquired Pavlovian conditioning normally (effect of
8 *Trial*, $F(2, 94) = 444.59$, $p < 0.001$). No drug treatments were administered during USAA,
9 and groups were matched based on USAA performance for drug assignment during PIT
10 testing. A split-plot repeated-measures ANOVA on acquisition data from this phase
11 (middle panel of Figure 1) similarly confirmed that learning proceeded normally during
12 this phase as well (effect of *Block*, $F(4, 100) = 62.03$, $p < 0.001$). No significant
13 interaction or main effect was found in this analysis.

14 Mean percent time freezing scores to the USAA context during the first 5 minutes of
15 sessions 1, 5, 10 and 15 of USAA are presented in the lower panel of Figure 1. A
16 repeated-measures ANOVA on these data confirmed that context freezing was
17 comparable for each group entering the test phase, (effect of *Session*, $F(3, 60) = 9.63$, p
18 < 0.01 , *Session x Group* interaction, $F(6, 60) = 1.45$, $p = 0.21$, effect of *Group* $F(2, 20) =$
19 0.31 , $p = 0.74$.

20

21

Figure 1 here

22

1 *Effects of a beta-adrenergic receptor antagonist and agonist on PIT and CS-elicited*
2 *freezing*

3 Shuttling data from the PIT test phase are presented in terms of responses per
4 minute during the Pre CS and CS periods for each group (Fig. 2A). Following exclusion
5 of poor performing subjects, final group sizes were eight, nine and nine for groups
6 treated with vehicle, propranolol and procaterol, respectively. A split-plot repeated-
7 measures ANOVA on these data found that PIT strength varied among the groups (effect
8 of *Interval*, $F(1, 23) = 54.33$, $p < 0.01$, *Interval x Group* interaction, $F(2, 23) = 14.71$, $p <$
9 0.01). The main effect of *Group* was also significant, $F(2, 23) = 15.12$, $p < 0.01$. Using
10 post hoc procedures that utilize a pooled error term to increase power for detecting
11 interactive effects (Rodger 1974), PIT (i.e., significant Pre-CS versus CS difference) was
12 observed for vehicle ($F(1,7) = 11.6$, $p < 0.05$) and propranolol ($F(1,8) = 72.5$, $p < 0.05$),
13 but not procaterol treated subjects ($F(1,8) = 0.86$, $p > 0.05$). Direct comparison of vehicle
14 and propranolol CS means revealed a significant effect ($F(2,15) = 8.34$, $p < 0.05$)
15 confirming that while procaterol impaired PIT, propranolol facilitated the transfer effect.

16 Freezing to the CS during PIT tests can be seen in Figure 2B for each group. A one-
17 way ANOVA on these data showed a significant effect of *Group*, $F(2, 23) = 4.97$, $p =$
18 0.017 , and Bonferonni corrected post hoc tests found that procaterol and propranolol
19 treatments did not significantly influence freezing relative to vehicle subjects ($ps > 0.05$).
20 Although freezing during PIT testing was significantly higher in procaterol than
21 propranolol treated subjects ($p < 0.05$), both groups showed statistically equal freezing
22 relative to controls. To provide a more accurate measure of conditioned freezing the CS
23 was also tested outside of the avoidance context. As was the case with transfer testing,

1 this was done following systemic drug treatment (see Figure 2C). Data were averaged
2 over the three test trials and were analyzed with a one-way ANOVA. Freezing
3 significantly differed among the groups, $F(2, 23) = 8.15$, $p = 0.002$, and post hoc tests
4 showed that subjects treated with procaterol displayed higher levels of freezing than all
5 other groups ($p < 0.05$). Vehicle and propranolol treated subjects showed similar levels
6 of freezing to the CS. In summary, these findings suggest that although crucial for
7 expression of conditioned defensive responses (freezing), NE activity must be inhibited
8 during PIT.

9

10

Figure 2 here

11

12 **Activation of locus coeruleus or locus coeruleus cell terminals in central**
13 **amygdala using designer receptors impairs PIT.**

14 Our systemic treatment data suggest that NE plays an inhibitory role in PIT. NE is
15 expressed in the brainstem LC and also plays an important role in aversive processing
16 (Murchison et al, 2004; Ferry & Quirarte, 2012; Gertner & Thomas, 2006). We therefore
17 sought to test the hypothesis that NE from the LC can affect PIT. Gq-coupled hM3Dq
18 DREADDs (Rogan and Roth, 2011) were targeted to NE-expressing LC neurons using
19 the synthetic promoter PRSx8 prior to behavioral training (see Vazey & Aston-Jones
20 2014). In order to activate the designer receptors, clozapine-N-oxide (CNO) was
21 administered before PIT testing, first systemically (IP) and then later, intracranially, into
22 the CeA (see Figure 3A). The latter strategy is possible due to the finding that DREADDs
23 are transported to axon terminals, and can be directly activated with intracranial infusions

1 (Mahler et al., 2014; Stachniak, Ghosh & Sternson, 2014; Zhu & Roth, 2014). Excitation
2 of LC via CNO treatment was expected to produce increased NE release and thus give
3 rise to effects on behavior similar to the agonist procateterol in Experiment 1, ultimately
4 reducing transfer. Local CNO infusions in CeA were expected to eliminate PIT via
5 projection neurons from LC-CeA. CeA was targeted because it is known to be crucial for
6 expression of both PTC and PIT (LeDoux et al., 2000; Campese et al., 2014).

7 A repeated-measures ANOVA on USAA data revealed normal acquisition (effect of
8 *Block*, $F(4, 28) = 7.08$, $p < 0.001$). Eight subjects met the training criteria and proceeded
9 to the test phase of the study. Data from the PIT testing phase are presented in Figure
10 3B below in terms of responses per minute during the Pre CS and CS intervals. These
11 data were analyzed using a repeated-measures ANOVA which found that systemic and
12 intracranial CNO treatment both impaired PIT relative to vehicle treatment using these
13 routes (effect of *Interval*; Pre vs CS: $F(1,7) = 8.94$, $p < 0.05$, *Drug*; Vehicle vs CNO: F
14 $(1,7) = 16.19$, $p < 0.05$, Interaction ($F(1,7) = 14.23$, $p < 0.05$). The main effect of
15 Treatment route (i.e., intracranial versus systemic) was not significant ($F(1,7) = 0.054$, p
16 $= 0.82$) and this factor did not interact with any other factor (*Drug x Route*; $F(1,7) = 0.41$,
17 $p = 0.54$, *Interval x Route*; $F(1,7) = 0.72$, $p = 0.43$, *Drug x Interval x Route*; $F(1,7) = 0.4$,
18 $p = 0.85$). Follow up tests found that the *Drug x Interval* interaction was due to significant
19 differences between the Pre and CS intervals following Vehicle (Pre: $M = 1.48$ 95% CI
20 $[1.11\ 1.79]$; CS: $M = 2.85$ 95%CI $[2.3\ 3.4]$) but not CNO treatment (Pre: $M = 1.36$ 95% CI
21 $[0.95\ 1.76]$; CS: $M = 1.53$ 95%CI $[0.88\ 2.19]$).

22 In the final phase, tests for CS-elicited freezing were conducted outside of the
23 avoidance context following systemic treatment with vehicle and CNO in a

1 counterbalanced manner. These data are presented in terms of percent time freezing to
2 the CS following vehicle and CNO treatment for each subject (Fig. 3E). Analysis of these
3 data found that systemic treatment with CNO significantly increased freezing compared
4 to vehicle treatment ($t(7) = 4.28, p < 0.01$).

5 In order to confirm that CNO alone did not impair PIT behavior, eight non-operated
6 rats received systemic treatment with CNO prior to PIT testing (Figure 3C). A clear
7 facilitative effect on shuttle responding by the CS was found in these subjects ($t(7) =$
8 $2.90, p < 0.05$), indicating CNO alone had not affected PIT. Six additional subjects
9 received CeA cannulations following testing for PIT. After recovery these subjects were
10 tested again with CNO and vehicle infusions into CeA using counterbalanced
11 assignments as described above. Data from the postoperative tests are presented in
12 Figure 3D and show that CNO infusions into CeA had no effect on PIT relative to vehicle
13 infusions in the absence of viral expression. An *Interval* (Pre vs CS) x *Test* (Vehicle 1
14 and 2, CNO 1 and 2) ANOVA found a significant effect of *Interval* ($F(1, 5) = 6.79, p =$
15 0.048) with no effect of *Test* ($F(3, 15) = 0.17, p = 0.92$), and no interaction between the
16 two factors ($F(3, 15) = 0.31, p = 0.82$).

17 Together with the systemic drug treatments, these data suggest that NE from LC
18 plays an inhibitory role in CS-elicited active behaviors. Specifically, excitation of LC-CeA
19 connections eliminates PIT and reinstates freezing, nullifying the impact of avoidance
20 learning on CS-elicited behavior. Therefore, in order to express PIT, NE release in CeA
21 must be blocked.

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Figures 3 & 4 here

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2 **Discussion**

3 The underlying psychological nature of avoidance behavior is not very well
4 understood (see LeDoux et al., 2016). While this form of learning is sensitive to elements
5 of negative reinforcement such as response feedback and shock omission (Kamin 1956),
6 other non-reinforcement based accounts have been proposed. For example, avoidance
7 may reflect a special class of unconditioned Pavlovian behavior (Bolles 1970; 1972)
8 rather than response contingent learning. However, this unresolved issue should not
9 prevent analysis of the effect of the CS on avoidance behavior. PIT is a robust
10 phenomenon that shows a new and unique function of the CS in aversive motivation.

11 The findings of Experiment 1 demonstrate normal facilitation of avoidance by the
12 aversive CS (i.e., PIT) in control subjects. Relative to this group, treatment with systemic
13 propranolol (beta-receptor antagonist) enhanced PIT while procaterol (beta-receptor
14 agonist) impaired the transfer effect. These findings suggest that NE, acting on beta-
15 adrenergic receptors (BARs), plays an important role in the expression of aversive PIT.
16 While the response feedback cue was presented throughout the test phase this was not
17 likely germane to the effects reported above. Rescorla (1968) has shown that long
18 duration presentations of the feedback cue can suppress avoidance behavior through
19 conditioned inhibitory properties. PIT involves enhanced rates of avoidance responding
20 during the excitatory aversive Pavlovian CS despite continued response contingent
21 feedback cue presentations. Therefore, the effects we report here are likely related to
22 processing the aversive CS and not the feedback stimulus since the modulatory effect on
23 behavior was dependent on the shock-paired tone. It may be that responding at this

1 point in training is not sensitive to feedback (i.e., conditioned inhibition) and is more
2 habitual. Thus, while important for avoidance acquisition, feedback may be of little
3 consequence during transfer. More work would be needed to address this possibility.

4 Despite this effect on shuttling during PIT, freezing to the CS during PIT for both
5 procaterol- and propranol-treated subjects (while different from one another) was
6 comparable to subjects treated with vehicle. However, in a test better geared to measure
7 CS-elicited freezing without interference from the avoidance response, procaterol
8 treatment significantly increased freezing compared to both vehicle and propranolol
9 treated subjects. This result suggests that NE transmission to BARs may also be
10 important for the expression of CS-elicited freezing.

11 Based on findings from Pavlovian conditioning (Bush, Caparosa, Gekker & LeDoux
12 2010) and inhibitory avoidance (Ferry, Parrot, Marien, Lazarus, Cassel & McGaugh,
13 2015) one could expect that freezing might be attenuated by propranolol treatment.
14 Indeed the finding of the Experiment 1 that procaterol suppresses PIT and promotes
15 freezing CRs supports this idea. If NE release related to CS-processing promotes
16 freezing, then antagonizing BARs should reduce freezing and increase avoidance. The
17 current study did not find reduced freezing in propranolol compared to vehicle subjects,
18 but BAR antagonism did enhance PIT. This is likely because control subjects in this
19 study also showed very little freezing to the CS, as is commonly observed in aversive
20 PIT (Campese et al., 2013). Many published reports find that over the course of
21 avoidance training, freezing CRs are attenuated as avoidance behavior emerges
22 (LeDoux et al., 2016). This is true when avoidance training includes an explicit signal
23 (Cain & LeDoux 2007; Choi et al., 2010; Moscarello & LeDoux 2014) as well as in

1 unsigned avoidance learning paradigms such as that used in the current paper where
2 freezing to the context is reduced over training (see figure 1C: Lazaro-Munoz et al.,
3 2010; Campese et al., 2013). However, because CS-elicited freezing was already low for
4 the control group in the current study after avoidance, any potential impact of propranolol
5 treatment on freezing was obscured by this floor effect. In the absence of USAA learning,
6 propranolol treatment may reduce CS-elicited freezing, though this was not directly
7 evaluated in the studies reported here. Nevertheless, the current findings suggest that
8 changes to NE neuromodulation at BARs during CS-processing is an important factor
9 underlying the transition from passive to active defensive responding.

10 The results of Experiment 2 demonstrate that activation of LC using designer
11 receptors impairs aversive PIT. This treatment also increased freezing to the CS in a
12 non-avoidance context. Subjects showed no changes in shuttling response rates during
13 the CS following LC activation via systemic treatment with CNO, but they showed normal
14 facilitation when tested following treatment with vehicle. These findings are in agreement
15 with those from Experiment 1, where systemic procaterol was found to eliminate PIT. LC
16 activation with DREADDs produced a behavioral effect similar to procaterol treatment on
17 both shuttling and freezing. This effect was specific to hM3Dq activation and not due to
18 nonspecific effects of CNO (MacLaren et al., 2016). The non-operated control group
19 treated with CNO prior to transfer showed intact PIT (see figure 3C). Thus, the effects
20 reported herein are likely due to the activity of designer receptors in NE releasing LC
21 neurons and not CNO alone. While some question has arisen about the nature of ligand-
22 receptor interactions with CNO and DREADDs (Gomez et al., 2017) the findings with

1 control subjects further suggest that peripheral effects of the metabolite clozapine were
2 not responsible for the behavioral effects seen in the study above.

3 PIT was also eliminated when CNO was infused directly into the CeA, onto the
4 terminals of hM3Dq-expressing LC cells. These same animals showed significant
5 enhancement of responding by the CS when tested following saline infusions. The
6 parameters used for intracranial infusions were based on previous studies that suggest
7 this volume is restricted to CeA. Therefore, it is not likely that CNO spreading to Basal
8 amygdala (BA), which also receives LC projections, produced this effect. Furthermore,
9 we have demonstrated that BA is not needed for aversive PIT (Campese et al., 2014).
10 Controls for intracranial treatments were included and showed that CNO infusions into
11 CeA do not impair PIT without viral expression. CeA is required for normal PIT
12 (Campese et al., 2014) and general disruption of processing in this region by infusions or
13 cannulations would be expected to impair transfer. PIT was reduced following surgery,
14 but not by CNO infusions into CeA relative to vehicle treatment. It should be noted that
15 these subjects were not given retraining after surgery, as LC-viral subjects had been.
16 Thus, the reduction in transfer seen in controls following surgery also likely reflects
17 extinction of PIT over repeated tests. Overall, the results with these controls suggests
18 that the impairment in PIT seen following CNO infusions in LC-viral subjects was due to
19 the effect of CNO on designer receptors (but see Gomez et al., 2017).

20 This result suggests that that NE transmission between LC and CeA is selectively
21 blocked during PIT. Published findings provide evidence that NE release is involved in
22 aversive memory expression (Tully & Bolshakov 2010; Murchison et al, 2004; Ferry &
23 Quirarte, 2012; Gertner & Thomas, 2006). Thus, a possible change to CS-processing

1 that arises due to USAA training may be suppression of CS-related NE signaling during
2 retrieval. If CS-elicited freezing requires NE, then this would be necessary to release the
3 animal from response competition and permit the activation of downstream structures
4 that control the shuttle response specifically. The source of this regulation is currently
5 unknown. Some studies have found that regulation of the CeA by prefrontal cortex is
6 involved in acquisition of instrumental avoidance (Moscarello & LeDoux, 2013). PFC is
7 also known to inhibit serotonin-related activity in the Dorsal Raphe nuclei (Jankowski &
8 Sesack, 2004; Warden, Selimbeyoglu, Mirzabekov, Lo, Thompson, Kim, Adhikari, Tye,
9 Frank & Deisseroth, 2012), perhaps it has a similar effect on NE release by LC.
10 Therefore, future studies will explore the possibility that this pathway regulates
11 transmission of NE between LC and CeA in some way.

12 It should be noted that in studies of avoidance learning, poor performance results in
13 the exclusion of subjects. The goal of the studies reported here was to identify key neural
14 components of transition from passive to active behavior when faced with a threat.
15 Because poor performers do not make this transition they cannot be studied to identify
16 these neural circuits. However, they can be used to test hypotheses about the underlying
17 mechanisms. For example, Lazaro-Munoz et al., (2010) found that CeA lesions rescued
18 USAA in poor performing subjects, while Choi et al., (2010) found similar effects in
19 signaled avoidance. These findings suggest that excessive CS-elicited activity in CeA
20 may underlie poor performance. Our current findings suggest that NE signaling in this
21 region may be an important part of this, with poor performers possessing high NE levels
22 and good performers having less transmitted to CeA during recall. This possibility should
23 be further explored by testing the ability of propranolol to rescue avoidance in poor

1 performers. Furthermore, because propranolol increased transfer in Experiment 1, this
2 would suggest that the exclusion criteria used here did not result in a sample of subjects
3 with floor levels of NE signaling. If the exclusion criteria had produced this, no significant
4 effects of antagonism should have been observed. This was not the case. While it can
5 be argued that comparable freezing between vehicle and propranolol treated subjects
6 may be evidence of this, studies of avoidance in general, and PIT specifically suggest
7 otherwise. These studies show that good performing subjects have no baseline deficits
8 in conditioned freezing. Indeed, freezing to the avoidance context during USAA training
9 is normal in Experiment 1. What distinguishes poor from good performers is the ability to
10 reduce this freezing and gradually transition to active responding (see Lazaro-Munoz et
11 al., 2010).

12 In summary, these data clearly show that NE is involved in the expression of aversive
13 Pavlovian threat learning, as assessed via PIT. This effect requires inhibition of
14 noradrenergic modulation in CeA by projections from LC. This change to CS processing
15 may arise as a direct result of USAA learning in order to suppress standard freezing CRs
16 to the CS. More work is needed to address this possibility.

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1 **References**

- 2 Blanchard, DC, & Blanchard, RJ (1972). Innate and conditioned reactions to threat in rats with
3 amygdaloid lesions. *Journal of Comparative and Physiological Psychology*, 81(2), 281-
4 290. doi: 10.1037/h0033521
- 5 Bolles, RC (1970). Species-specific defense reactions and avoidance learning. *Psychol.*
6 *Rev.* 77, 32–48. doi: 10.1037/h0028589
- 7 Bolles RC (1972) Reinforcement, expectancy, and learning. *Psychol Rev* 79(5):394–409
- 8 Bolles, RC; Fanselow, MS (1980). A perceptual–defensive–recuperative model of fear
9 and pain. *Behavioral and Brain Sciences*, Vol 3(2), Jun 1980, 291-323.
10 <http://dx.doi.org/10.1017/S0140525X0000491X>
- 11 Bush DE, Caparosa EM, Gekker A, Ledoux J. (2010). Beta-adrenergic receptors in the lateral
12 nucleus of the amygdala contribute to the acquisition but not the consolidation of
13 auditory fear conditioning. *Front Behav Neurosci.* 2010 Oct 26;4:154. doi:
14 10.3389/fnbeh.2010.00154. eCollection 2010.
- 15 Campese, V., McCue, M., Lazaro-Munoz, G., Ledoux, J. E., & Cain, C. K. (2013). Development
16 of an aversive Pavlovian-to-instrumental transfer task in rat. *Frontiers in Behavioral*
17 *Neuroscience*, 7, 176. doi: 10.3389/fnbeh.2013.00176
- 18 Campese, V. D., Kim, J., Lazaro-Munoz, G., Pena, L., LeDoux, J. E., & Cain, C. K. (2014).
19 Lesions of lateral or central amygdala abolish aversive Pavlovian-to-instrumental transfer
20 in rats. *Frontiers in Behavioral Neuroscience*, 8, 161. doi: 10.3389/fnbeh.2014.00161
- 21 Campese VD, Gonzaga R, Moscarello JM, LeDoux JE. (2015). Modulation of
22 instrumental responding by a conditioned threat stimulus requires lateral and

- 1 central amygdala. *Front Behav Neurosci*. 2015 Oct 30;9:293. doi:
2 10.3389/fnbeh.2015.00293. eCollection 2015.
- 3 Cardinal RN, Parkinson JA, Hall J, Everitt BJ. (2002). Emotion and motivation: the role of the
4 amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev*. 2002
5 May;26(3):321-52.
- 6 Corbit, L. H., & Balleine, B. W. (2005). Double dissociation of basolateral and central amygdala
7 lesions on the general and outcome-specific forms of Pavlovian-instrumental transfer.
8 *The Journal of Neuroscience*, 25(4), 962-970. doi: 10.1523/JNEUROSCI.4507-04.2005.
- 9 Corbit, L. H., & Balleine, B. W. (2011). The general and outcome-specific forms of Pavlovian-
10 instrumental transfer are differentially mediated by the nucleus accumbens core and
11 shell. *The Journal of Neuroscience*, 31(33), 11786-11794. doi:
12 10.1523/JNEUROSCI.2711-11.2011
- 13 Ferry B, Parrot S, Marien M, Lazarus C, Cassel JC, McGaugh JL. (2015). Noradrenergic
14 influences in the basolateral amygdala on inhibitory avoidance memory are
15 mediated by an action on α 2-adrenoceptors. *Psychoneuroendocrinology*. 2015
16 Jan;51:68-79. doi: 10.1016/j.psyneuen.2014.09.010. Epub 2014 Sep 19.
- 17 Ferry B, and Quirarte G (2012). Role of Norepinephrine in Modulating Inhibitory
18 Avoidance Memory Storage: Critical Involvement of the Basolateral Amygdala.
19 *The Amygdala: A Discrete Multitasking Manager*, Edition: INTECH, Chapter: 8,
20 Publisher: INTECH, Editors: Barbara Ferry, pp.203-230. DOI: 10.5772/53246
- 21 Gertner MJ, and Thomas SA, (2006). The role of norepinephrine in spatial reference and

- 1 spatial working memory. 08 June 2006. CUREJ: College Undergraduate
2 Research Electronic Journal, University of Pennsylvania,
3 <http://repository.upenn.edu/curej/18>.
- 4 Gomez JL, Bonaventura J, Lesniak, Mathews WB, Sysa-Shah P, Rodriguez LA, Ellis RJ, Richie
5 CT, Harvey BK, Dannals RF, Pomper MG, Bonci A, Michaelides M (2017).
6 Chemogenetics revealed: DREADD occupancy and activation via converted clozapine.
7 Science 04 Aug 2017: Vol. 357, Issue 6350, pp. 503-507 DOI:
8 10.1126/science.aan2475
- 9 Holmes, N. M., Marchand, A. R. & Coutureau, E. (2010). Pavlovian to instrumental transfer: A
10 neurobehavioural perspective. *Neuroscience & Biobehavioral Reviews*, 34(8), 1277-1295.
11 doi: 10.1016/j.neubiorev.2010.03.007
- 12 Jankowski MP, Sesack SR. (2004). Prefrontal cortical projections to the rat dorsal raphe
13 nucleus: ultrastructural features and associations with serotonin and gamma-
14 aminobutyric acid neurons. *J Comp Neurol*. 2004 Jan 19;468(4):518-29.
- 15 Johansen, J. P., Cain, C. K., Ostroff, L. E., & LeDoux, J. E. (2011). Molecular mechanisms of
16 fear learning and memory. *Cell*, 147(3), 509-524. doi: 10.1016/j.cell.2011.10.009
- 17 Kamin, L. J. (1956). The effects of termination of the CS and avoidance of the US on avoidance
18 learning. *Journal of Comparative and Physiological Psychology*, 49(4), 420-424. doi:
19 10.1037/h0088011
- 20 Kravets, J.L., Reyes, B.A.S., Unterwald, E.M. Van Bockstaele E.J. (2015). Direct targeting of
21 peptidergic amygdalar neurons by noradrenergic afferents: linking stress-integrative
22 circuitry. *Brain Struct Funct* 220: 541. doi:10.1007/s00429-013-0674-8
- 23 Lazaro-Munoz, G., LeDoux, J. E., & Cain, C. K. (2010). Sidman instrumental avoidance initially

- 1 depends on lateral and basal amygdala and is constrained by central amygdala-
2 mediated Pavlovian processes. *Biological Psychiatry*, 67(12), 1120-1127. doi:
3 10.1016/j.biopsych.2009.12.002
- 4 Laurent V, Bertran-Gonzalez J, Chieng BC, Balleine BW. (2014). δ -opioid and
5 dopaminergic processes in accumbens shell modulate the cholinergic control of
6 predictive learning and choice. *J Neurosci*. 2014 Jan 22;34(4):1358-69. doi:
7 10.1523/JNEUROSCI.4592-13.2014.
- 8 LeDoux JE, Schiller D, Cain C (2009) Emotional Reaction and Action: From Threat
9 Processing to Goal-Directed Behavior. In: *The Cognitive Neurosciences*, 4th
10 Edition (Gazzaniga MS, ed), pp 905-924. Cambridge: MIT Press.
- 11 LeDoux JE (2015). *Anxious: Using the Brain to Understand and Treat Fear and Anxiety*.
12 Penguin, New York, NY.
- 13 MacLaren DAA, Browne RW, Shaw JK, Radhakrishnan SK, Khare P, España RA, Clark
14 SD, (2016). Clozapine-n-oxide administration produces behavioral effects in Long-
15 Evans rats - implications for designing DREADD experiments. *eneuro* Oct 2016,
16 ENEURO.0219-16.2016; DOI: 10.1523/ENEURO.0219-16.2016
- 17 Mahler SV, Vazey EM, Beckley JT, Keistler CR, McGlinchey EM, Kaufling J, Wilson SP,
18 Deisseroth K, Woodward JJ, Aston-Jones G (2014). Designer receptors show role
19 for ventral pallidum input to ventral tegmental area in cocaine seeking. *Nat*
20 *Neurosci*. Apr;17(4):577-85. doi: 10.1038/nn.3664. Epub 2014 Mar 2.
- 21 Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA. (2004). A distinct
22 role for norepinephrine in memory retrieval. *Cell*. 2004 Apr 2;117(1):131-43.
- 23 Moscarello, J. M., & LeDoux, J. E. (2013). Active avoidance learning requires prefrontal

- 1 suppression of amygdala-mediated defensive reactions. *The Journal of Neuroscience*,
2 33(9), 3815-3823. doi: 10.1523/JNEUROSCI.2596-12.2013
- 3 McCue, M. G., LeDoux, J. E., & Cain, C. K. (2014). Medial amygdala lesions selectively block
4 aversive Pavlovian-instrumental transfer in rats. *Frontiers in Behavioral Neuroscience*, 8,
5 329. doi: 10.3389/fnbeh.2014.00329
- 6 Ostlund SB, Wassum KM, Murphy NP, Balleine BW, Maidment NT. (2011). Extracellular
7 dopamine levels in striatal subregions track shifts in motivation and response cost during
8 instrumental conditioning. *J Neurosci*. 2011 Jan 5;31(1):200-7. doi:
9 10.1523/JNEUROSCI.4759-10.2011.
- 10 Overmier, J. B., & Payne, R. J. (1971). Facilitation of instrumental avoidance learning by prior
11 appetitive Pavlovian conditioning to the cue. *Acta Neurobiologiae Experimentalis*, 31(4),
12 341-349.
- 13 Overmier, J. B., & Brackbill, R. M. (1977). On the independence of stimulus evocation of fear
14 and fear evocation of responses. *Behaviour Research and Therapy*, 15(1), 51-56. doi:
15 10.1016/0005-7967(77)90087-0
- 16 Patterson, J., & Overmier, J. B. (1981). A transfer of control test for contextual associations.
17 *Animal Learning & Behavior*, 9(3), 316-321. doi: 10.3758/Bf03197837
- 18 Rescorla, R. A., & Lolordo, V. M. (1965). Inhibition of avoidance behavior. *Journal of*
19 *Comparative and Physiological Psychology*, 59(3), 406-412. doi: 10.1037/h0022060
- 20 Rescorla, R. A. (1968). Pavlovian conditioned fear in Sidman avoidance learning. *J.*
21 *Comp. Physiol. Psychol.* 65, 55–60. doi: 10.1037/h0025412
- 22 Rodger, R. S. (1974). Multiple contrasts, factors, error rate, and power. *British Journal of*
23 *Mathematical & Statistical Psychology*, 27, 179 - 198.

- 1 Rogan SC, Roth BL. (2011). Remote control of neuronal signaling. *Pharmacol Rev.* 2011
2 Jun;63(2):291-315. doi: 10.1124/pr.110.003020. Epub 2011 Mar 17.
- 3 Shiflett MW, Balleine BW. (2011). Molecular substrates of action control in cortico-striatal
4 circuits. *Prog Neurobiol.* 2011 Sep 15;95(1):1-13. doi:
5 10.1016/j.pneurobio.2011.05.007. Epub 2011 Jun 17.
- 6 Sacco, T. & Sacchetti, B. Role of secondary sensory cortices in emotional memory
7 storage and retrieval in rats. *Science* 329, 649–656 (2010).
- 8 Sengupta A, Winters B, Bagley EE, McNally GP, (2016). Disrupted Prediction Error Links
9 Excessive Amygdala Activation to Excessive Fear. *J Neurosci.* 2016 Jan
10 13;36(2):385-95. doi: 10.1523/JNEUROSCI.3670-15.2016.
- 11 Stachniak, T. J., Ghosh, A. & Sternson, S. M. Chemogenetic synaptic silencing of neural
12 circuits localizes a hypothalamus-midbrain pathway for feeding behavior. *Neuron*
13 **82**, 797-808, doi:10.1016/j.neuron.2014.04.008 (2014).
- 14 Tully K, Bolshakov VY. (2011). Emotional enhancement of memory: how norepinephrine
15 enables synaptic plasticity. *Mol Brain.* 2010 May 13;3:15. doi: 10.1186/1756-
16 6606-3-15.
- 17 Vazey EM, Aston-Jones G (2014). Designer receptor manipulations reveal a role of the
18 locus coeruleus noradrenergic system in isoflurane general anesthesia. *Proc Natl*
19 *Acad Sci U S A.* 2014 Mar 11;111(10):3859-64. doi: 10.1073/pnas.1310025111.
20 Epub 2014 Feb 24.
- 21 Warden MR, Selimbeyoglu A, Mirzabekov JJ, Lo M, Thompson KR, Kim SY, Adhikari A,

- 1 Tye KM, Frank LM, Deisseroth K. (2012). A prefrontal cortex-brainstem neuronal
2 projection that controls response to behavioural challenge. *Nature*. 2012 Dec
3 20;492(7429):428-32. doi: 10.1038/nature11617.
- 4 Wassum KM, Ostlund SB, Balleine BW, Maidment NT. (2011). Differential dependence
5 of Pavlovian incentive motivation and instrumental incentive learning processes
6 on dopamine signaling. *Learn Mem*. 2011 Jun 21;18(7):475-83. doi:
7 10.1101/lm.2229311. Print 2011.
- 8 Weisman, R. G., & Litner, J. S. (1969). Positive conditioned reinforcement of Sidman
9 avoidance behavior in rats. *Journal of Comparative and Physiological Psychology*,
10 68(4), 597-603. doi: 10.1037/h0027682
- 11 Zhu, H. & Roth, B. L. Silencing Synapses with DREADDs. *Neuron* **82**, 723-725,
12 doi:10.1016/j.neuron.2014.05.002 (2014).
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1 **Captions**

2 **Figure 1: Experiment 1 Training Data.** Percent time freezing data to the CS during the
3 PTC phase are presented in the upper panel for each group. Mean avoidance shuttle
4 responses from the USAA phase are presented in the middle panel for 3-session blocks
5 of training for each group. Freezing data to the USAA context for the first 5-minute of
6 sample sessions (1, 5, 10 and 15) are presented in the lower panel for each group in
7 terms of percent time.

8

9 **Figure 2: Experiment 1 Test Data.** A: Experimental design, IP stands for intraperitoneal
10 propranolol, proclaterol or vehicle treatments administered prior to tests. B: Shuttling data
11 during the PIT testing phase. These data are presented in terms of responses per minute
12 for each group. Asterisks refer to statistical significance between the Pre and CS periods
13 while the hash indicates significance between vehicle and propranolol treated subjects
14 during the CS (all at the 0.05 alpha level). C: Freezing during the CS presentations in the
15 PIT testing phase are presented in terms of percent time. The asterisk denotes
16 significant differences in freezing between propranolol and proclaterol treated subjects.
17 D: Freezing during the CS in the follow up test for CS-elicited freezing conducted in the
18 non-avoidance context. The asterisk signifies statistically significant differences in
19 freezing between proclaterol treated subjects and all other groups.

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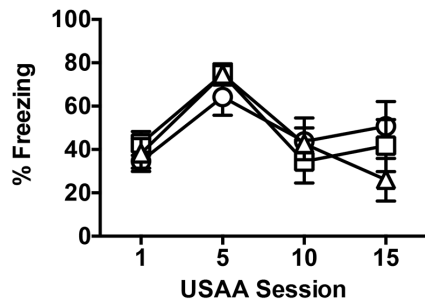
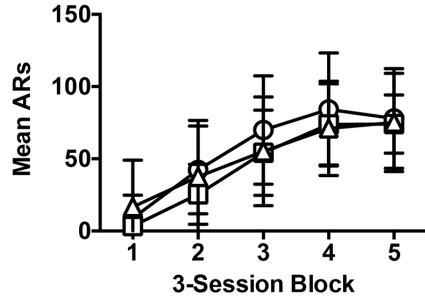
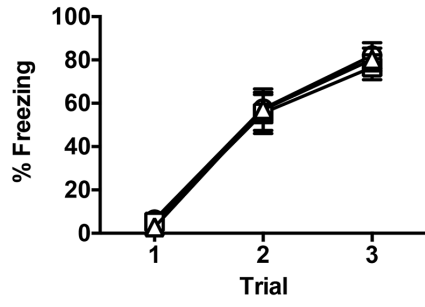
21 **Figure 3: Experiment 2.** A: Design for Experiment 2 training and transfer tests. B: Data
22 from PIT tests preceded by IP and IC CNO/Vehicle treatments are presented as a
23 function of drug in terms of responses per minute during the Pre and CS intervals. C:

1 Data from control subjects that received IP CNO treatment before PIT testing but were
2 not expressing hM3Dq receptors are also presented in terms of responses per minute.
3 D: Postoperative PIT test data from subjects treated with intracranial vehicle and CNO
4 before sessions without hM3Dq expression. E: The data from the follow up tests for CS-
5 elicited freezing are presented in terms of percent time freezing and are also expressed
6 as a function of pre-session drug treatment. Asterisks indicate significance at the 0.05
7 alpha level, and the hash signifies that the Pre CS levels differed pre and post surgery,
8 but only during vehicle tests.

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10 **Figure 4: Experiment 2 Histology.** A: Minimum (red) and maximum (green) viral
11 expression in LC. Values are mm relative to the interaural line. B: Viral expression in LC
12 (HA-tag - red), DBH staining (green) and a merge of the two (yellow; scale bar = 500 μ m).
13 C: Infusion sites for intracranial CNO treatment are presented relative to Bregma (mm).
14 D: Axon terminal expression of DREADD in CeA (HA-tag (red); scale bar for lower
15 magnification = 500 μ m, scale bar for higher magnification = 100 μ m). HA-
16 immunostaining in CeA is indicated by white arrows.

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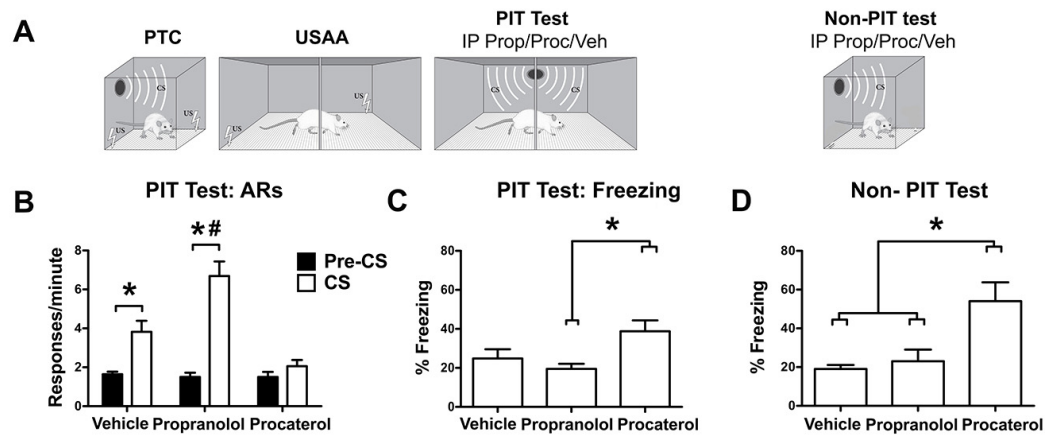
1 **Figure 1**



○ Vehicle □ Propranolol △ Procaterol

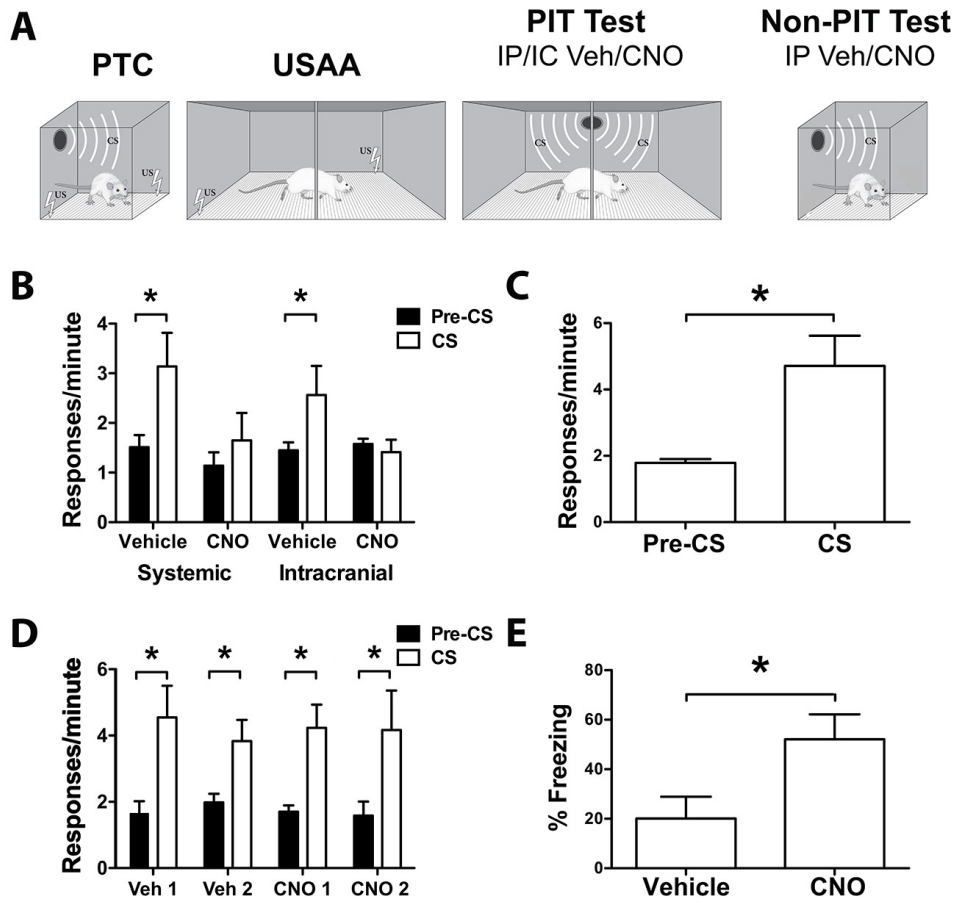
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1 **Figure 2**



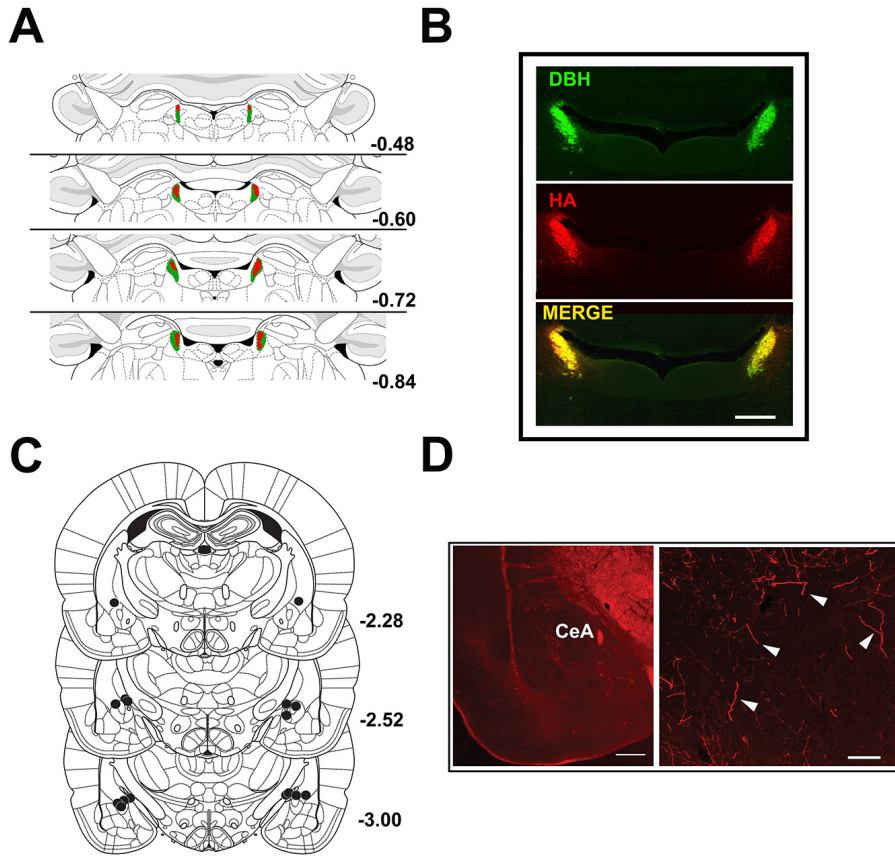
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1 **Figure 3**

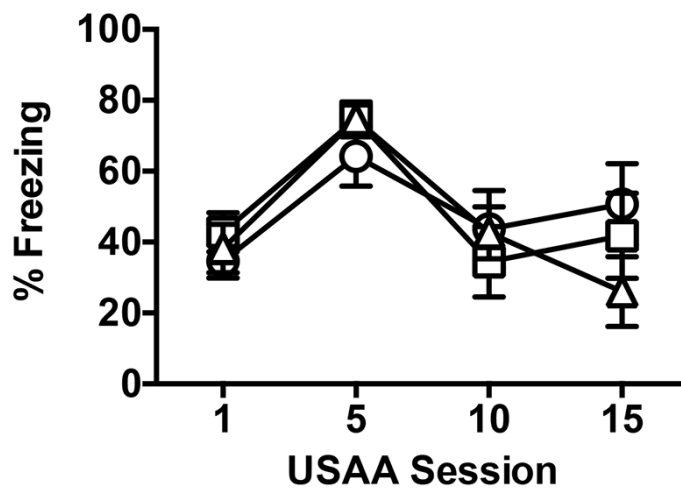
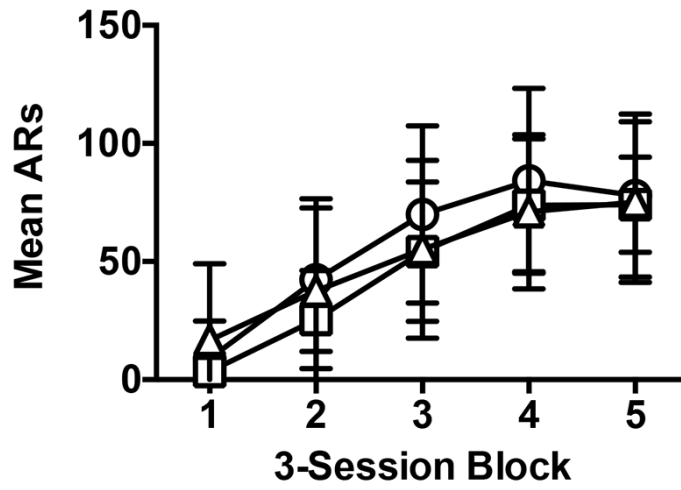
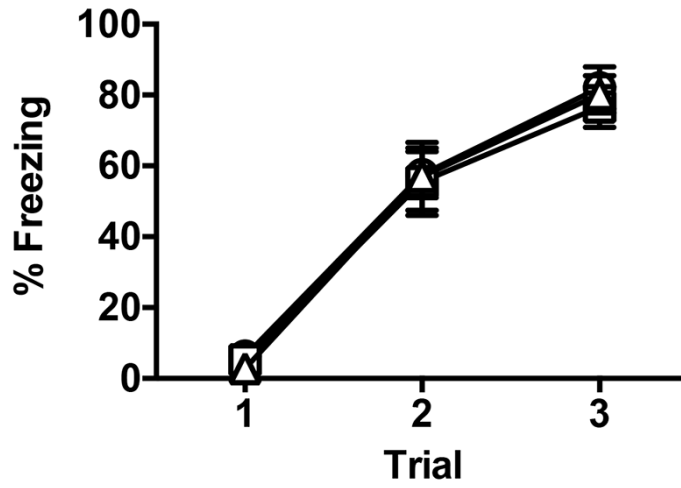


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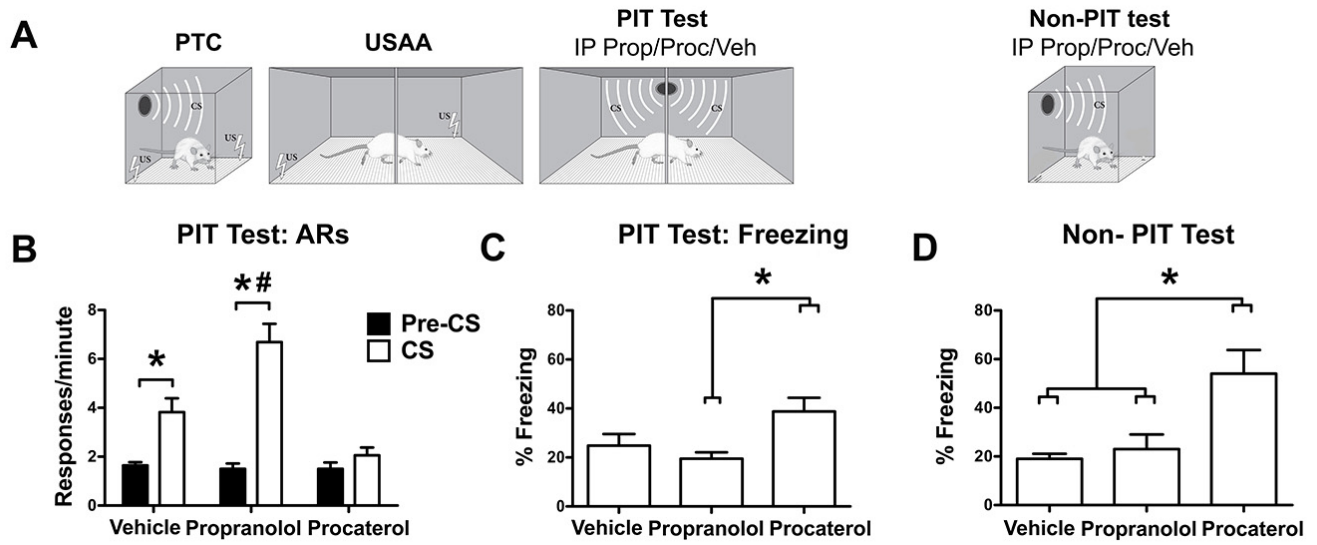
1 **Figure 4**

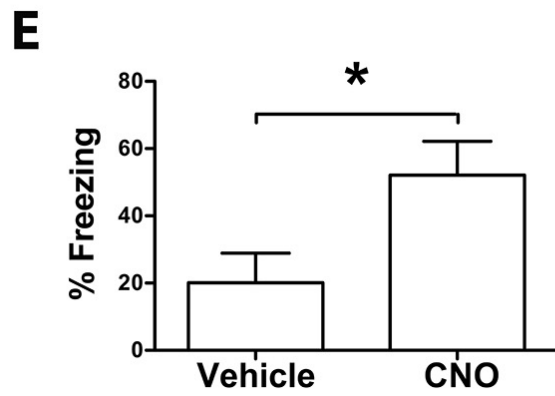
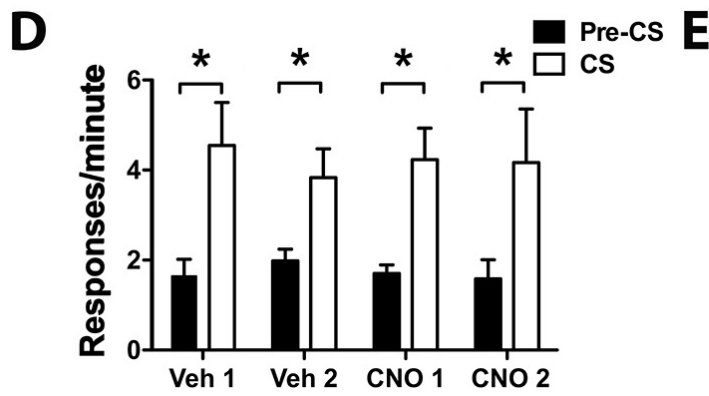
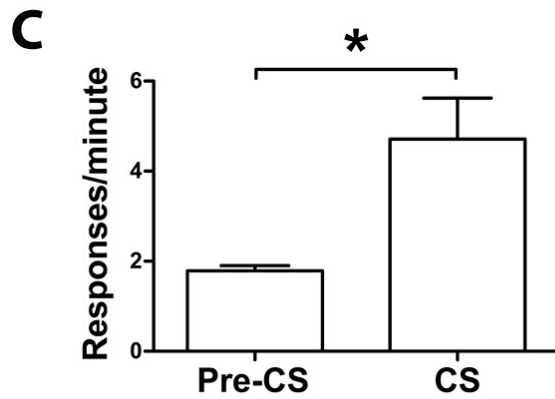
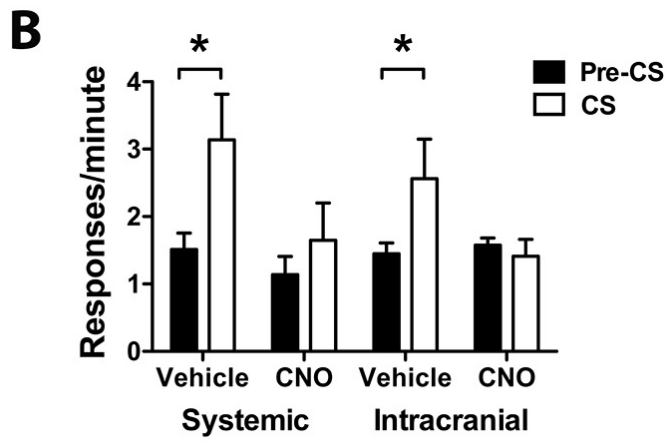
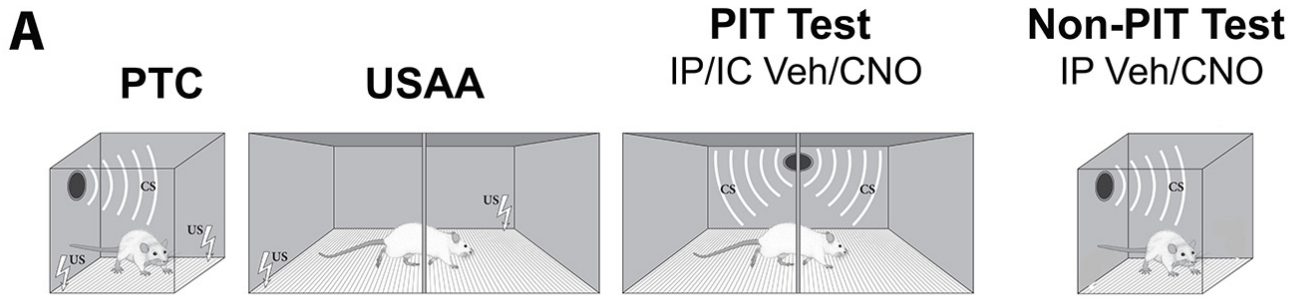


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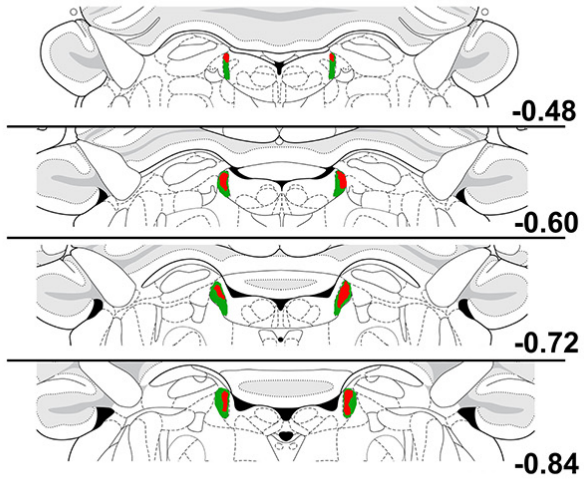


○ Vehicle □ Propranolol △ Procaterol

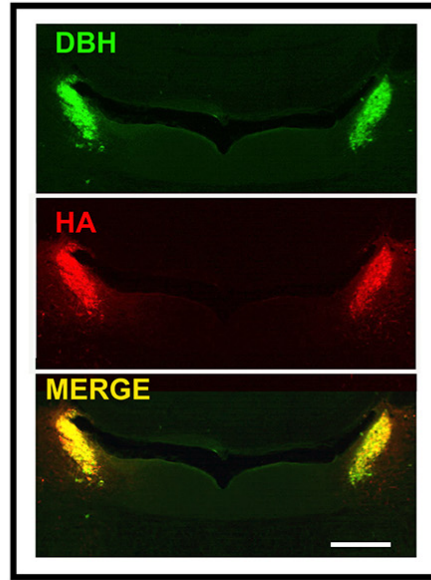




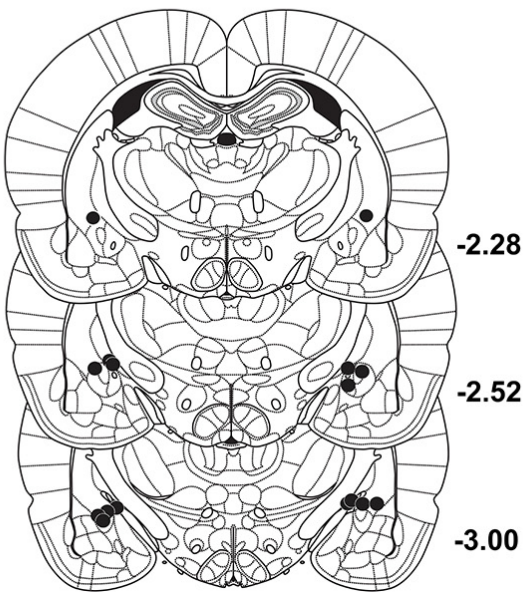
A



B



C



D

