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Neurokinin-1 receptor antagonism attenuates neuronal activity triggered by stress-induced reinstatement of alcohol seeking

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ABSTRACT

Substance P (SP) and its cognate neurokinin-1 receptor (NK1R) are involved in alcohol-related behaviors. We have previously reported that NK1R antagonism attenuates stress-induced reinstatement of alcohol seeking and suppresses escalated alcohol self-administration, but does not affect primary reinforcement or cue-induced reinstatement. Here, we administered an NK1R antagonist or vehicle prior to footshock-induced reinstatement of alcohol seeking, and mapped the resulting neuronal activation using Fos immunohistochemistry. As expected, vehicle treated animals exposed to footshock showed induction of Fos immunoreactivity in several regions of the brain stress circuitry, including the amygdala (AMG), nucleus accumbens (NAC), dorsal raphe nucleus (DR), prefrontal cortex (PFC), and bed nucleus of the stria terminalis (BNST). NK1R antagonism selectively suppressed the stress-induced increase in Fos in the DR and NAC shell. In the DR, Fos-induction by stress largely overlapped with tryptophan hydroxylase (TrpH), indicating activation of serotonergic neurons. Of NAC shell neurons activated during stress-induced reinstatement of alcohol seeking, about 30% co-expressed dynorphin (DYN), while 70% co-expressed enkephalin (ENK). Few (<1%) activated NAC shell neurons coexpressed choline acetyltransferase (ChAT), which labels the cholinergic interneurons of this region. Infusion of the NK1R antagonist L822429 into the NAC shell blocked stress-induced reinstatement of alcohol seeking. In contrast, L822429 infusion into the DR had no effect, suggesting that the influence of NK1R signaling on neuronal activity in the DR is indirect. Taken together, our results outline a potential pathway through which endogenous NK1R activation mediates stress-induced alcohol seeking.

1. Introduction

Addiction is a chronic relapsing disorder, and prevention of relapse is one of the most challenging aspects of its treatment. In patients with alcoholism, relapse is frequently triggered by stressors (Witkiewitz and Marlatt, 2004; Sinha, 2007), but no therapeutics currently available are able to reduce the risk of stress-induced relapse. Reinstatement of drug seeking following extinction has been extensively used to model relapse-like behavior in rodents (for recent review see Bossert et al., 2013), and can be used for discovery and validation of novel therapeutic targets. In the reinstatement model, animals are first trained to self-administer the drug until response rates stabilize, after which drug is removed and operant responding is extinguished. In this model, reinstatement of responding on the previously active lever is induced by stimuli with correlates that trigger relapse in humans including a non-contingent drug injection, presentation of cues previously paired with drug availability, and exposure to stressors such as intermittent footshock.

Using the reinstatement model, we have previously identified the neurokinin 1 receptor (NK1R) as a candidate target for prevention of stress-induced relapse. We showed that L822429, a specific NK1R antagonist with high affinity for the rat receptor (Ebner et al., 2008; Singewald et al., 2008), selectively suppresses stress-induced reinstatement of alcohol seeking as well as escalated self-administration, but does not affect cue-induced

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reinstatement or baseline self-administration (Schank et al., 2011, 2013a, 2013b). However, because these studies used systemic injections of L822429, the neurocircuitry that mediates the effects of NK1R antagonism on stress-induced reinstatement remains unknown.

The immediate-early gene *c-fos* is widely used as a marker of neuronal activation. Using *in situ* hybridization to measure c-fos mRNA, Funk and colleagues previously mapped the neurocircuitry that mediates stress-induced neuronal activity following exposure to stressors that trigger reinstatement of alcohol seeking, namely footshock or yohimbine, and by stressors that fail to reinstate extinguished responding for alcohol (Funk et al., 2006). This study identified a set of structures including the NAC shell, BNST, paraventricular nucleus of the hypothalamus (PVN), locus coeruleus (LC), DR, and the central nucleus of the AMG (CeA) as the core network that is activated by stimuli that also trigger relapse-like behavior. It is presently unknown whether NK1R antagonism blocks stress-induced reinstatement through actions with specific nodes within this network.

Here, we carried out experiments to identify brain regions through which NK1R antagonism may act to prevent relapse-like behavior. First, we used immunohistochemistry (IHC) for Fos protein to identify activated neurons in a set of brain regions known to be influenced by stressors. After identifying stress-responsive brain regions that are sensitive to NK1R antagonism, we applied a double labeling strategy to identify the affected neuronal subtypes within these structures. Second, we used intracranial microinfusion of L822429 to determine if region-specific injections of the antagonist within structures identified by the Fos-mapping study can block stress-induced reinstatement. We report that the NAC shell is a major site of action through which NK1R activation drives stress-induced reinstatement of alcohol seeking.

2. Materials and methods

2.1. Animals

Male Wistar rats (175–225 g, Charles River, Wilmington, MA) were allowed at least 1 week habituation, after which they were handled daily for another week prior to experiments. Rats were housed on reverse light cycle (on 20:30, off 08:30), and testing took place during the dark phase. Food and water were available *ad libitum*, except when stated. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism and of the University of Georgia.

2.2. Stress-induced reinstatement

Reinstatement experiments were carried out as described (Hansson et al., 2006; Cippitelli et al., 2010; Schank et al., 2011). Briefly, animals were trained to acquire stable rates of lever pressing for 10% alcohol (v/v, in water) using a saccharin fading procedure. Alcohol solution was delivered on an FR1 schedule into a drinking receptacle in a 0.1 ml volume during 30 min sessions. Each alcohol delivery was followed by a 5 s timeout interval during which responses were recorded but not reinforced. A cue light was illuminated above the active lever during timeout. The self-administration phase continued for 14–16 days, when response rates had stabilized (<15% variability over 3 days). After this phase, extinction conditions were in place for 15–19 sessions. Thirty minute extinction sessions were run under the same conditions as self-administration, but no alcohol was delivered following active lever presses. At the conclusion of the extinction phase, all rats exhibited less than 20 active lever responses during the 30 min extinction session. In the reinstatement phase, rats (n = 12–19/group) were injected with vehicle or L822429 (30 mg/kg, i.p., dissolved in 2-hydroxypropyl β-cyclodextrin) and 60 min later were exposed to 15 min of intermittent footshock (0.5 s shock, 0.6 mA, mean off time 45 s) immediately before the reinstatement session. Reinstatement responding was then measured under extinction conditions for 30 min. Ninety minutes after the start of the session, rats were removed from the chambers, deeply anesthetized with pentobarbital (100 mg/kg, i.p., dissolved in sterile water), and transcardially perfused with cold saline followed by cold 4% paraformaldehyde. After rats were decapitated, the brains were removed, post-fixed for 2 h in 4% paraformaldehyde and dehydrated in 30% sucrose prior to freezing. Behavioral data from reinstatement experiments were analyzed using repeated measures two-way ANOVA with the between subjects factors of session and drug treatment. Post-hoc group comparisons were performed using Newman–Keuls tests.

2.3. Footshock sensitivity testing

To determine if L822429 treatment could influence shock sensitivity or nociceptive response, rats (n = 4–5/group) were injected with vehicle or L822429 (30 mg/kg, i.p., dissolved in 2-hydroxypropyl β-cyclodextrin) and 60 min later were exposed to footshock, starting at 0.08 mA and increasing by intensities of 0.02 mA (0.5 s shock, 30 s between shocks). The animal's sensitivity to footshock was assessed by recording the lowest voltage at which the animal showed a behavioral response to the shock, including flinching, freezing, or vocalization. A one-way ANOVA, with injection group as the factor, was used to test for differences in footshock sensitivity flinch thresholds.

2.4. Immunohistochemistry

DAB staining: Brains from half of the test animals were sectioned and stained for Fos expression using DAB visualization. Floating sections were first washed 3 times for 5 min in 1 × PBS at room temperature. After 1 h incubation in PBS containing 3% normal goat serum/0.2% Triton-X 100 at room temperature, tissue was incubated for 48 h with rabbit anti-Fos primary antibody (Santa Cruz Biotechnologies, sc-52, 1:4000) at 4 °C. Tissue was then washed in 1 × PBS, and incubated for 2 h in biotinylated goat anti-rabbit secondary antibody (1:600) at room temperature. After this step, tissue was washed in PBS and stained with DAB chromagen using an ABC kit (Vector Labs) according to the manufacturer's instructions. Sections were examined under light microscope and images were taken at 20 × magnification for quantification of Fos-positive cells using Bioquant software (Nashville, TN). Fos immunoreactive cell counts were taken in multiple brain regions including the PFC, NAC, BNST, DR, and AMG.

2.5. Immunofluorescence

Brains from the remaining rats were sectioned and double stained using specific primary antibodies and secondary antibodies linked to Alexaflour fluorophores. Floating sections were first washed in 1 × TBS, then incubated in TBS with 0.2% Triton-X 100 for 20 min. After another round of washes, tissue was incubated with primary antibodies for 48 h at 4 °C. Tissue from the DR was co-incubated with rabbit anti-Fos (Santa Cruz Biotechnologies, sc-52, 1:1000) and sheep anti-TrpH (Millipore, AB144P, 1:2000). For ChAT and Fos colabeling, tissue from the NAC shell was co-incubated with rabbit anti-Fos (1:1000) and goat anti-ChAT (Millipore, AB1541, 1:250). Tissue was then washed and incubated with

secondary antibodies for 2 h at room temperature. Secondary antibodies tagged with fluorophores of different wavelengths (Alexaflour, Invitrogen: donkey anti-goat 555, 1:300; donkey anti-rabbit 488, 1:300 or 1:1000; donkey anti-sheep 568, 1:1000) were used to distinguish primary antibodies on double labeled sections. For Fos and peptide colabeling, NAC shell tissue was co-incubated with goat anti-Fos (1:500, Santa Cruz Biotechnologies, sc-52G) and guinea pig anti-prodynorphin (Neuromics, 1:200) or rabbit anti-preproenkephalin (Neuromics, 1:500). Tissue was then washed and incubated with secondary antibodies for 2 h at room temperature. Secondary antibodies tagged with fluorophores of different wavelengths (Invitrogen: donkey anti-goat 488; donkey anti-goat 633; donkey anti-rabbit 488; Millipore: donkey anti-guinea pig 647; all at 1:500 dilution) were used to distinguish primary antibodies on double labeled sections. After secondary antibody incubation, sections were washed and then coverslipped using vectashield mounting medium, imaged on a Leica confocal microscope at 40 × or 63 × , and cell counts were obtained using Bioquant.

2.6. Intracranial infusion

Rats were trained to self-administer 10% (v/v) alcohol on an FR1 reinforcement schedule with 5 s timeout, as above. After 7 days of baseline self-administration, rats were implanted with bilateral cannulas directed at the NAC or unilateral cannulae directed at the DR. Treatment groups were matched for baseline responses. Rats were implanted with 26 gauge guides (PlasticsOne, Roanoke VA) under isoflurane anesthesia. Coordinates for NAC were AP:+1.7 mm, ML: +/− 2.8 mm, DV: −7.0 mm, 10° angle; injectors extended 1 mm beyond cannula tip for a final DV coordinate of −8.0 mm. Coordinates for the DR were AP: −7.8 mm, ML: +/− 2.5 mm, DV: −6.1 mm, 20° angle; injectors extended 1 mm beyond the cannula tip, for a final DV coordinate of −7.1 mm. Half of the unilateral DR cannulae were implanted through the right hemisphere of the brain and the other half into the left hemisphere. Guides were cemented in place and sealed with an obturator. Following 1 week of recovery, self-administration sessions were restarted and continued for 10 days, until stable response levels were reached and responding was then extinguished, as above.

Rats received intracranial infusions (vehicle or L822429, 7.5 μg in 0.5 μl/side, delivered over 1 min, with the injector left in place for 1 min following infusion) 5–10 min before stress-induced reinstatement sessions (intermittent footshock, 0.5 s shock, 0.6 mA). For Intra-NAC infusion, ondansetron treatment (2 μg in 0.5 μl/side) was also included. After reinstatement, rats underwent extinction sessions for 2–3 days prior to retesting using the opposite treatment. This was continued in a randomized, counterbalanced order until each rat received each treatment infusion (2 test days for DR; 3 test days for NAC shell). After behavioral experiments were completed, cresyl violet was injected, rats decapitated, brains removed, snap frozen on dry ice, and stored at −20 °C. Brains were sectioned through the target regions, and cannula placement was determined by an experimenter blind to treatment. Rats were removed from analysis if they became ill during the course of the experiment or if both cannulae were misplaced.

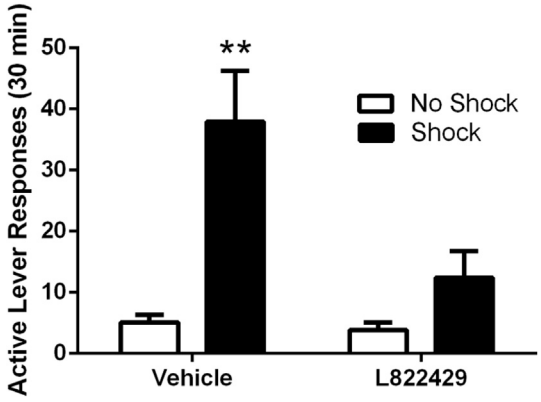

Fig. 1. L822429 attenuates stress-induced reinstatement of alcohol seeking. Footshock exposure induced an increase in active lever responding during the reinstatement test that was blocked by administration of 30 mg/kg L822429. **p < 0.01 compared to all other groups. Main effects of shock ($F(1,55) = 11.7$, $p < 0.01$), pretreatment ($F(1,55) = 4.9$, $p = 0.03$), and interaction ($F(1,55) = 4.0$, $p = 0.05$). N = 12–19/group.

Table 1
Flinch threshold after L822429 or vehicle injection. Rats were injected intraperitoneally with L822429 or vehicle and tested 60 min later. Table shows the footshock intensity at which shock stimulus elicited a behavioral response. L822429 injection had no effect on footshock sensitivity.

Injection	Flinch threshold (mA)	SEM	N
L822429	0.195	0.009	5
Vehicle	0.188	0.005	4

2.7. Statistics

Behavioral data from the reinstatement experiment with systemic L822429 injection and Fos positive cell counts were analyzed using repeated measures two-way ANOVA with the two between subjects factors of session and drug treatment. Post-hoc group comparisons were performed using Newman–Keuls test. Final group sizes for statistical analysis of Fos IHC may differ between brain regions due to tissue loss from specific brain regions during sectioning or staining. Intracranial infusions were analyzed using repeated measures one way ANOVA with the main effect of drug treatment and post-hoc group comparisons were performed using Newman–Keuls test.

2.8. Drugs

L822429 was synthesized by Drs. Cheng and Rice (Chemical Biology Research Branch, NIDA), dissolved in 45% w/v 2-hydroxypropyl β-cyclodextrin (Sigma, St. Louis, MO), and pH was neutralized using 1N NaOH. Systemic injections were in 2 ml/kg, i.p. and intracranial infusions were given at 0.5 μl/side. Ondansetron (Tocris, St. Louis, Missouri) was dissolved in the same vehicle and also infused at 0.5 μl/side.

Table 2
Fos staining after footshock-induced reinstatement. Rats were perfused following reinstatement sessions, brains were removed, sectioned, and stained for Fos protein. Table shows F and p values for each factor in a two way ANOVA analysis. Abbreviations: NAC core, nucleus accumbens core; NAC shell, nucleus accumbens shell; BNST, bed nucleus of the stria terminalis; dPFC, dorsal prefrontal cortex; vPFC, ventral prefrontal cortex; DR, dorsal raphe nucleus; CeA, central nucleus of the amygdala; MeA, medial nucleus of the amygdala; BLA, basolateral nucleus of the amygdala.**p < 0.01, *p < 0.05,#<p < 0.10.

Region	Shock	Drug	Interaction
NAC core	$F = 38.1$, $p < 0.01^{**}$	$F = 2.5$, $p = 0.12$	$F = 2.7$, $p = 0.11$
NAC shell	$F = 5.4$, $p = 0.03^{*}$	$F = 5.4$, $p = 0.03^{*}$	$F = 2.9$, $p = 0.10^{\#}$
BNST	$F = 10.0$, $p < 0.01^{**}$	$F = 1.6$, $p = 0.22$	$F = 1.1$, $p = 0.31$
dPFC	$F = 9.1$, $p < 0.01^{**}$	$F = 3.2$, $p = 0.09^{\#}$	$F = 1.1$, $p = 0.31$
vPFC	$F = 6.7$, $p = 0.02^{*}$	$F = 1.8$, $p = 0.19$	$F = 0.8$, $p = 0.39$
DR	$F = 11.0$, $p < 0.01^{**}$	$F = 5.4$, $p = 0.03^{*}$	$F = 5.2$, $p = 0.03^{*}$
CeA	$F = 11.4$, $p < 0.01^{**}$	$F = 0.6$, $p = 0.46$	$F = 0.3$, $p = 0.60$
MeA	$F = 5.0$, $p = 0.03^{*}$	$F = 0.1$, $p = 0.74$	$F = 0.3$, $p = 0.59$
BLA	$F = 0.9$, $p = 0.35$	$F = 0.5$, $p = 0.48$	$F = 0.9$, $p = 0.35$

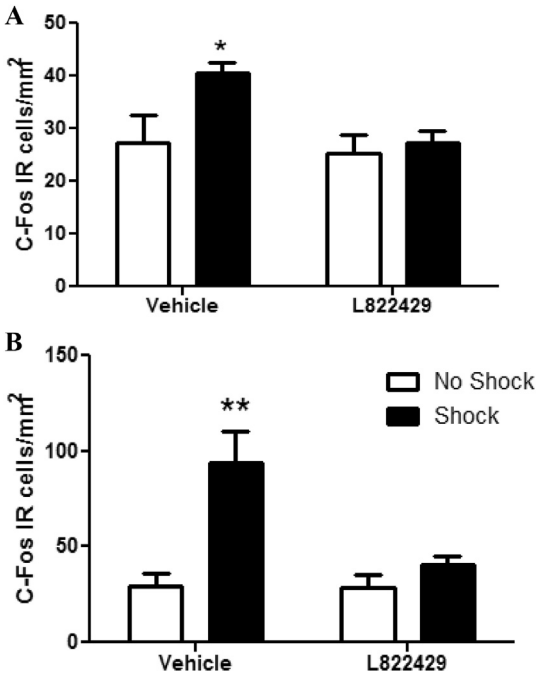

Fig. 2. Fos expression in the NAC shell and DR following stress-induced reinstatement of alcohol seeking. Rats were perfused following reinstatement sessions, brains were removed, sectioned, and stained for Fos protein. There was an increase Fos positive cells in the vehicle-shock group relative to all other groups for both regions. NAC shell shown in (A) and DR shown in (B). *$p < 0.05$; **$p < 0.01$.

3. Results

3.1. Footshock-induced reinstatement

After self-administration training and extinction, rats were exposed to 15 min of intermittent footshock (0.6 mA, 0.5 s duration, mean off time 45 s) prior to a reinstatement session that took place under extinction conditions. Footshock exposure induced an increase in active lever responding during the reinstatement test that was blocked by administration of 30 mg/kg L822429 (Fig. 1). Specifically, two way ANOVA showed main effects of shock ($F(1,55) = 11.7$, $p < 0.01$) and pretreatment ($F(1,55) = 4.9$, $p = 0.03$), as well as a shock × pretreatment interaction ($F(1,55) = 4.0$, $p = 0.05$). Post-hoc testing indicated that the vehicle-shock group showed significantly increased lever pressing when compared to all other groups ($p < 0.01$ for all comparisons).

3.2. Footshock sensitivity testing

There was no effect of injection of L822429 on flinch threshold (Table 1; $F_{(1,8)} = 0.482$, $p = 0.51$), indicating that antagonist treatment did not affect nociceptive sensitivity to footshock. Sensitivity of L822429-treated rats (mean ± SEM; 0.195 ± 0.009 mA) did not differ from flinch threshold of vehicle-treated rats (0.188 ± 0.005 mA).

3.3. Fos expression

Ninety minutes after the start of the reinstatement session, rats (N = 5–9/group) were perfused and brains were collected for Fos IHC. Fos positive cells were counted in a range of brain regions previously shown to mediate stress-induced reinstatement (Table 2). Two way ANOVA for each brain region revealed a main effect of shock in the NAC core ($F(1,24) = 38.1$, $p < 0.001$), NAC shell ($F(1,24) = 5.4$, $p = 0.03$), BNST ($F(1,24) = 10.0$, $p < 0.01$), dorsal PFC ($F(1,23) = 9.1$, $p < 0.01$), ventral PFC ($F(1,23) = 6.7$, $p = 0.02$), DR ($F(1,22) = 11.0$, $p < 0.01$), CeA ($F(1,24) = 11.4$, $p < 0.01$), and the medial nucleus of the AMG (MeA; $F(1,24) = 5.0$, $p = 0.03$), but not the basolateral nucleus of the AMG (BLA; $F(1,24) = 0.9$, $p = 0.35$).

A main effect of L822429 pretreatment was found for two brain regions: the NAC shell ($F(1,24) = 5.4$, $p = 0.03$) and the DR ($F(1,22) = 5.4$, $p = 0.03$). L822429 pretreatment main effect also reached a trend level of significance for the dorsal PFC ($F(1,23) = 3.2$, $p = 0.09$). The drug pretreatment × shock interaction effect reached significance in the DR ($F(1,22) = 5.2$, $p = 0.03$) and was trend-level in the NAC shell ($F(1,24) = 2.9$, $p = 0.10$). Post-hoc comparisons in these two regions indicated a significant increase in

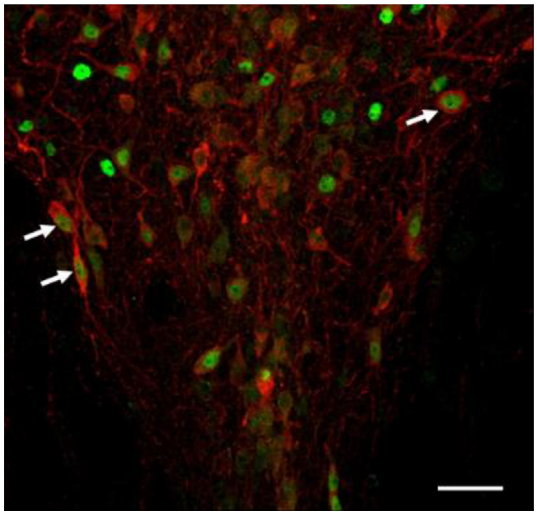

Fig. 3. Fos and TrpH double labeling in the DR. DR sections were labelled for both Fos and TrpH, which marks serotonergic neurons. Confocal microscope image of DR section from vehicle-treated, shock exposed group at 40 × magnification. Fos staining is shown in green and TrpH staining in red. Approximately 43% of Fos positive cells in the vehicle-shock treated group co-labeled for TrpH. Examples of double labeled cells are indicated with white arrows. Scale bar 50 μm.

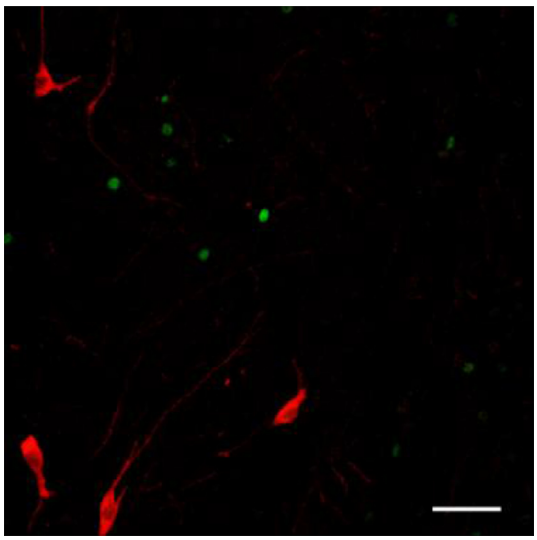

Fig. 4. Fos and ChAT double labeling in the NAC shell. NAC shell sections were labelled for both Fos and ChAT, which marks cholinergic interneurons. Confocal microscope image of NAC shell from vehicle-treated, shock-exposed group at 40 × magnification. Fos staining is shown in green and ChAT staining in red. Less than 1% of Fos positive cells in the vehicle-shock treated group co-labeled for ChAT. Scale bar 50 μm.

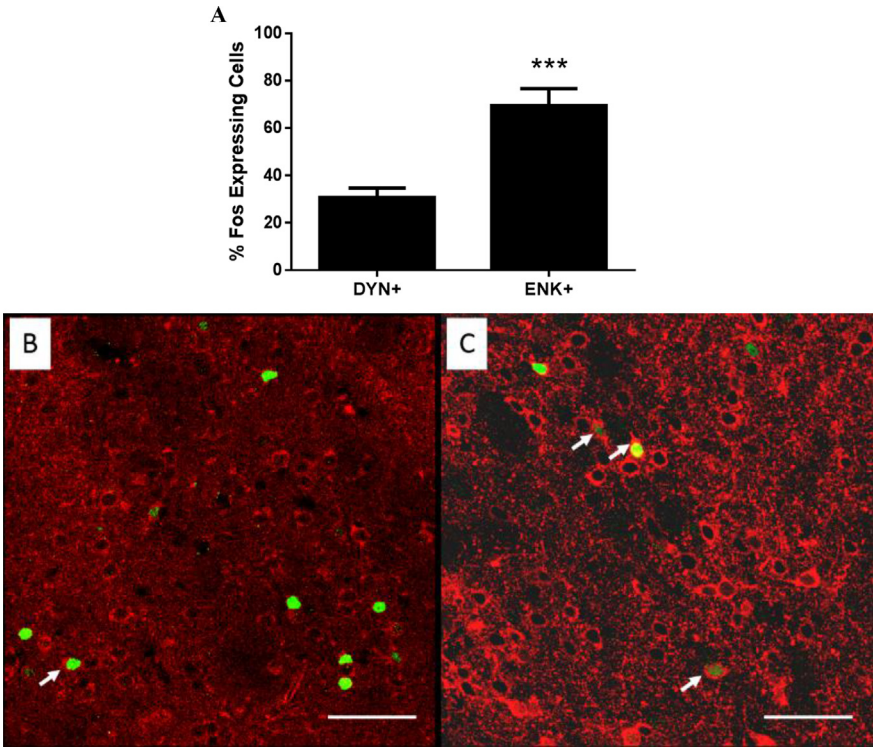

Fig. 5. A majority of NAC shell Fos activation is observed in enkephalinergic MSNs. (A) The percentage of Fos positive cells that co-labeled for PENK was significantly greater than percentage of Fos positive cells co-labeling for PDYN. (B) PDYN and Fos co-labeling is shown in NAC shell from vehicle-treated, shock exposed group at 63 × magnification. PDYN labeling is shown in red and Fos labeling is shown in green. (C) PENK and Fos co-labeling is shown in NAC shell from vehicle-treated, shock-exposed group at 63 × magnification. PENK labeling is shown in red and Fos labeling is shown in green. Examples of double labeled cells are indicated with white arrows. ***$p < 0.001$. Scale bar 50 μm.

Fos expression in the vehicle-shock group compared to all other groups in the NAC shell ($p < 0.05$ for all comparisons; Fig. 2A) and DR ($p < 0.01$ for all comparisons; Fig. 2B).

There was no significant main effect of drug treatment in the NAC core ($F(1,24) = 2.5$, $p = 0.12$), BNST ($F(1,24) = 1.6$, $p = 0.22$), ventral PFC ($F(1,23) = 1.8$, $p = 0.19$), CeA ($F(1,24) = 0.6$, $p = 0.46$), MeA ($F(1,24) = 0.1$, $p = 0.74$), or BLA ($F(1,24) = 0.5$, $p = 0.48$). No significant interaction was detected in the NAC core ($F(1,24) = 2.7$, $p = 0.11$), BNST ($F(1,24) = 1.1$, $p = 0.31$), dorsal PFC ($F(1,23) = 1.1$, $p = 0.31$), ventral PFC ($F(1,23) = 0.8$, $p = 0.39$), CeA ($F(1,24) = 0.3$, $p = 0.60$), MeA ($F(1,24) = 0.3$, $p = 0.59$), or BLA ($F(1,24) = 0.9$, $p = 0.35$).

3.4. Double labeling

Tissue from a subset of rats ($n = 6–10$/group) from the reinstatement experiments were used for double labeling immunofluorescence histochemistry. The objective of these experiments was to assess the activation of specific cell types within the DR and NAC shell. We first focused on two cell types within these regions that are known to be regulated by NK1Rs: the serotonergic projection neurons of the DR, and the cholinergic interneurons of the striatum (of which the NAC shell is a part).

First, DR tissue was double labeled for Fos and the serotonergic marker TrpH. We found that in the vehicle-shock group, which exhibited reinstatement of active lever pressing after footshock exposure, 43% of Fos positive cells co-stained for TrpH (Fig. 3). Next, we double labeled for Fos and the cholinergic marker choline acetyltransferase (ChAT) and found that in the vehicle-shock group, 0.7% of Fos positive cells co-stained for ChAT (Fig. 4).

This lack of effect of NK1R antagonism on cholinergic interneurons of the NAC shell was rather unexpected, given the presence of NK1Rs on these cells. However, it is important to note that these neurons make up a small portion of the cells that are found in the striatum. The vast majority of striatal neurons are GABAergic medium spiny neurons (MSNs) that consist of two major subtypes: 1.) those that express dynorphin (DYN), SP, and the dopamine type-1 receptor (D1R), and 2.) those that express enkephalin (ENK) with the dopamine type-2 receptor (D2R). Therefore, we analyzed NAC shell tissue from the vehicle-shock group for double labeling of Fos with markers for these two subtypes of MSNs: prodynorphin (PDYN) or proenkephalin (PENK), the precursor peptides for DYN and ENK, respectively. We found that, of the Fos positive cells in the NAC shell, 69% co-labeled for PENK and 31% colabeled for PDYN (Fig. 5A–C). While the activation was not exclusively found in PENK positive neurons, this subtype of MSN was preferentially activated. Comparing the percentage of PENK-expressing to PDYN-expressing Fos positive cells using t-test, there was a significant difference ($t(16) = 4.9$, $p < 0.001$).

3.5. Intracranial infusion

In these experiments, rats were implanted with intracranial cannulae directed at the NAC shell or DR and underwent reinstatement testing as above.

First, rats ($n = 17$) implanted with cannulae aimed at the DR were trained to self-administer alcohol, extinguished, and subjected to reinstatement by exposure to 15 min of intermittent footshock, as above. Either vehicle or L822429 was infused directly into the DR prior to footshock exposure and reinstatement responding was compared to responding on the last day of extinction. One way repeated measures ANOVA revealed a main effect of treatment ($F(2,32) = 8.4$, $p = 0.001$; Fig. 6A). Post-hoc comparisons indicated that there was a significant increase in

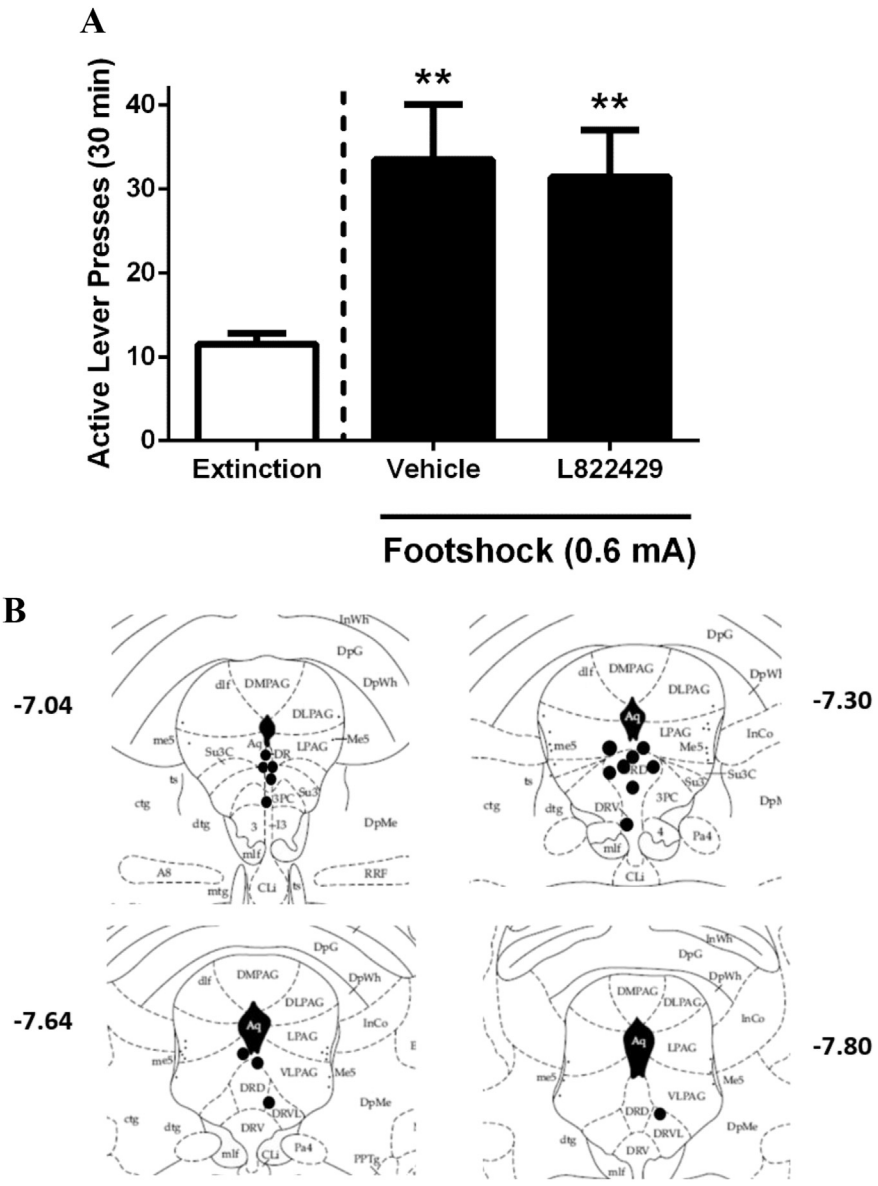

Fig. 6. DR microinfusion of L822429 has no effect on stress-induced reinstatement of alcohol seeking. (A) Footshock stress significantly increased lever pressing relative to extinction responding, indicating a stress-induced reinstatement. L822429 infusion had no effect on footshock-induced reinstatement. Cannula placements shown in (B). **$p < 0.01$ compared to extinction responding.

responding after shock exposure with both vehicle ($p = 0.002$) and L822429 ($p = 0.002$) pretreatment. However, responding following vehicle and L822429 pretreatment did not differ from each other ($p = 0.73$). Cannula placements for DR infusions are shown in Fig. 6B.

Next, we implanted rats ($n = 10$) with cannulae aimed at the NAC shell, and performed the reinstatement experiment as above delivering vehicle, L822429, or the 5HT3 antagonist ondansetron to this region prior to shock exposure and reinstatement testing. One way repeated measures ANOVA revealed a main effect of treatment ($F(3,27) = 8.7$, $p < 0.001$; Fig. 7A). Post-hoc comparisons indicated that there was a significant increase in responding following footshock in the vehicle treated ($p = 0.007$) and ondansetron-treated ($p < 0.001$) groups when compared to extinction level of responding. There was no significant difference between extinction responding and reinstatement with L822429 pretreatment ($p = 0.18$). Post-hoc tests also revealed that there was a trend-level decrease in reinstatement responding between the vehicle and L822429 treated groups ($p = 0.06$). Cannula placements for NAC shell infusion are shown in Fig. 7B.

4. Discussion

The objective of the present experiments was to identify brain regions through which NK1R antagonism suppresses stress-induced relapse to alcohol seeking. First, we replicated our previous findings demonstrating that systemic injection of a NK1R antagonist can attenuate stress-induced reinstatement of alcohol seeking. Control experiments confirmed that this behavioral effect was not due to altered nociceptive sensitivity to shock following NK1R antagonism. Next, we used Fos mapping to identify brain regions where stress-induced neuronal activity is NK1R dependent. This identified two candidate sites, the DR and NAC shell. Double labeling studies with Fos and specific neuronal subtype

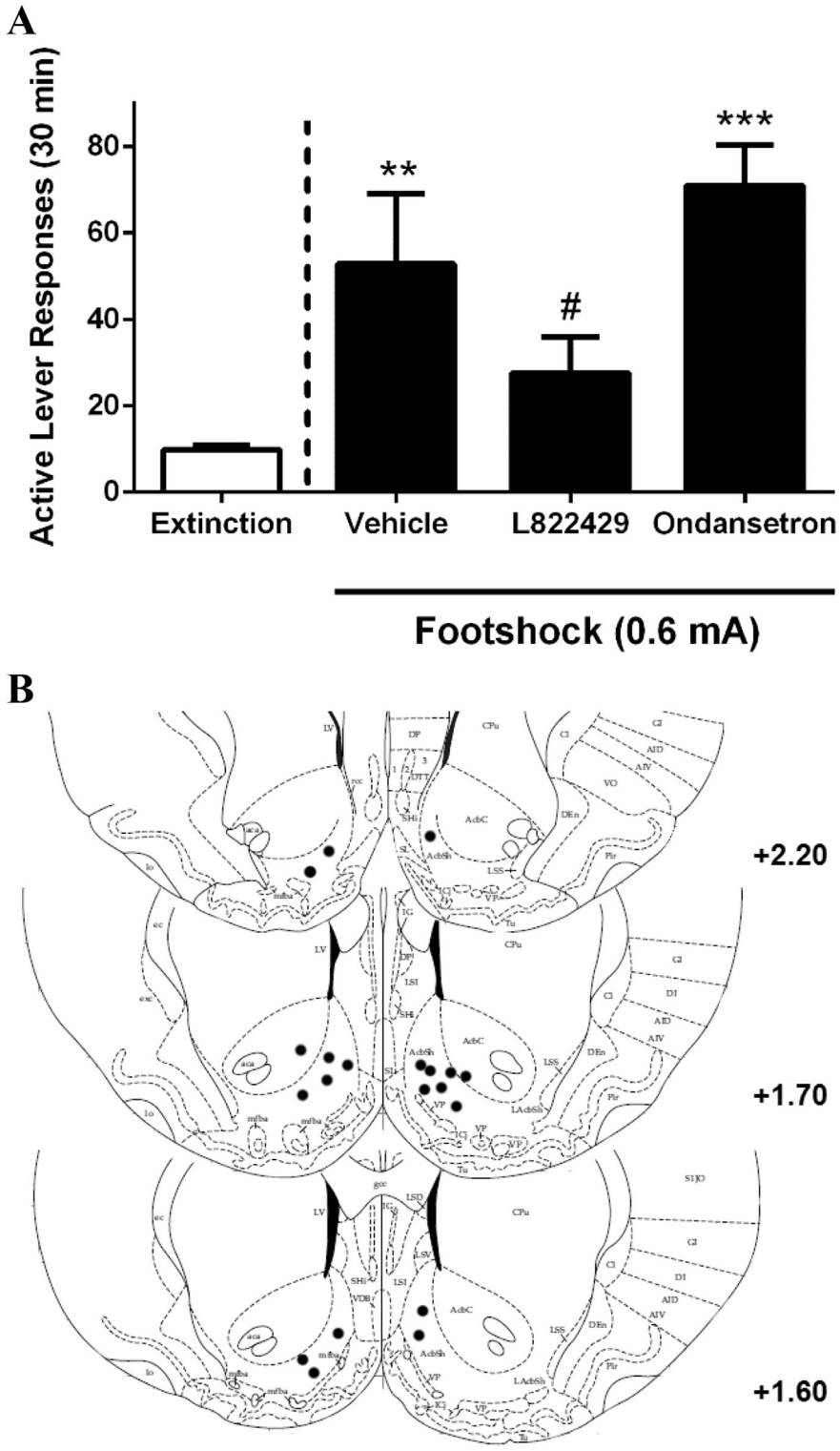

Fig. 7. NAC microinfusion of L822429 attenuates stress-induced reinstatement of alcohol seeking. (A) Footshock stress significantly increased lever pressing relative to extinction responding, indicating a stress-induced reinstatement. While ondansetron infusion had no effect on reinstatement lever responding, L822429 infusion significantly attenuated footshock-induced reinstatement. Cannula placements shown in (B). $^{**}p < 0.01$, $^{***}p < 0.001$ compared to extinction responding. #p = 0.06 compared to reinstatement responding in vehicle treated group.

markers suggested that the serotonergic projection neurons of the DR are involved in the expression of stress-induced relapse, and that enkephalinergic MSNs are preferentially activated in the NAC shell in association with this behavior. Site specific infusion of an NK1R antagonist into the NAC shell suggested that the influence of NK1R in this region is direct, since this treatment blocked the expression of stress-induced reinstatement. In contrast, the involvement of the NK1R in DR during expression of reinstatement is likely to be indirect, since antagonist infusion into this region had no effect.

The NAC shell has long been considered a component of the extended amygdala stress circuitry that mediates stress-elicited drug seeking and escalated self-administration. In terms of reinstatement, it has been proposed that the NAC shell and related structures feed into the "final common pathway" that drives reinstatement behavior (Kalivas and Volkow, 2005). In our dissection of the neuronal subtypes within the NAC shell that mediate the NK1R antagonist effect on stress-induced reinstatement of alcohol seeking, we found that cholinergic interneurons, which have been shown to express NK1Rs, are not likely to be involved in this particular behavior. Instead, both DYN and ENK expressing MSNs of the NAC shell are activated during stress-induced reinstatement, with the ENK subtype being preferentially (but not exclusively) involved. Consistent with our IHC data, direct infusion of L822429 into the NAC shell blocked stress-induced reinstatement. Because both the DR and NAC shell were found to play a role in this behavior, and because the DR sends serotonergic projections to the NAC shell (Vertes, 1991; Waselus et al., 2006; Chang et al., 2011), we tested whether a 5HT3 receptor antagonist, ondansetron, delivered into the NAC shell would prevent reinstatement. We chose to examine ondansetron because it has been suggested that 5HT3 receptors influence alcohol responses (Tomkins et al., 1995; Yoshimoto et al., 1996; Le et al., 2006; Umathe et al., 2009), and ondansetron has shown clinical efficacy in alcohol addiction (Roache et al., 2008; Johnson et al., 2013). However, ondansetron administration into the NAC did not affect stress-induced relapse, suggesting that 5HT3 receptors in the NAC shell do not mediate this behavior. A DR to NAC shell projection could still be involved in the expression of this behavior. Some 5HT projections of the DR co-express SP (Chan-Palay et al., 1978; Magoul et al., 1986; Baker et al., 1991; Sergeyev et al., 1999), and SP release from terminals of these neurons could activate postsynaptic NK1Rs on NAC shell MSNs. Alternatively, the family of 5HT receptors consists of several subtypes, and a subtype other than 5HT3, such as 5HT2C, may contribute to the expression of reinstatement (Yoshimoto et al., 2012). Ultimately, tract tracing and further pharmacological studies will be required to determine the functional role of this pathway in reinstatement of alcohol seeking.

Similar to the effects observed in the NAC shell, Fos activation in the DR was stimulated by footshock-induced reinstatement, and this phenomenon was attenuated by NK1R antagonism. However, intra-DR infusion of L822429 did not suppress stress-induced reinstatement of alcohol seeking. Taken together, these observations suggest the possibility that another NK1R containing region, not examined in the present study, may project to the DR to influence its activity in a NK1R-dependent manner. A candidate region in this context is the locus coeruleus, a major noradrenergic nucleus that contains NK1Rs and sends significant projections to the DR (Peyron et al., 1996; Chen et al., 2000; Ma and Bleasdale, 2002; Kim et al., 2004). Another candidate is the lateral habenula, which contains NK1Rs and influences DR activity (Conley et al., 2002; Bernard and Veh, 2012; Yang et al., 2014; Quina et al., 2015; Zhao et al., 2015). Finally, our data are suggestive of the possibility that a projection from the NAC shell to the DR could play a functional role in the expression of stress-induced relapse, but it is presently unclear if such a projection exists.

5. Conclusions

In summary, our findings replicate earlier work indicating a role of the NK1R in stress-induced reinstatement of alcohol seeking, and extend these findings by identifying brain sites that mediate this effect. We propose that two of the critical regions that are affected by NK1R activity and influence stress-induced reinstatement of alcohol seeking are the NAC shell and DR. Based on site-specific infusions, we hypothesize that the effects of the NK1R in the NAC shell are direct and preferentially influence enkephalinergic MSNs, whereas the influence of the NK1R on the DR in the expression of this specific behavior is indirect.

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