SUPPLEMENTARY METHODS

Behavioral equipment

Operant training and testing was in sixteen identical operant chambers (Med Associates Inc., St Albans, VT, USA; 30.5×29.2×24.1 cm) housed in sound-attenuating cubicles. Each operant chamber was equipped with two retractable levers positioned laterally to a liquid cup receptacle. Locomotor activity was assessed using six identical standard locomotor activity testing chambers (Med Associates Inc., St Albans, VT, USA; 44.5×44.5×30.5 cm).

Alcohol self-administration

32 operant- and drug-naive rats (group 1, see Supplementary Figure 1) were trained to self-administer 20% (v/v) alcohol without sucrose/saccharin fading as described previously (Augier *et al*, 2017; Augier *et al*, 2014). Briefly, rats were first trained on a fixed ratio 1 (FR1) 5 second time-out (TO) schedule to self-administer 20% alcohol during 30 minute sessions. Two levers were extended to mark the onset of the session and to signal alcohol availability. Pressing once on the lever associated with alcohol (active) was reinforced by the delivery of a volume of 100 microliters of 20% alcohol in water in the adjacent drinking well and initiated a concomitant 5 second time-out period signaled by the illumination of the cue-light above the lever. Responses on the inactive lever and during the time-out period were recorded but had no programmed consequences. Sessions were conducted 5 - 6 days a week until stabilization of performance (defined as a minimum of 15 sessions and no change greater than 15 % in the total number of reinforcers earned during the last 3 sessions). A total of 20 FR1 sessions were necessary to reach this criterion. Once a stable self-administration baseline was reached, the fixed ratio was increased and a total of 22 FR2 sessions were performed. Rats were injected with saline during the last 8 FR2 sessions to habituate them to

injections. Starting on day 43, rats were tested in a randomized counterbalanced order in a within-subjects design across the five ADX71441 doses (0, 1, 3, 10 and 30 mg/kg), administered one hour before the respective self-administration session. Between each dosing cycle, rats were allowed to washout the drug with at least three consecutive self-administration sessions. Thus, at the end of the test, all rats had been injected with each of the five doses.

Cue-induced reinstatement

After returning to baseline during 4 consecutive sessions on FR2, responding was extinguished during 17 sessions. During extinction, conditions were identical to baseline self-administration except that active lever presses resulted in neither alcohol delivery nor activation of the light cue. Rats were tested for reinstatement if they decreased their active lever presses to fewer than 15 during the last 3 stabilized sessions of extinction. Two rats didn't achieve this criterion and were excluded from the analysis. For cue-induced reinstatement, a small droplet of alcohol was added on the drinking well associated with alcohol to serve as an odor cue and the test was preceded by 1 minute of intermittent presentation of the cue-light (the light was illuminated for 5 seconds every 20 seconds). During the test itself, active lever presses resulted in contingent illuminations of the cue-light previously paired with drug delivery, but alcohol was not delivered.

Stress-induced reinstatement

Following the cue-induced reinstatement test, responding was re-extinguished during a total of 17 sessions. To prepare for stress-induced reinstatement, rats were habituated for 15 minutes in the self-administration chambers immediately preceding extinction sessions for the last 4 days of extinction. For stress-induced reinstatement, rats received 15 minutes of intermittent footshock (0.5 s shock, 0.6 mA, mean intershock interval 40 s) immediately

preceding the reinstatement test, in accordance with previous experiments conducted in our lab (Augier *et al*, 2016). Two rats were excluded from the analysis for not meeting the extinction criterion.

Progressive ratio schedule of reinforcement

The motivation of the animals to consume alcohol was assessed using a progressive ratio schedule (Hodos, 1961). Conditions were identical to baseline self-administration except that the response requirement to receive a single alcohol reinforcer was increased within-session according to the following formula: 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32…. The self-administration session terminated once 30 minutes had elapsed without a reinforcer. The breakpoint is defined as the last completed response requirement during the progressive ratio test.

Saccharin self-administration

Conditions were similar to alcohol self-administration. Briefly, rats were trained to self-administer 0.2% saccharin in 30 minute sessions under a FR2 5s time-out schedule of reinforcement. A total of 9 FR2 sessions were performed. Once a stable self-administration baseline was reached, rats were tested in a randomized counterbalanced order in a within-subjects design across the four ADX71441 doses (0, 1, 3 and 10 mg/kg), given one hour before the respective self-administration session. Rats were allowed to washout the drug between each dosing cycle with at least three consecutive self-administration sessions.

Locomotor activity

Locomotor activity was measured in a separate group of rats (group 2, n=18) during 30 minute daily sessions to match the length of the self-administration session. Rats were first tested during 3 consecutive sessions until their baseline locomotor activity was established.

They were then tested in a randomized counterbalanced order in a within-subjects design across the five ADX71441 doses (0, 1, 3, 10 and 30 mg/kg), given one hour before the respective session. Between each dosing cycle, rats were allowed to washout the drug with at least two consecutive locomotion sessions.

Brain tissue processing

A separate group of rats (group 4, n=21) was trained on alcohol self-administration, subjected to extinction and stress-induced reinstatement sessions using procedures identical to those described above, and was then used for the c-Fos mapping study. Rats were removed from the chambers 90 minutes after the start of the session, deeply anesthetized with pentobarbital (100 mg/kg i.p. dissolved in sterile water) and transcardially perfused, first with 100 ml saline followed immediately with 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS). Following perfusion, rats were decapitated, brains removed and post-fixed for two hours in 4% PFA followed by dehydration in 30% sucrose in 0.1M PBS for 48 hours at 4°C. Brains were subsequently frozen in powdered dry ice and stored at -80°C until further use. Coronal sections (20 µm) of the medial prefrontal cortex (mPFC), Nucleus Accumbens Shell (NAcSh), Bed Nucleus of Stria Terminalis (BNST), Central Amygdala (CeA), Basolateral Amygdala (BLA) and Dorsal Raphe Nucleus (DRN) were obtained on a cryostat (Leica CM3050), collected in cryoprotectant solution and stored at -20 °C. The brain regions were chosen in accordance with a previous investigation from our lab (Schank *et al*, 2015)

Immunohistochemistry

Free-floating sections were first washed 3 times for 10 min in 1X PBS at room temperature. Tissue was incubated for 1 h in 3% normal goat serum in 0.1 M PBS containing 0.2% Triton X-100 (PBS-Tx) and then incubated for 48 hours at 4°C with anti-c-Fos primary antibody (rabbit c-Fos sc-52, Lot E0514, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:4000 in

1% NGS in PBS-Tx. Sections were then washed in PBS and incubated with a secondary antibody (1:200, goat anti-rabbit vector ABC Elite Kit, PK-6101, Vector Laboratories, Burlingame, CA) for 2 h. Sections were again washed in PBS and incubated in avidin-biotin-peroxidase complex (Elite kit, PK-6101, Vector Laboratories) in PBS for 1 h and washed in PBS.

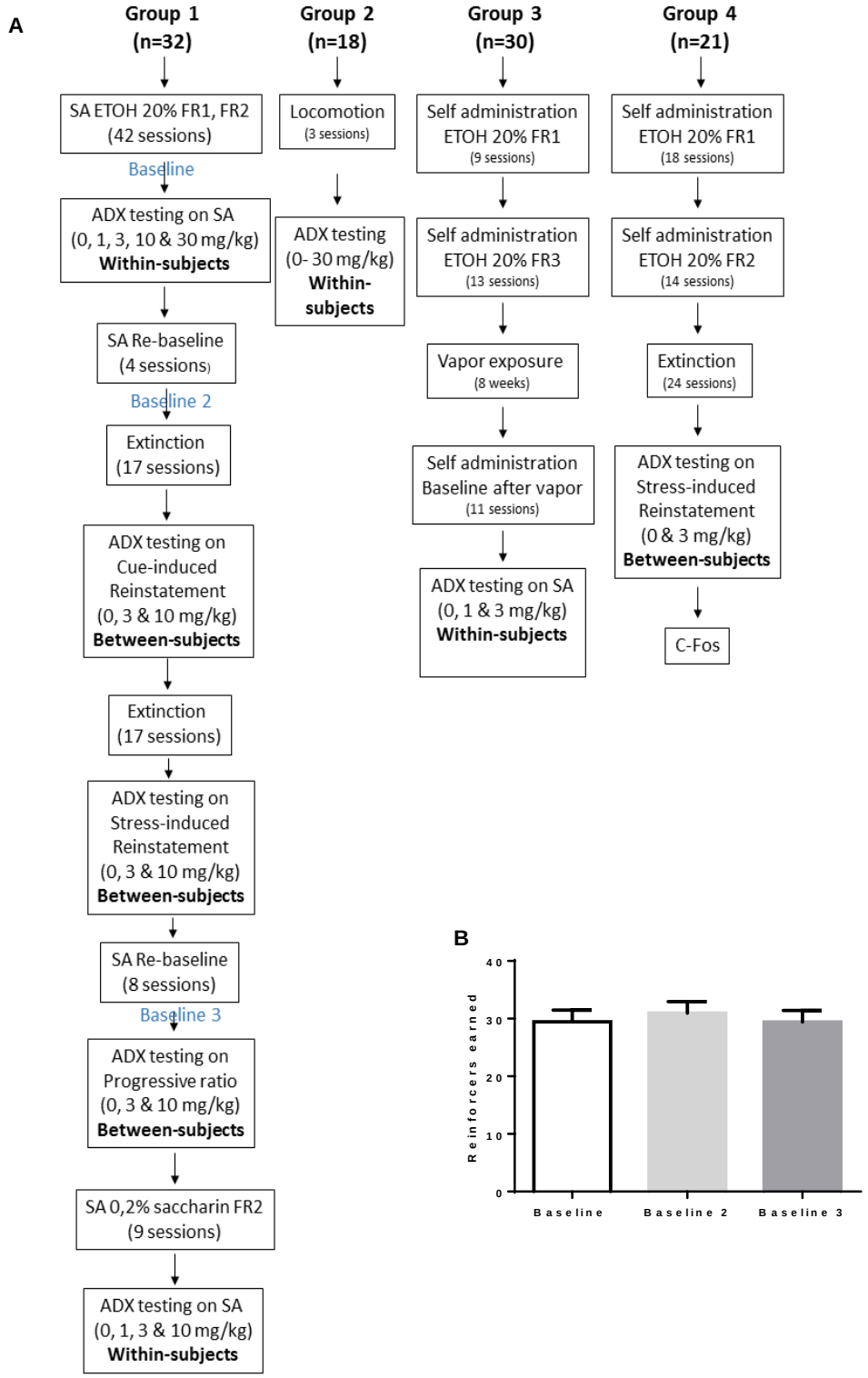
Sections were developed in 3,3'-diaminobenzidine (DAB) for approximately 3 min, washed in PBS, mounted on gelatine-coated slides and air-dried. The tissue was processed the next day through alcohol-xylene for light microscopy examination and coverslipped. All washing and incubation steps were on a shaker.

Quantification of c-Fos immunoreactivity

Images were obtained on a Leica light microscope DM6000CS (Leica Microsystem Inc., Bannockbern, IL, USA) at 40X magnification and BioQuant imaging software (R&M Biometrics, Nashville, TN) was used for cell density analysis. Two coronal sections (two levels), both left and right side, were analyzed for c-Fos positive cells for every region (coordinates: mPFC - Bregma: +3.20 to +2.70; NAcSh – Bregma: +2.20 to +1.20 mm; BNST – Bregma: -0.30 to -0.92 mm; CeA and BLA – Bregma: -1.80 to -2.80 mm and DRN – Bregma: -7.30 to -8.30 mm; (Paxinos and Watson, 1998)). Results are depicted as the nr of positively stained cells per square mm.

<u>Supplementary Fig. 1</u> Experimental timeline.

A: Schematic representation of the timeline of the experiment. Four groups of rats were independently tested in this study as shown above. A detailed description of the training and tests can be found in the methods section **B:** Repeated drug and reinstatement tests did not affect baseline self-administration (group 1, n=32, $F_{2,62}$=0.51, p = 0.60; eta^2=0.016)



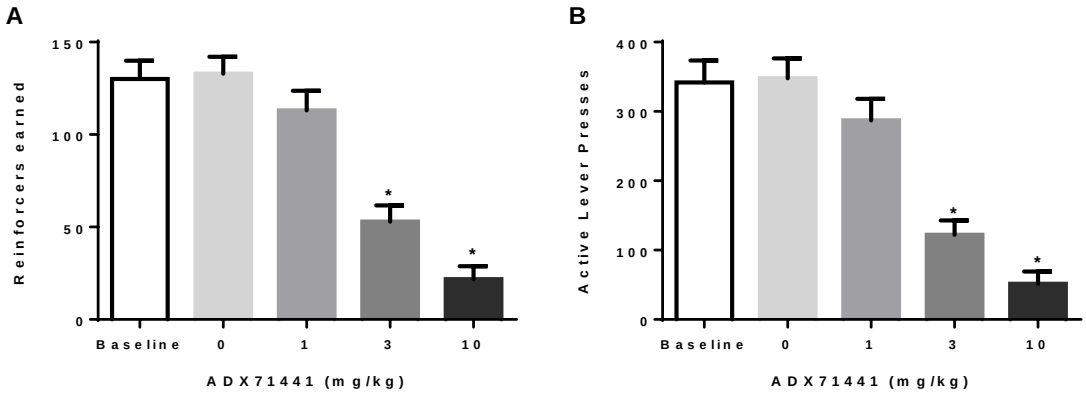
Supplementary Tab. 1 Measurement of Blood Alcohol Concentrations (mg/dl)

Mean BACs (±SEM) after 8 weeks of chronic intermittent alcohol vapor exposure (n=14)

Week	1	2	3	4	5	6	7	8	Mean
BAC (mg/dl)	272.9 ± 31.7	349.5 ± 34.0	196.8 ± 17.1	149.2 ± 20.8	286.2 ± 47.2	190.1 ± 33.4	160.6 ± 33.5	202.9 ± 22.4	232.8 ± 20.3

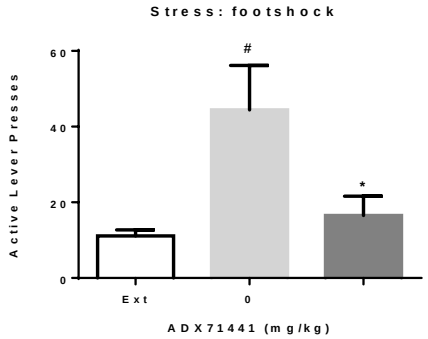
<u>Supplementary Fig. 2</u> ADX71441 decreases 0.2% operant responding for saccharin

A: Mean reinforcers (±SEM) earned during a 30-minute FR2 self-administration session of 0.2% saccharin following either vehicle or ADX71441 treatment (1,3 or 10 mg/kg) (n=32) (* = p<0.001) **B:** Mean active lever presses (±SEM) completed during a 30-minute FR2 self-administration session of 0.2% saccharin following either vehicle or ADX71441 treatment (1,3 or 10 mg/kg) (n=32) (* = p<0.001)



<u>Supplementary Fig. 3</u> ADX71441 attenuates stress-induced

Mean number of non-reinforced lever presses (±SEM) during the 30-minute test for stress-induced reinstatement following either saline or ADX71441 at a dose of 3 mg/kg (n=10-11 by groups) (* = p<0.01 compared to vehicle, # = p<0.01 compared to extinction)

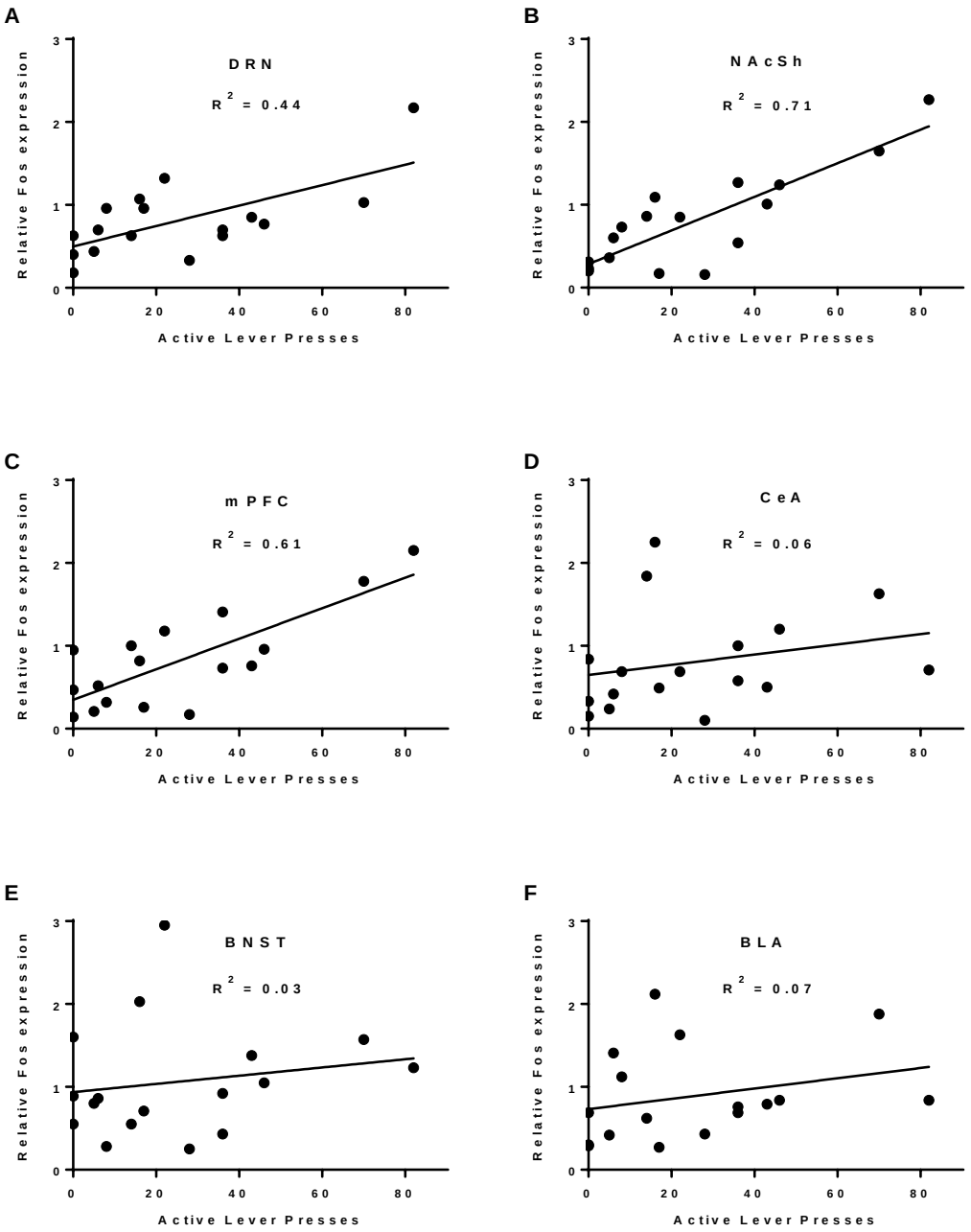


Supplementary Tab. 2. Correlation matrix of c-Fos immunoreactivity in individual brain structures analyzed following exposure to footshock stress in a stress-induced reinstatement test. Significant correlations are highlighted in red.

Structure	NacSh	mPFC	CeA	BNST	BLA
DRN	**R^2= 0.61;p<0.001**	**R^2=0.50;p<0.01**	**R^2=0.09;p=0.25**	**R^2=0.17;p=0.10**	**R^2=0.21;p=0.06**
NacSh		**R^2=0.80;p<0.0001**	**R^2=0.25;p=0.04**	**R^2=0.09;p=0.25**	**R^2=0.25;p=0.04**
mPFC			**R^2=0.21;p=0.07**	**R^2=0.17;p=0.10**	**R^2=0.16;p=0.11**
CeA				**R^2=0.06;p=0.33**	**R^2=0.40;p<0.01**
BNST					**R^2=0.34;p<0.05**

<u>Supplementary Fig. 4</u> Correlations of c-Fos expression in individual brain structures analyzed and stress-induced alcohol-seeking.

There was a significant and positive correlation in the DRN (R^2 =0.44, p<0.001, panel A), NAcSh (R^2 =0.71, p<0.0001, panel B), mPFC (R^2 =0.61, p<0.001, panel C) but not in CeA (R^2 =0.06, p=0.34, panel D), BNST (R^2 =0.03, p=0.50, panel E) or BLA (R^2 =0.07, p=0.30, panel F).



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