Nucleus Incertus Corticotrophin Releasing Factor 1 Receptor Signaling Regulates Alcohol Seeking in Rats

Leigh C. Walker[1,2,a], Hanna E. Kastman[1,2,a], Jan A. Koeleman[1,3], Craig M. Smith[1,2,4], Christina J. Perry[1,2], Elena V. Krstew[1], Andrew L. Gundlach[1,2,b], and Andrew J. Lawrence[1,2,b,*]

1 The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria 3052, Australia

2 Florey Department of Neuroscience and Mental Health, The University of Melbourne, Victoria 3010, Australia

3 Institute for Interdisciplinary Studies, University of Amsterdam, Amsterdam 1098 XH, The Netherlands

4 School of Medicine, Deakin University, Geelong, Victoria 3216, Australia

a. Co-first authors, LCW and HEK contributed equally to this work

b. Co-senior authors, ALG and AJL jointly supervised this research

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***Correspondence**: Andrew J Lawrence

Email: andrew.lawrence@florey.edu.au

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ABSTRACT

Alcoholism is a chronic relapsing disorder and stress is a key precipitant of relapse. The nucleus incertus (NI) is highly responsive to corticotrophin releasing factor (CRF) and psychological stressors, receives a CRF innervation and expresses CRF_1 and CRF_2 receptor mRNA. Furthermore, the ascending NI relaxin-3 system is implicated in alcohol seeking in rats. Therefore, in alcohol-preferring (iP) rats, we examined the effect of bilateral injections into the NI of the CRF_1 receptor antagonist, CP376395 or the CRF_2 receptor antagonist, astressin-2B on yohimbine-induced reinstatement of alcohol seeking. Using qPCR analysis of NI micropunches we assessed the effects of chronic alcohol consumption on gene expression profiles for components of the relaxin-3 and CRF systems. Bilateral intra-NI injections of CP376395 (500 ng/0.25 µL) attenuated yohimbine-induced reinstatement of alcohol seeking. In contrast, intra-NI injections of astressin-2B (200 ng/0.25 µL) had no significant effect. In line with these data, CRF_1, but not CRF_2, receptor mRNA was upregulated in the NI following chronic ethanol intake. Relaxin family peptide 3 receptor (RXFP3) mRNA was also increased in the NI following chronic ethanol. Our qPCR analysis also identified CRF mRNA within the rat NI, and the existence of a newly-identified population of CRF-containing neurons was subsequently confirmed by detection of CRF immunoreactivity in rat and mouse NI. These data suggest NI neurons contribute to reinstatement of alcohol seeking, via an involvement of CRF_1 signaling. Furthermore, chronic ethanol intake leads to neuroadaptive changes in CRF and relaxin-3 systems within rat NI.

INTRODUCTION

Alcohol use disorders (AUD) account for approximately 3.8% of deaths and 4.6% of disease and injury burden in developed countries (Rhem *et al*, 2009). Current first line pharmacotherapeutics for alcoholism are inadequate, with low compliance, adverse side effects and high rates of relapse (Jupp and Lawrence, 2010). Up to 90% of addicted individuals relapse within 12 months of abstinence (DeJong, 1994), most within 3 months (Sinha, 2008). Stress plays a key role in drug/alcohol abuse and is a major trigger of relapse (Koob et al, 2014). The neurocircuitry and brain chemistry underlying stress and addiction are the subject of ongoing investigation (Mantsch *et al*, 2016), although both involve corticotrophin releasing factor (CRF) (Koob *et al*, 2014). CRF binds to two G-protein-coupled receptors (CRF-receptor 1 (CRF_1) and CRF-receptor 2 (CRF_2)), plus CRF binding protein (CRF-BP) (Bale and Vale, 2004). CRF is expressed widely throughout the brain and implicated in the acquisition, maintenance and relapse of alcohol seeking (Lodge and Lawrence, 2003; Koob *et al*, 2014).

The pontine nucleus incertus (NI) is anatomically divided into the pars compacta (NIc) and pars dissipata (NId) (Goto *et al,* 2001). In rats, the NI was first characterized by its dense CRF receptor expression (Potter *et al*, 1994) and sensitivity to exogenous CRF (Bittencourt and Sawchenko, 2000). Afferent and efferent connections of the NI suggest it is a likely site of integration for behavioral planning and modulation of cognitive processes (Ma *et al,* 2013). NI neurons are predominantly γ-aminobutyric acid (GABA)ergic, and the NI contains the largest population of neurons producing the neuropeptide relaxin-3 in rat (Ma *et al*, 2007),

mouse (Smith *et al*, 2010) and macaque (Ma *et al*, 2009). The ascending relaxin-3/RXFP3 (relaxin family peptide 3 receptor) system regulates stress effects on alcohol preference in mice (Walker *et al*, 2015) and reinstatement of alcohol (but not sucrose) seeking in rats (Ryan *et al*, 2013).

NI relaxin-3 neurons co-express CRF_1 receptors (Tanaka *et al*, 2005; Ma *et al*, 2013), and are activated by psychological stress and intracerebroventricular (icv) CRF (Tanaka *et al*, 2005). Electrophysiological studies confirmed that icv CRF excites relaxin-3 neurons within the NI (Ma *et al*, 2013). Pretreatment with the CRF_1 receptor antagonist antalarmin attenuated swim-stress induced elevations of relaxin-3 mRNA (Banerjee *et al*, 2010), while CRF depolarization of relaxin-3 NI neurons was prevented by NBI35965 (Ma *et al*, 2013). These data suggest relaxin-3 neurons within the NI integrate information related to stressful stimuli in a CRF_1-dependent manner.

Chronic alcohol exposure is associated with changes in CRF expression in the paraventricular nucleus of the hypothalamus (Rivier *et al*, 1990), basolateral amygdala (BLA) (Falco *et al*, 2009), and central amygdala (CeA) (Zhou *et al*, 2013; Sommer *et al*, 2008). In alcohol dependent and certain alcohol-preferring rats CRF receptor expression is also altered in the BLA and medial amygdala (Sommer *et al*, 2008; Hansson et al, 2006). Given the roles of CRF and relaxin-3 systems in stress-induced alcohol seeking (Le *et al*, 2000; Ryan *et al*, 2013), and the functional interaction between CRF and relaxin-3 systems within the NI, we assessed whether a CRF receptor-mediated pathway in the NI was implicated in alcohol seeking. We also examined the impact of chronic ethanol intake on gene expression within the rat

NI.

MATERIALS AND METHODS

All studies were performed in accordance with the Prevention of Cruelty to Animals Act (2004), under the guidelines of the NHMRC Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia (2013) and approved by The Florey Animal Ethics Committee. Adult male alcohol-preferring rats (iP) rats were obtained from in-house breeding. Parental stock came from Professor T.K. Li (while at Indiana University, USA). Rats were pair-housed under ambient conditions (21ºC) and maintained on a 12 h light/dark cycle (lights on at 0700h), with access to chow and water *ad libitum*. Post-surgery, rats were single housed. B6(Cg)-Crhtm1(cre)Zjh/J (Crh-IRES-Cre) mice and B6.Cg-Gt(ROSA)26Sortm14(CAG-TdTomato)Hze/J; (Ai14) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA; stock number 012704 and 007914 respectively). Upon arrival, mice were backcrossed with a C57BL/6J breeder line, and mutants identified using polymerase chain reaction (PCR) procedures provided by the supplier. Homozygous Crh-IRES-Cre and Ai14 mice were bred to produce heterozygous Crh-IRES-Cre**x**Ai14 progeny (Cusulin et al, 2013). Mice were housed on a 12 h light/dark cycle (lights on at 7:00) with ad libitum access to food and water.

Alcohol self-administration

Male iP rats were trained to self-administer ethanol (10% (v/v)) under operant conditions using a fixed ratio of 3 (FR3; 3 lever presses for one 100μl reward delivery) during 20-min sessions (Lawrence et al, 2006). For each session, total

ethanol and water responses were recorded, and the difference in fluid in the ethanol reservoir between the beginning and end of the session was recorded to ensure correct calibration of the delivery system, and consumption of ethanol. Operant conditioning chambers supplied by Med Associates (St Albans, VT, USA) were employed. Each chamber was housed individually in sound-attenuation cubicles and chambers were connected to a computer running Med-PC IV software (Med Associates) to record data. Within the chambers, a house light provided soft illumination during operant conditioning sessions. Retractable levers (exerted during operant conditioning sessions) were placed below a stimulus light and adjacent to a fluid receptacle. Availability of ethanol was conditioned by the presence of an olfactory cue (S+; one drop of vanilla essence (Queen Foods, Alderley, QLD, Australia) placed directly underneath the ethanol-paired lever of the operant conditioning chamber). A 1-s light stimulus (CS+) occurred when the FR3 requirement was obtained with both the ethanol-paired and water-paired lever. Rats underwent an extended period of alcohol self-administration (~7 weeks, 5 consecutive days per week), followed by surgery.

Stereotaxic implantation of cannulae into NI

Rats were anesthetized with isoflurane (5% (v/v) induction, 2% maintenance), positioned in a stereotaxic frame (Stoelting Co. Wood Dale, USA), and the scalp shaved and cleaned (proviodine-iodine 10% (w/v); Orion Laboratories, Arkles Bay, NZ). A small incision was made to expose the skull. Four pits were drilled into the skull and screws (1.4 mm diameter and 2 mm length; Mr Specs, Parkdale, Australia) inserted. A hole was drilled through the skull and a single stainless steel 26 gauge

bilateral cannula cut 7.3 mm below the pedestal (PlasticsOne, Roanoke, USA) was implanted relative to bregma: anteroposterior, −9.8 mm; mediolateral, ±0.3 mm; and dorsoventral, −7.8 mm, with the incisor bar set at -9.2 mm from the flat skull position to flex the head (Paxinos and Watson, 1986). Cannulae were fixed in place using dental cement (Vertex-Dental, Zeist, The Netherlands). Patency was maintained by inserting a dummy, which projected 1.5 mm beyond the tip (PlasticsOne). Meloxicam (3 mg/kg, i.p.) was administered for analgesia, plus antibiotic (Baitryl, 3 mg/kg, i.p.). Rats recovered for 7 days.

Yohimbine-induced reinstatement of alcohol seeking

After recovery, rats reacquired ethanol responding to pre-surgical levels before extinction training. During extinction training no cues were present and there was no programmed response following task completion. Extinction sessions continued until mean responding on the ethanol-paired lever was <15 lever presses for 3 consecutive days. Subsequently, rats underwent a yohimbine-induced reinstatement session whereby yohimbine (Tocris Bioscience, Bristol, UK) dissolved in distilled water was injected (1 mg/kg i.p.) 30 min prior to test (Ryan *et al*, 2013). During this session, rats received intra-NI infusions of either vehicle or treatment directly prior to reinstatement testing in a randomized manner. Subsequently rats received 2 days of ethanol reacquisition to re-stabilize ethanol consumption and were then re-extinguished (Lawrence *et al*, 2006). A second reinstatement test was performed where rats received the alternate intra-NI treatment in a counterbalanced manner. Cohort 1 (n = 9) received 0.25 μL vehicle and 500 ng/0.25 μL per side of the CRF_1 antagonist CP376395 (Tocris Bioscience), while Cohort 2 (n = 10) received 0.25 μL

vehicle and 200 ng/0.25 µL per side of the CRF$_2$ antagonist astressin-2B (Tocris Bioscience). Additional rats whose injections fell outside of the NI were used as anatomic controls: CP376395 (n = 15) or astressin-2B (n = 7).

Nucleus incertus infusions

Bilateral NI infusions were made using 40 cm polyethylene connectors (PlasticsOne) attached to 1 µL microsyringes (SGE Analytical Science, Ringwood, Australia). 0.25 µL of vehicle or drug were infused bilaterally (0.25 µL/min) by an automated syringe pump (Harvard Apparatus, Holliston, USA). The injectors were left in place for 2 min after infusion. After completion of behavioral testing, rats were anesthetized with pentobarbitone (100 mg/kg i.p., Virbac, Milperra, Australia). Correct cannula positioning was verified in each rat by infusing methylene blue (0.25 µL/side). Rats were euthanized (pentobarbitone (100 mg/kg i.p.)), brains collected, frozen over liquid nitrogen and sectioned for injection site validations, which were performed by an investigator blinded to the identity of the tissue.

Drugs

CP376359 and astressin-2B were purchased from Tocris Bioscience and dissolved in normal saline (0.9% NaCl). The dose of CP376395 was selected based on previous studies, which showed microinjections into the rat VTA to attenuate cocaine seeking, without inducing any non-specific motor impairments (Blacktop et al, 2011). The dose of astressin-2B was chosen based on previous studies (Tran et al, 2014).

Two-bottle free-choice drinking

A separate cohort (n = 21) of individually housed male iP rats were acclimatized to drinking from two bottles of tap water. 14 rats then had one bottle replaced with ethanol (10% v/v), while the remaining n = 7 rats received tap water only. Over the subsequent ~125 days, fluid consumption volumes were calculated daily between 0930 and 1100 h. The position of bottles was altered randomly. Ethanol was removed 24 h prior to culling to avoid acute pharmacological effects of ethanol on mRNA. Rats were anesthetized with pentobarbitone (100 mg/kg i.p.) decapitated and the brains rapidly dissected and frozen over liquid nitrogen (-80 °C).

Tissue preparation and qPCR

NI bilateral micro-punch samples (diameter: 0.3 mm, thickness: 400 μm) were taken relative to bregma: anteroposterior, −9.72 mm; mediolateral, ±0.3 mm; and dorsoventral, -7.6 mm (Paxinos and Watson, 1986) over dry ice using micro-dissection needles (ProSciTech, Kirwan, Australia). Micropunches were homogenized using QIAzol Lysis Reagent (Qiagen, Venlo, The Netherlands) and RNA purified using the RNeasy Micro Kit (Qiagen). RNA yields, concentration and purity were measured using a Nanodrop 2000C UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For each sample, 0.3 μg of total RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription reagents (Thermo Fisher Scientific) with random hexamers. Reverse transcription was performed on a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA): 10 min at 25°C, 30 min at 42°C, 5 min at 95°C and hold at 4°C. cDNA products were stored at -20°C until use. See Table 1 for primer sequences.

qPCR was performed on a ViiA^{TM} 7 Real Time PCR System (Applied Biosystems). Reactions were performed in triplicate in optical 384-well plates (Applied Biosystems): 10 min at 95°C for polymerase activation followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each reaction contained 2 µL of cDNA, 5 µL of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), sense and antisense primers (0.5 µL, each 20 µM) made up to 10 µL with DEPC water. Data were analysed using 7500 Fast System Sequence Detection Software. Expression levels of mRNA transcripts encoding relaxin-3 (*Rln3*), RXFP3 (*Rxfp3*), CRF1 (*Crhr1*), CRF2 (*Crhr2*), CRF-binding protein (*Crhbp*), and CRF (*Crh*) were assessed. Target gene expression was normalized to the expression of the internal control gene encoding GAPDH (*Gapdh*) using the $2^{-\Delta\Delta CT}$ method as described (Scmittgen *et al*, 2008), and mean relative gene expression calculated for each target.

iP rat immunohistochemistry

Ethanol naïve adult male iP rats (350 g; *n* = 2) were anaesthetized with isoflurane (5% induction, 2% maintenance) and placed into a stereotaxic frame (Stoelting Co.). 80 µg colchicine (Sigma-Aldrich, Schnelldorf, Germany) dissolved in 5 µL of 0.9% NaCl was injected into the lateral ventricle (anteroposterior, −0.9 mm; mediolateral, -1.3 mm; and dorsoventral, −3.8 mm from bregma) via a needle connected to a 10 µL Hamilton syringe (Harvard Apparatus). Meloxicam (3 mg/kg, i.p.) was administered for analgesia. After 24 h rats were deeply anaesthetized with pentobarbitone (100 mg kg; i.p.) and transcardially perfused with 400 ml of 0.1 M phosphate buffered saline (PBS; 137 mM NaCl, 2.7 KCl, 11.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) followed by 500 ml of 1% paraformaldehyde (PFA; Sigma-Aldrich) with 15% (v/v) picric acid

(Sigma-Aldrich) (Gunn *et al,* 2013) in PBS. Rats were then decapitated, brains removed and submerged in fixation solution for 24 h at 4°C. Brains were then transferred to 20% (w/v) sucrose in PBS for 24 h at 4°C. Coronal sections (50 μm) through the NI were cut on a cryostat at −18°C (Cryocut 1800; Leica Microsystems, Heerbrugg, Switzerland) and collected into PBS.

Sections were preincubated (10% v/v normal goat serum (NGS) in PBS containing 0.5% TritonX-100) for 1 h at room temperature (RT). Sections were then incubated in a primary antibody mix of 1:2000 dilution rabbit anti-CRF (PBL#C70) (Sawchenko *et al*, 1984) and 1:5 dilution mouse monoclonal anti-Relaxin-3 (HK4–144–10) (Tanaka *et al*, 2005), with 2% NGS in PBS containing 0.1% TritonX-100 for 48 h at 4°C. Sections were washed 3 × 5 min in PBS and incubated in biotinylated donkey anti-rabbit IgG (1:500, Vector Laboratories, Burlingame, USA, BA-1000) for 2h. Following 3 × 5 min washes in PBS, sections were incubated in avidin–biotin complex (ABC; VectaStain Elite; Vector Laboratories), followed by 3 × 5 min washes in PBS and 2 h incubation in a mix of 1:400 dilution donkey anti-mouse-Alexa488 secondary antibody (Life Technologies, Carlsbad, USA, A-21202) and streptavidin-Alexa594 (Life Technologies, S-11227).

Crh-IRES-CrexAi14 mouse immunohistochemistry

Mice (9 weeks, n = 2) were anaesthetized (pentobarbitone, 80 mg kg; i.p.) and transcardially perfused with 50 ml of 0.1 M PBS followed by 50 ml of 4% PFA in PBS. Mice were then decapitated, brains removed and submerged in PFA for 1 h at 4°C. Brains were then transferred to 20% (w/v) sucrose in PBS for 24 h at 4°C. Coronal

sections (40 μm) through the NI were cut on a cryostat at −18°C (Cryocut 1800; Leica Microsystems) and collected into PBS. Sections were pre-blocked (10% v/v normal goat serum (NGS) in PBS containing 0.5% TritonX-100) for 1 h at RT. Sections were then incubated with rabbit anti-DsRed (1:2000, Clonetech, 632496) (Krashes et al, 2011) and mouse monoclonal anti-Relaxin-3 (1:5) (HK4–144–10) (Tanaka *et al*, 2005) with 2% NGS in PBS containing 0.1% TritonX-100 overnight at RT. Sections were washed 3 × 5 min in PBS and incubated in donkey anti-mouse-Alexa488 (1:400, Life Technologies, Carlsbad, USA, A-21202) and donkey anti-rabbit-Alexa594 (1:400, Life Technologies, A-21207) for 2 h at RT.

Sections were washed 3 × 5 min, mounted on glass microscope slides with fluorescence mounting medium (DAKO, Carpentaria, USA). Micrographs were obtained using a confocal microscope (Leica DM LB2, Leica Microsystems).

Statistical analysis

Behavioral data were analyzed by repeated measures (RM) two-way ANOVA with Bonferroni post hoc analysis using the statistical software SPSS (Version 21; IBM Corp., Armonk, NY, USA). GraphPad Prism (Version 5; GraphPad Software, Inc., La Jolla, CA, USA) was used for t-test statistical comparisons of qPCR data and to generate graphs. Data are reported as mean ± SEM, significance set at $p < 0.05$. EXT data are reported as an average of the last 3 days before reinstatement test.

RESULTS

Ethanol self-administration

During the last week of ethanol (10%v/v) self-administration rats averaged 90 ± 5.3 ethanol-paired lever presses (0.56 ± 0.3 g/kg/session ethanol intake), and 6.3 ± 0.3 water-paired lever presses (0.02 ± 0.003 g/kg/session water intake) which results in pharmacologically relevant blood alcohol levels (Roberts et al., 1999).

Nucleus incertus CRF_1 receptor antagonism attenuates yohimbine-induced reinstatement of alcohol seeking

We investigated the effect of the CRF_1 receptor antagonist, CP376395 on yohimbine-induced reinstatement of alcohol seeking in rats. RM two-way ANOVA revealed a significant main effect of both treatment ($F_{(2,7)} = 21.925$, $p < 0.001$) and lever ($F_{(1,8)} = 41.261$, $p < 0.001$) and a significant treatment x lever interaction ($F_{(2,8)} = 23.165$, $p < 0.001$). Bonferroni post hoc analysis showed yohimbine to induce a robust reinstatement of alcohol seeking in vehicle-treated animals (EXT vs. VEH, $p < 0.001$; Fig 1B), while CP376395 injected directly into the NI significantly reduced this reinstatement of alcohol seeking (VEH vs. CP376395, $p = 0.036$; Fig 1A). No differences in latency to ethanol reward were observed between VEH and CP376395-treated rats (Student's t-test; $p = 0.356$). Time course data revealed a significant decrease in responding from 5 - 10 min during the reinstatement test (RM two-way ANOVA; $p < 0.001$; Fig 1C)

To assess anatomic specificity injections were made immediately adjacent to the NI and/or within the fourth ventricle (4V) as controls. RM two-way ANOVA revealed an overall effect of both treatment ($F_{(2,13)} = 39.194$, $p < 0.001$) and lever ($F_{(1,14)} = 111.858$, $p < 0.001$) and a significant treatment x lever interaction ($F_{(2,13)} = 39.562$, $p < 0.001$). Bonferroni post hoc analysis confirmed yohimbine administration induced

reinstatement of alcohol seeking in vehicle-treated rats (EXT vs. VEH, p < 0.001; Fig. 1B). However, CP376395 control injections had no effect on yohimbine-induced reinstatement of alcohol seeking (p = 0.218; Fig. 1B), suggesting a localized effect within the NI. Injection sites were validated histologically for all CP376395-treated rats (Fig. 1D).

Nucleus incertus CRF_2 receptor antagonism does not attenuate yohimbine-induced reinstatement of alcohol seeking

We also investigated the effect of the CRF_2 receptor antagonist, astressin-2B, on yohimbine-induced reinstatement of alcohol seeking. RM two-way ANOVA revealed a significant main effect of both treatment ($F_{(2,8)}$ = 44.510, p < 0.001) and lever ($F_{(1,9)}$ = 138.703, p < 0.001) and a significant treatment x lever interaction ($F_{(2,8)}$ = 44.010, p < 0.001). Bonferroni post hoc analysis showed yohimbine to induce a robust reinstatement of alcohol seeking in vehicle-treated rats (EXT vs. VEH, p < 0.001; Fig 2A), however astressin-2B injected directly into the NI had no effect on reinstatement of alcohol seeking (VEH vs. astressin-2B, p = 0.177; Fig 2A). No difference in latency to reward was observed between astressin-2B and VEH-treated animals (Student's t-test; p = 0.250). There were no time course differences during reinstatement (Fig 2C).

Anatomic specificity was also tested for astressin-2B, with injections made immediately adjacent to the NI as controls. RM two-way ANOVA revealed an overall effect of both treatment ($F_{(2,5)}$ = 37.934, p < 0.001) and lever ($F_{(1,6)}$ = 86.049, p < 0.001) and a significant treatment x lever interaction ($F_{(2,5)}$ = 26.453, p < 0.001). Once again, Bonferroni post hoc analysis showed yohimbine administration induced

reinstatement of alcohol seeking in vehicle-treated rats (EXT Vs. VEH, p < 0.001; Fig. 2B). However, astressin-2B control injections had no effect on yohimbine-induced reinstatement of alcohol seeking (p = 0.218; Fig. 1B). Injection sites were validated histologically for all astressin-2B-treated rats (Fig. 2D).

Chronic alcohol consumption increases CRF_1 and RXFP3 receptor expression within the nucleus incertus

To assess changes in CRF and relaxin-3 system-related gene expression within the NI, male adult iP rats were provided a choice between either water and 10% ethanol, or water alone, for ~125 days. After an initial ~25 day period in which rats with access to alcohol displayed escalating rates of consumption and preference, over the remaining ~100 days consumption remained stable. During the last week of ethanol consumption rats averaged 4.1 ± 0.5 g/kg/day ethanol intake, with an alcohol preference of 79.2 ± 5.3% (Fig. 3).

Rats were culled and tissue that encompassed the NI was collected (Fig. 4A) for qPCR analysis. Importantly, high levels of relaxin-3 mRNA were detected within every individual tissue sample, confirming correct tissue targeting. Chronic ethanol consumption did not alter relaxin-3 expression, relative to water controls (Student's t-test; p = 0.414; Fig. 4B). Expression of mRNA encoding the cognate receptor for relaxin-3, RXFP3, was significantly increased in rats following long-term voluntary ethanol intake compared to water controls (Student's t-test; p = 0.012; Fig. 4C). CRF_1 receptor gene expression was significantly increased following chronic ethanol intake (Student's t-test; p = 0.037; Fig. 4D). In contrast, no changes in the expression of mRNA encoding CRF_2 receptors or CRF-BP were observed (Student's t-test; p =

0.704 and p = 0.394; Fig. 4E and 4F). CRF mRNA was detected within the NI but not altered by chronic ethanol intake (Student's t-test; p = 0.114; Fig. 4G).

A population of CRF-positive neurons within the NI

To confirm the presence of CRF-containing neurons within the NI, we employed immunohistochemistry with a validated CRF antibody (Sawchenko *et al*, 1984) and Crh-IRES-CrexAi14 transgenic mice (Cusulin et al, 2013). Barrington's nucleus was included as a positive control for CRF-like-IR specificity. A high density of cells displaying CRF-like-IR was evident within Barrington's nucleus, however CRF-like-IR-positive soma were largely absent in adjacent regions concordant with previous reports (Swanson *et al*, 1983; Fig. 6A, 7A). Importantly however, cell soma with punctate cytoplasmic CRF-like-IR were observed throughout the rostrocaudal axis of the iP rat and the Crh-IRES-CrexAi14 mouse NI (Table 2, 3; Fig. 5, 6, 7; CRF-like IR red circles; relaxin-3-like IR green circles), which was identified via double-labeling with relaxin-3 (Tanaka *et al*, 2005; Ma *et al*, 2013). While a small number of CRF-positive neurons co-express relaxin-3 (Table 2, 3; Fig. 6, 7), the majority do not (Table 2, 3; Fig. 6, 7). CRF-positive cells were distributed throughout both the pars compacta and dissipata regions of the NI and more numerous towards the caudal extent of the NI (Table 2, 3).

DISCUSSION

Our results demonstrate that CRF_1 receptor antagonism in the NI via local microinjection of CP376395 attenuates yohimbine-induced reinstatement of alcohol

seeking in iP rats. Importantly, injections of the same dose of CP376395 immediately adjacent to the NI had no effect, suggesting the effect is specifically associated with actions on NI neurons and not CRF_1-positive neurons in adjacent areas. In contrast, intra-NI injections of astressin-2B did not alter alcohol seeking at the dose employed, suggesting a lesser role for CRF_2 signaling within the NI in the regulation of this behavior, despite CRF_2 expression within this region. Importantly, the attenuation of alcohol seeking after intra-NI CP376395 confirms that under our experimental paradigm, CRF is released within the NI and contributes to the observed behavior.

Our findings are in accordance with immunohistochemical, electrophysiological and behavioral studies that demonstrate CRF and/or stress act via CRF_1 to activate relaxin-3 containing neurons within the NI (Tanaka *et al*, 2005; Banerjee *et al*, 2010; Ma *et al*, 2013). In combination with previous studies which demonstrated that relaxin-3/RXFP3 signaling within the BNST promotes yohimbine-induced alcohol seeking (Ryan *et al*, 2013), our data are consistent with the hypothesis that stress activates CRF input(s) to the NI, which acts upon CRF_1 receptors to drive ascending relaxin-3 pathways innervating the BNST (and likely other stress-relapse circuitry) to contribute to reinstatement of alcohol seeking. It is important to note that intra-NI CP376395 attenuated but did not prevent yohimbine-induced reinstatement of alcohol seeking. This is to be expected given the complex circuitry and multiple neurotransmitters/neuropeptides known to contribute to stress-induced alcohol seeking (Mantsch et al 2016). Nevertheless, our findings suggest that CRF_1 receptor signaling within the NI is involved in this process, and that the NI therefore apparently interfaces with the established stress-relapse circuitry. In this regard, the BNST is a likely candidate where ascending relaxin-3 neurons modulate relapse-like behavior

(Ryan et al., 2013).

The exact mechanisms involved in yohimbine-induced reinstatement of operant responding still require elucidation. A recent study showed that yohimbine reinstates lever pressing in rats that were previously trained with lever pressing resulting in cue presentation with or without reward delivery and therefore may not necessarily act as a stressor model (Chen et al, 2015). It is however noteworthy that yohimbine-induced reinstatement of ethanol seeking can be prevented by treatments that do not impact upon yohimbine-induced reinstatement of sucrose seeking (Ryan et al., 2013), suggesting that cue reactivity is not the only factor behind a yohimbine-induced reinstatement of reward seeking. Importantly, yohimbine induces anxiety and the subjective measures of alcohol intoxication in healthy humans (McDougle et al, 1995), as well as craving in human alcoholics (Umhau et al., 2011) and opiate addicts (Greenwald et al, 2013). Based on this property yohimbine has been suggested to represent an ideal cross-species probe for translational addiction research (See & Waters, 2010). Yohimbine produces a robust and stable reinstatement of alcohol seeking in rats (Le et al, 2000). Importantly in the context of the present study, yohimbine shares a commonality with footshock stress-induced reinstatement of alcohol seeking in that both are critically dependent upon extrahypothalamic CRF_1 receptor signaling (Marinelli et al., 2007). Moreover, yohimbine administration also induces c-fos and CRF mRNA in limbic regions to a similar degree as foot-shock (Funk et al, 2006). Therefore, while yohimbine may act via more than one mechanism to precipitate reinstatement of alcohol seeking it is nevertheless a useful tool to assess the involvement of CRF_1 receptors in

reinstatement following extinction. Note also that yohimbine specifically reinstated responding on the lever associated with alcohol and not the lever paired with water.

CRF$_1$ antagonists effectively attenuate both footshock- and yohimbine- induced reinstatement of alcohol seeking (Le *et al*, 2000; Marinelli *et al*, 2007). Interestingly, existing evidence suggests that CRF$_1$ antagonists are more effective in attenuating stress-induced reinstatement in rats with increased stress sensitivity, such as post-dependent rats or behaviorally selected lines that prefer alcohol (Hansson *et al*, 2006; Gehlert *et al*, 2007). It has been proposed that this behavior may be mediated by the upregulation of CRF$_1$ receptor gene expression. For example Marchigian Sardinian alcohol-preferring (msP) rats display increased CRF$_1$ receptor mRNA expression in limbic regions compared with Wistar rats, and unlike non-dependent Wistar rats, msP rats are susceptible to attenuation of drinking after treatment with a CRF$_1$ receptor antagonist (Hansson *et al*, 2006). Consistent with this theory, iP rats also have enhanced CRF/stress responsivity (Ehler *et al*, 1992).

Our molecular data demonstrate that CRF$_1$, but not CRF$_2$, receptor gene expression was upregulated within the NI following chronic voluntary ethanol consumption and confirms that chronic ethanol intake modulates expression of CRF system components within the rat NI. Our findings also provide further support that CRF$_1$ and CRF$_2$ receptors may have differential roles with regards alcohol intake/seeking. For example, administration of a CRF$_1$ receptor antagonist, CP154546, or the CRF$_2$ receptor agonist, urocortin 3, dose-dependently reduced binge-like ethanol intake in C57BL/6J mice (Lowery *et al*, 2010). In addition, urocortin 3 reduces stress-related behavior and ethanol self-administration in ethanol-dependent rats following acute

withdrawal (Valdez *et al*, 2004). Chronic intermittent ethanol exposure in mice upregulates CRF_1, but not CRF_2, receptor transcripts in the CeA (Eisenhardt *et al*, 2015). In line with this, we did not detect changes in CRF_2 receptor mRNA expression within the NI following chronic ethanol consumption; however, the mRNA encoding CRF_1 receptors was upregulated. Given our behavioral data, it is possible that increased CRF_1 receptor expression within the NI may contribute to a heightened CRF/stress response, leaving rats more susceptible to reinstate. However methodological differences must be considered. Different drinking paradigms were employed when examining the role of CRF receptors in operant behavior compared to mRNA regulation following long-term voluntary ethanol consumption. Our operant studies were designed to model relapse-like behavior and examine the role of CRF_1 receptor signaling within the NI in this regard. On the other hand, our chronic ethanol consumption model was designed to assess whether molecular adaptations to relaxin-3 and/or CRF systems occurred within the NI after long-term intake. Both of these paradigms model features of human AUD and therefore are different, but also complementary, means to study alcohol-related problems. Therefore, the fact that intra-NI CRF_1 receptor antagonism behaviorally reduced alcohol seeking while chronic ethanol consumption regulated CRF_1 receptor mRNA expression within the NI provides strong support for a role of NI CRF signaling in aspects of alcohol use and abuse.

The NIc and NId receive innervation from similar afferent sources, including the medial prefrontal cortex, lateral hypothalamus, lateral habenula and BNST (Goto *et al,* 2001), plus a recently revealed CRF input from the lateral preoptic area (Ma *et al*, 2013). Previous studies evaluating CRF distribution in the brainstem have shown

scattered CRF-like-IR (Sutin and Jacobowitz, 1988). Our identification of CRF-positive neurons and fibers within the rat and mouse NI suggests both extrinsic and intrinsic sources of CRF apparently exist in this nucleus.

RXFP3 mRNA was significantly upregulated within the NI following chronic voluntary ethanol consumption. While RXFP3 may act as an autoreceptor on NI neurons, it is also possible that RXFP3 is expressed by adjacent neurons that receive relaxin-3 inputs. In contrast, relaxin-3 expression was unaltered by chronic alcohol consumption, which corroborates our previous study in which relaxin-3 expression (detected via *in situ* hybridization) was unaltered by chronic alcohol consumption in iP rat brain (Ryan *et al*. 2014). Notably relaxin-3 expression in the NI of iP rats correlated with alcohol consumption, suggesting that natural variations in expression may relate to the propensity to consume alcohol (Ryan *et al*. 2014).

CRF is implicated in stress-induced reinstatement acting in extrahypothalamic regions, including the ventral tegmental area (VTA) (Blacktop *et al*, 2011; Chen *et al*, 2014) and ventral BNST (Erb and Stewart, 1999); and in specific projections from the CeA to the BNST (Erb *et al*, 2001), and the BNST to the VTA (Silberman *et al*, 2013). The NI sends relaxin-3 containing efferent projections to all of these regions, and in particular, relaxin-3/RXFP3 signaling within the BNST promotes yohimbine-induced alcohol seeking (Ryan *et al*, 2013). The present studies reveal that CRF_1 receptor signaling within the NI is implicated in yohimbine-induced alcohol seeking, while chronic alcohol consumption increases RXFP3 and CRF_1 receptor expression within the NI. Furthermore, we reveal for the first time the existence of a population of CRF-positive neurons within the NI. Taken together, these findings give further credence

to the hypothesis that the NI/relaxin-3 system functionally interfaces with CRF/CRF$_1$ signaling to modulate stress-associated responses, including alcohol seeking.

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AUTHOR CONTRIBUTIONS

AJL, LCW and HEK contributed to the design and analysis of the study; AJL and ALG jointly supervised the project. LCW, HEK, EVK and CJP conducted all behavioral experiments; LCW, JAK, CMS conducted molecular experiments and performed related analysis; LCW and AJL wrote the manuscript. All authors reviewed the content and approved the final version of the manuscript.

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Figure Legends

Figure 1. Bilateral injections of CRF_1 antagonist CP376395 into the nucleus incertus attenuated yohimbine-induced reinstatement of alcohol seeking. (A) Ethanol-paired lever and water-paired lever presses following bilateral NI injections of CRF_1 antagonist CP376395 (n = 9). (B) Ethanol-paired lever and water-paired lever presses in anatomic controls for CP376395 infusion (n = 15). (C) Time course of lever pressing throughout reinstatement session for vehicle (V) or CP376395. (D) Neuroanatomical representation of injection sites for CP376395 treated rats. Green circles represent NI injections, red triangles represent anatomic controls. Data were analyzed by repeated measures two-way ANOVA with post-hoc Bonferroni multiple comparisons test and expressed as mean ± SEM. Extinguished rats (EXT), and rats that underwent yohimbine-induced reinstatement, pre-treated with either, vehicle (V) or 500 ng CP376395 (CP). EXT data are represented as combined values of the last 3 days of EXT prior to each reinstatement test. Yohimbine precipitated reinstatement of alcohol seeking (***$p < 0.001$), which was attenuated by CP76395 microinjection within the NI (#$p < 0.05$). Furthermore time course data revealed a significant difference in responding 5 - 10 min into the reinstatement test (***$p < 0.001$).

Figure 2. Bilateral injections of CRF_2 antagonist astressin-2B into the nucleus incertus does not attenuate yohimbine-induced reinstatement of alcohol seeking. (A) Ethanol-paired lever and water-paired lever presses following bilateral NI injections of CRF-R2 antagonist astressin-2B (n = 10). (B) Ethanol-paired lever and water-paired lever of anatomic controls following astressin-2B infusion (n = 7).

(C) Time course of lever pressing throughout reinstatement session for vehicle (V) or astressin-2B. (D) Neuroanatomical representation of injection sites for astressin-2B treated rats. Green circles represent NI injections, red triangles represent anatomic controls. Data were analysed by repeated measures two-way ANOVA with post-hoc Bonferroni multiple comparisons test and expressed as mean ± SEM. Extinguished rats (EXT), and rats that underwent yohimbine-induced reinstatement, pre-treated with either, vehicle (V) or 200 ng astressin-2B (A2B). EXT data are represented as combined values of the last 3 days of EXT prior to each reinstatement test. Yohimbine induced reinstatement of alcohol seeking (***$p < 0.001$), astressin-2B did not attenuate this behaviour; no differences in time course data were observed.

Figure 3. Two bottle choice fluid and ethanol consumption in iP rats. (A) Ethanol intake relative to body weight for ethanol-consuming iP rats (g/kg/day) and (B) 10% ethanol solution preference. All values are mean ± SEM, n = 14. Abbreviations: EtOH, ethanol.

Figure 4. Chronic ethanol intake modulates the expression of components of the relaxin-3 and CRF system in the iP rat NI. (A) Representative micropunch dissection of the NI. Relative mRNA expression of (B) relaxin-3, (C) RXFP3, (D) CRF_1, (E) CRF_2, (F) CRF-BP and (G) CRF in rats voluntarily administering water (n = 7) or ethanol (n = 14) for >125 days. Data were analyzed by unpaired student's t-test and expressed as mean ± SEM. Chronic alcohol consumption increased RXFP3 and CRF_1 receptor mRNA in the NI (*$p < 0.05$). Abbreviations: Cb, cerebellum; NI, nucleus incertus; 4V, fourth ventricle, CG, central gray.

Figure 5. CRF is expressed throughout the rat nucleus incertus. Schematic representation of CRF expression through the rostrocaudal axis of the rat nucleus incertus. Relaxin-3 positive neurons indicated in green, CRF positive neurons indicated in red. Distance from bregma is indicated for each section. Abbreviations: 4V, fourth ventricle; Cb, cerebellum; PDTg, posterodorsal tegmental nucleus.

Figure 6. CRF positive neurons are present within the nucleus incertus of the iP rat. (A) Low magnification confocal projection image of a section illustrating CRF positive neurons within the nucleus incertus and adjacent Barrington's nucleus of the iP rat. Overview of CRF positive neurons within the (B) NI compacta (NIc) and (C) NI dissipata (NId). Confocal projection of (D) CRF, (E) relaxin-3, and (F) overlay show both CRF positive/relaxin-3 negative cells and CRF negative/relaxin-3 positive cells. High magnification confocal projection of (G) CRF, (H) relaxin-3, and (I) overlay showing co-localization of CRF and relaxin-3. Relaxin-3 neurons (green) and CRF neurons (red), co-localisation indicated by arrowhead, while CRF positive / relaxin-3 negative neurons indicated by arrow. 4V, fourth ventricle; NIc, nucleus incertus compacta; NId, nucleus incertus dissipata; Bar, Barrington's nucleus; PDTg, posterodorsal tegmental nucleus.

Figure 7. CRF positive neurons are present within the nucleus incertus of the Crh-IRES-CrexAi14 mouse. (A) Low magnification confocal projection image illustrating CRF/TdT positive neurons within the NI and Barrington's nucleus of Crh-IRES-Cre**x**Ai14 mice. Overview of CRF positive neurons within the (B) NI compacta

(NIc) and (C) NI dissipata (NId) of Crh-IRES-Cre**x**Ai14 mice. High magnification confocal projection of CRF/TdT (D), relaxin-3 (E), and overlay (F), showing co-localization of CRF/TdT and relaxin-3 within the NIc of the Crh-IRES-Cre**x**Ai14 mouse. Relaxin-3 neurons (green) and CRF/TdT neurons (red), co-localisation indicated by arrowhead, while CRF positive / relaxin-3 negative neurons indicated by arrow. Abbreviations: TdT, TdTomato; NIc, nucleus incertus compacta; NId, nucleus incertus dissipata; Bar, Barrington's nucleus; PDTg, posterodorsal tegmental nucleus.

Table 1. List of primers used in quantitative PCR

Table 1.

Gene		
Relaxin-3	Forward primer	5'-CCCTATGGGGTGAAGCTCTG-3'
	Reverse primer	5'-GCTTCTCCATCAGCGAAGAA-3'
RXFP3	Forward primer	5'-AGGCCAGGGTACGGATCCT-3'
	Reverse primer	5'-CCAGTCCCAGGGCACAAAC-3'
CRF	Forward primer	5'-AAATGGCCAGGGCAGAGCAGT-3'
	Reverse primer	5'-TGGCCAAGCGCAACATTTCAT-3'
CRF$_1$	Forward primer	5'- TGCCAGGAGATTCTCAACGAA-3'
	Reverse primer	5'- AAAGCCGAGATGAGGTTCCAG-3'
CRF$_2$	Forward primer	5'-CTCATCAATTTTGTGTTTCTGTTCAA-3'
	Reverse primer	5'-CTGTACTGGATGGTCTCGGATGT-3'

CRF-BP	Forward primer	5'-GCCCAGTGAGTTCTCCACAGTT-3'
	Reverse primer	5'-CATGTGTGCAGGTTTTCAAAGC-3'
GAPDH	Forward primer	5'-CTACCCCCAATGTATCCGTTG-3'
	Reverse primer	5'-AGCCCAGGATGCCCTTTAGT-3'

Table 2. Number of corticotrophin releasing factor (CRF) positive neurons throughout the rostrocaudal axis of the iP rat nucleus incertus. Numbers shown as an average across two ethanol naïve iP rats. Sections cut at 50 μm in a 1 in 2 series from -9.30 mm to -10.0 mm relative to bregma. Abbreviations: NIc, nucleus incertus compacta; NId, nucleus incertus dissipata; CRF, corticotrophin releasing factor; RLX3, relaxin-3

Table 2.

Distance (bregma)	NIc		NId		Total	
	CRF	CRF/RLX3	CRF	CRF/RLX3	CRF	CRF/RLX3
- 9.3 mm	1.5	0.5	1.5	0	3	0.5
- 9.4 mm	3	0	3	0	6	0
- 9.5 mm	3.5	0	8.5	0	12	0
- 9.6 mm	2	0	6.5	0	8.5	0
- 9.7 mm	4.5	0.5	8	0	12.5	0.5
- 9.8 mm	9.5	0	10.5	0	20	0
- 9.9 mm	6.5	1	4	0	10.5	1
- 10.0 mm	4	0	6.5	0	10.5	0
Total	**34.5**	**2**	**48.5**	**0**	**83**	**2**

Table 3. Number of corticotrophin releasing factor (CRF) positive neurons throughout the rostrocaudal axis of the Crh-IRES-Cre+/−;Ai14+/− mouse nucleus incertus. Numbers shown as an average across two ethanol naïve Crh-IRES-Cre+/−xAi14+/− mice. Sections cut at 40 µm from 5.4 mm to 5.6 mm relative to bregma. Abbreviations: NIc, nucleus incertus compacta; NId, nucleus incertus dissipata; TdT, tdTomato; CRF, corticotrophin releasing factor; RLX3, relaxin-3

Table 3.

Distance (bregma)	NIc		NId		Total	
	CRF/TdT	CRF/TdT + RLX3	CRF/TdT	CRF/TdT + RLX3	CRF/TdT	CRF/TdT+ RLX3
- 5.40 mm	16.5	0.5	8.5	0	25	0.5
- 5.44 mm	17.5	1	10.5	0	28	1
- 5.48 mm	18	0	12.5	0	30.5	0
- 5.52 mm	20	1	13.5	0	33.5	1
- 5.56 mm	19.5	0	11	0	30.5	0
- 5.60 mm	19	0	18	0	37	0
Total	**110.5**	**2.5**	**75**	**0**	**184.5**	**2.5**

A

B

C

D
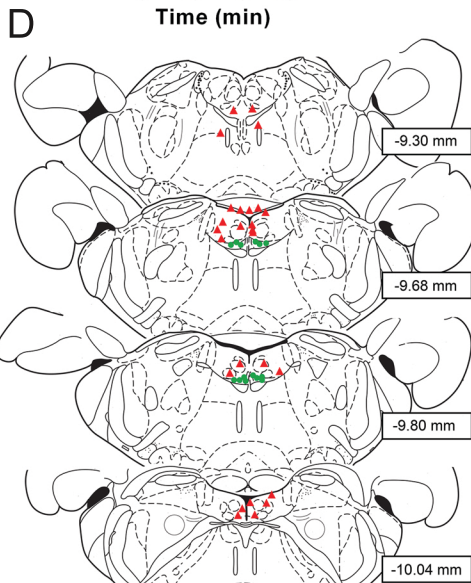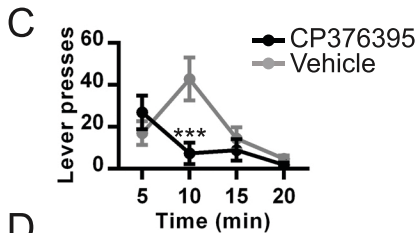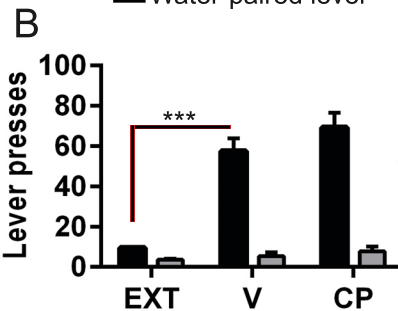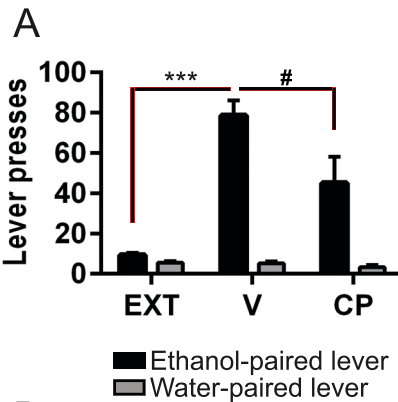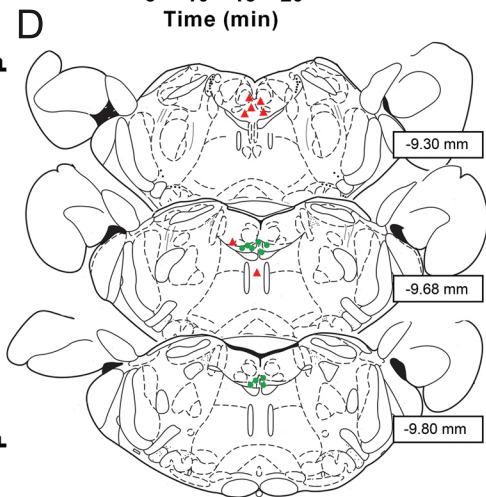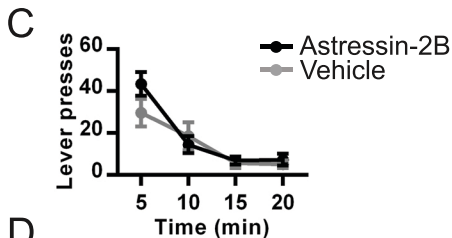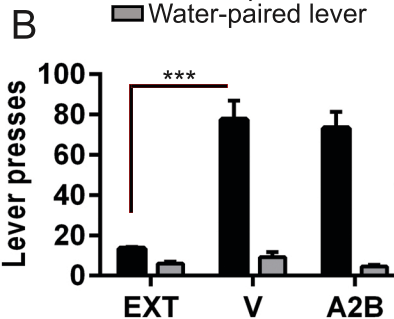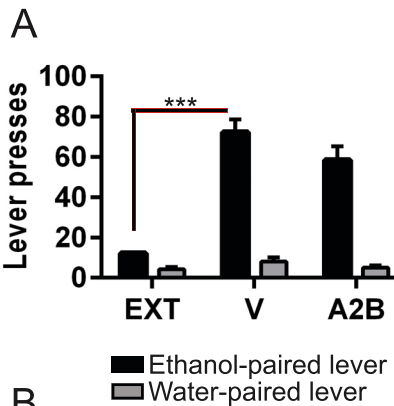
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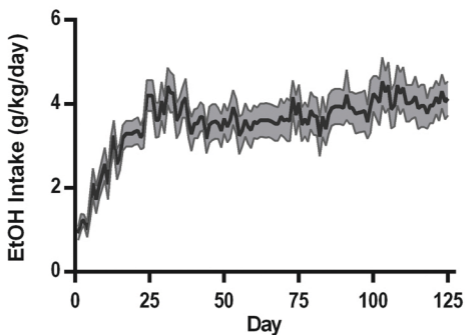
B EtOH Preference
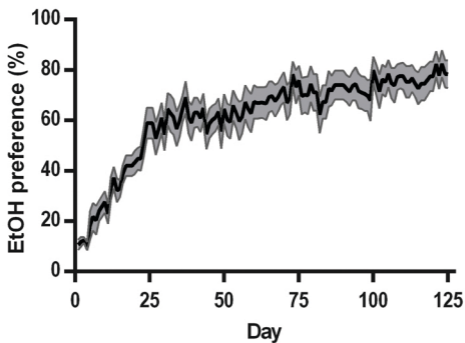
The panels are labeled: -9.3 mm, -9.7 mm, -9.4 mm, -9.8 mm, -9.5 mm, -9.9 mm, -9.6 mm, -10.0 mm. Structures labeled include 4V, PDTg, mlf, and Cb.
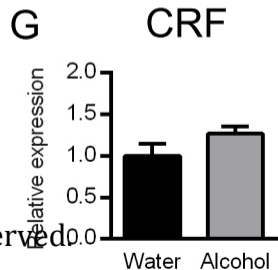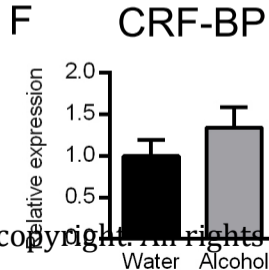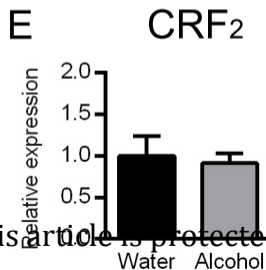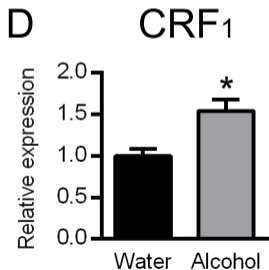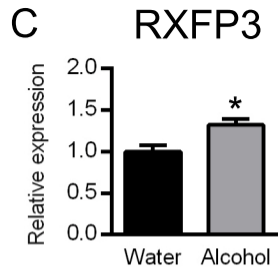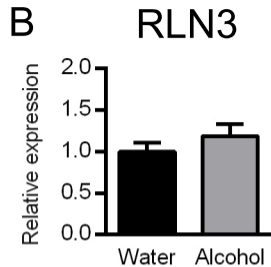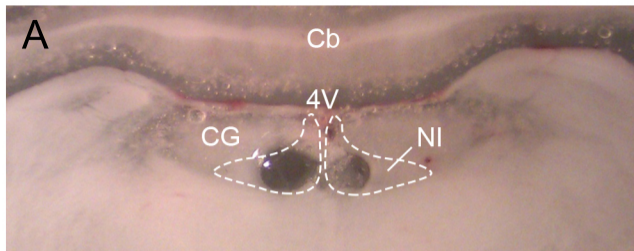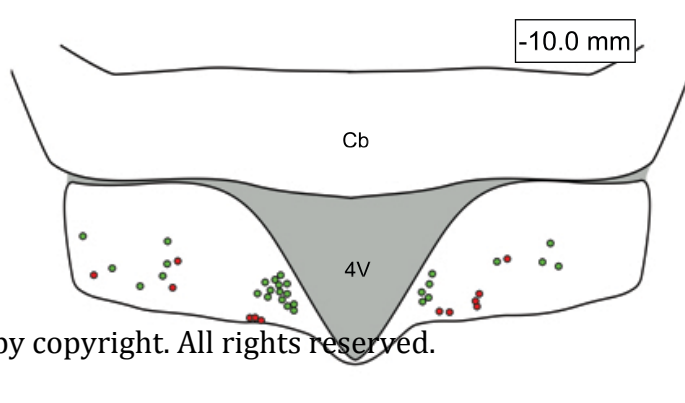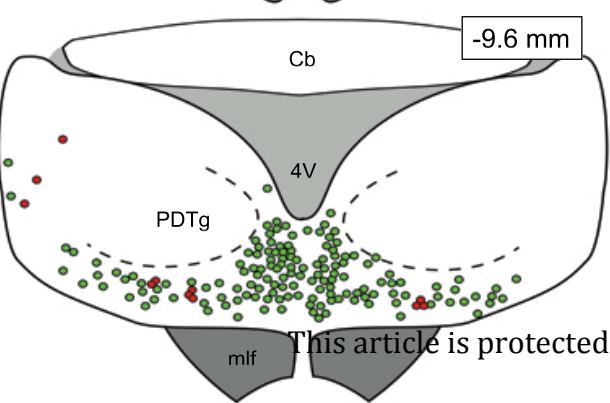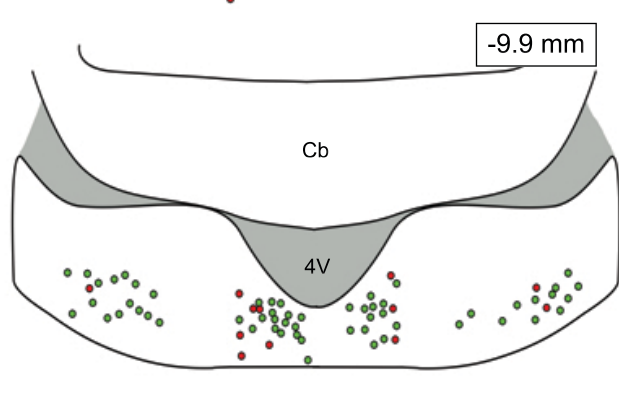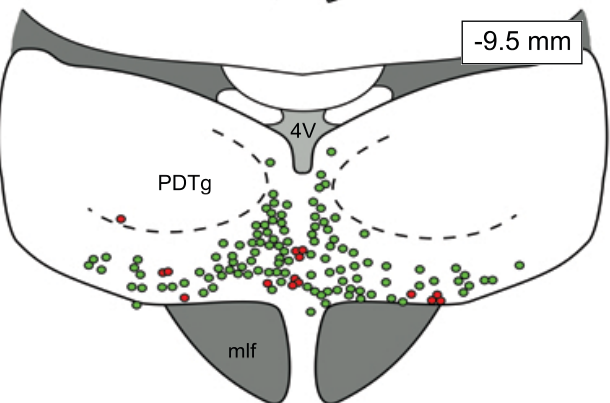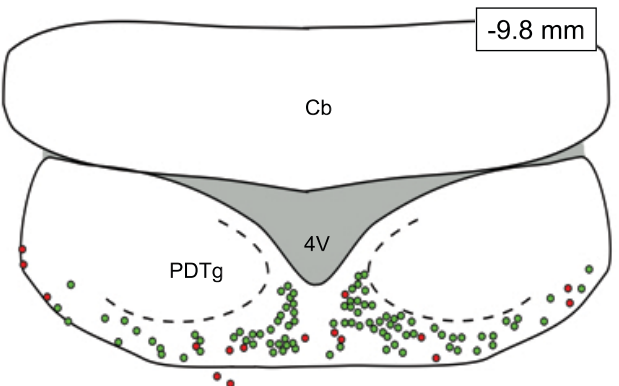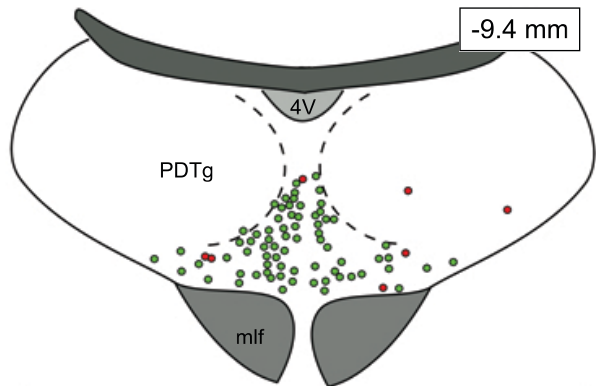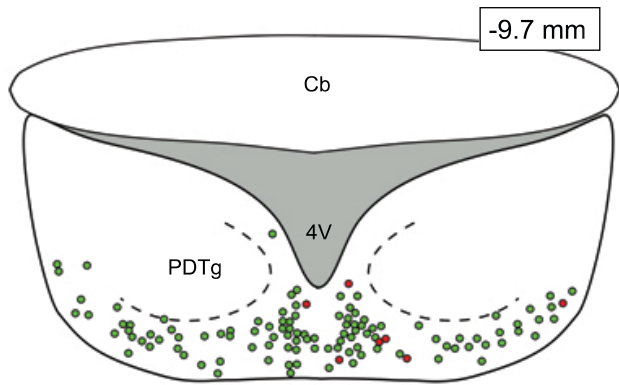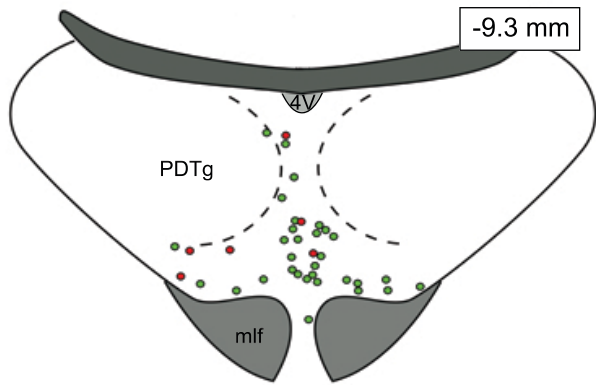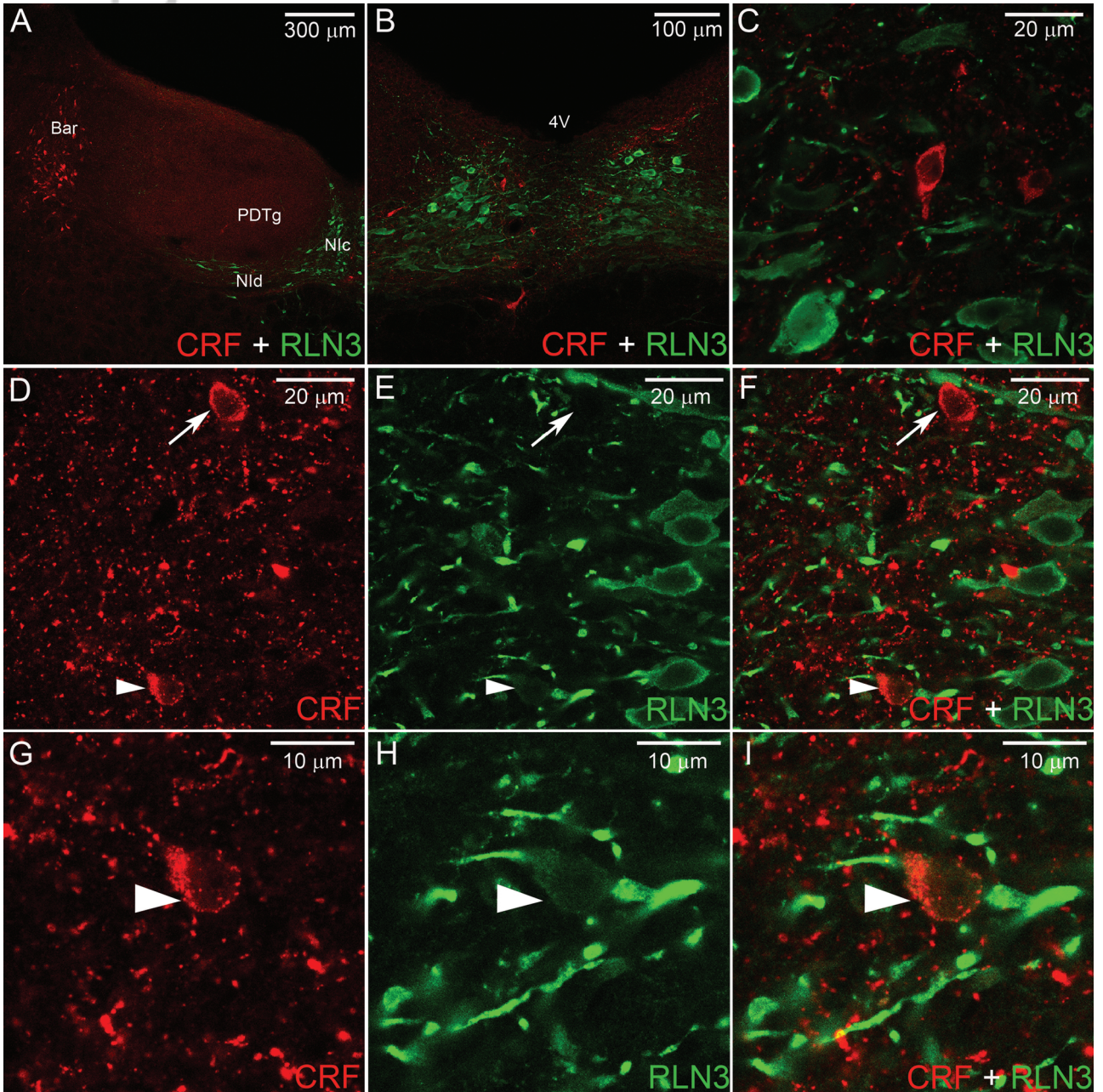
ADB_12426_F6.tif

ADB_12426_F7.tiff