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CENTRAL AMYGDALA RELAXIN-3/RXFP3 SIGNALLING MODULATES ALCOHOL-SEEKING IN RATS

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Abstract

Background and purpose

Alcohol use disorders are a leading cause of preventable deaths worldwide and stress is a major trigger of relapse. The neuropeptide relaxin-3 and its cognate receptor, relaxin family peptide receptor 3 (RXFP3), modulate stress-induced relapse to alcohol seeking in rats and while the bed nucleus of the stria terminalis has been implicated in this regard, the central nucleus of the amygdala (CeA) also receives a relaxin-3 innervation and CeA neurons densely express RXFP3 mRNA. Moreover, the CeA is consistently implicated in both stress and addictive disorders. Yohimbine precipitates relapse-like behaviour in rodents, although exactly how yohimbine induces relapse is unknown, possibly by increasing stress levels and inducing heightened cue reactivity.

Experimental Approach

In the current study, we examined the effects of yohimbine (1 mg/kg, i.p.) on anxiety-like behaviour in alcohol-experienced rats. Further, we assessed CeA neuronal activation following yohimbine-induced reinstatement of alcohol seeking, and the role of the relaxin-3/RXFP3 signalling within the CeA in yohimbine-induced reinstatement to alcohol seeking.

Key Results

Low dose yohimbine was anxiogenic in rats with a history of alcohol use. Furthermore, yohimbine-induced reinstatement of alcohol seeking increased Fos activation in CeA corticotrophin releasing factor (CRF), dynorphin (DYN) and GABA neurons compared to naïve and vehicle controls. Bilateral intra-CeA injections of the selective RXFP3 antagonist, R3(B1-22)R, attenuated yohimbine-induced reinstatement of alcohol seeking.

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Conclusions

Collectively, these data suggest the CeA is a node where yohimbine acts to induce reinstatement of alcohol seeking and implicate the relaxin-3/RXFP3 system within the CeA in this process.

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Abbreviations

AUD – Alcohol use disorders
BEC – blood ethanol concentration
BNST – bed nucleus of the stria terminalis
CeA – central nucleus of the amygdala
CRF – corticotrophin releasing factor
DALY - disability-adjusted life years
DYN – dynorphin
EXT - extinction
iP – Indiana alcohol preferring
IR - immunoreactivity
L/D – light/dark
NAV - naive
NDS – normal donkey serum
NI – nucleus incertus
OX - orexin
PFA - paraformaldehyde
REIN - reinstatement
RXFP3 – relaxin family peptide 3 receptor
VEH - vehicle

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Introduction

Addiction is a chronic relapsing disorder whereby addicts take drugs despite negative effects and consequences. Alcohol use disorders (AUDs) are a leading cause of preventable death worldwide, accounting for 5.5% of the global burden of disease and 4.6% of disability-adjusted life years (DALY) (World Health Organisation (WHO), 2014). Self-medication is a widely recognized hypothesis of drug and alcohol use, with almost 15% of individuals with an anxiety disorder also having a comorbid AUD (Robinson *et al.*, 2009). Stress-related disorders including anxiety can precipitate alcohol abuse and are a common symptom during withdrawal from alcohol dependence. At a population level, current therapeutics to treat AUDs suffer from low compliance, adverse side effects and high rates of relapse (Jupp *et al.*, 2010). Up to 90% of addicted individuals relapse within one year of abstinence (Dejong, 1994), most within three months (Sinha, 2008). Therefore a more detailed understanding of addiction neurobiology may help in the development of new therapeutic targets and treatment strategies to manage both anxiety and substance use disorders.

One potential target for pharmacotherapeutic development to treat AUDs is the cognate G-protein-coupled receptor for the conserved neuropeptide, relaxin-3, known as relaxin family peptide 3 receptor (RXFP3) (Bathgate *et al.*, 2002). Relaxin-3 is predominantly expressed in γ-aminobutyric acid (GABA) neurons in the hindbrain nucleus incertus (NI), which projects widely to forebrain areas, including the amygdala, bed nucleus of the stria terminalis (BNST), hippocampus, and lateral hypothalamus, which also express high levels of RXFP3 (Goto *et al.*, 2001; Ma *et al.*, 2007; Olucha-Bordonau *et al.*, 2012; Santos *et al.*, 2016). The NI relaxin-3/RXFP3 is implicated in stress and arousal related behaviours (Tanaka *et al.*, 2005; Banerjee *et al.*, 2010; Ma *et al.*, 2015; Walker *et al.*, 2017). In addition, central administration of the

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selective RXFP3 antagonist, R3(B1-22)R , attenuates both operant alcohol self-administration and stress-induced relapse to alcohol seeking (Ryan *et al.*, 2013), mediated by corticotrophin-releasing factor (CRF_1) receptor and orexin (OX_2) receptor signalling within the NI (Kastman *et al.*, 2016; Walker *et al.*, 2016). At a circuit level, the BNST has been implicated as a node in which relaxin-3/RXFP3 signalling facilitates alcohol seeking (Ryan *et al.*, 2013). However, since intra-BNST RXFP3 antagonism only partially attenuated stress-induced alcohol seeking, other brain regions are likely involved (Ryan *et al.*, 2013). Given that the central nucleus of the amygdala (CeA) is consistently implicated in both addiction and fear/anxiety-related conditions (see (Gilpin *et al.*, 2015) for review), receives afferent input from relaxin-3 neurons in the NI (Santos *et al.*, 2016) and contains high levels of RXFP3 mRNA (Ma *et al.*, 2007), we hypothesised that the CeA may constitute another node in which relaxin-3/RXFP3 signalling mediates alcohol seeking.

The CeA is a major component of the 'extended amygdala', a group of interconnected basal forebrain structures that constitute integral parts of the central stress system associated with the negative reinforcement of dependence and with neurotransmitters linked to the positive reinforcing effects of drugs of abuse (Koob *et al.*, 2005). This nucleus has been identified as a key neural substrate for alcohol drinking and alcohol dependence related behaviours (Koob *et al.*, 2010; Gilpin *et al.*, 2015) and is highly stress-sensitive (Singewald *et al.*, 2003; Funk *et al.*, 2006a; Funk *et al.*, 2016). Studies have reported activation of the CeA following footshock-induced reinstatement of alcohol seeking (Zhao *et al.*, 2006; Schank *et al.*, 2015); however, this is yet to be examined following yohimbine-induced reinstatement of alcohol seeking. Furthermore, multiple neuropeptide and neuromodulatory systems are dysregulated by alcohol, including CRF and dynorphin (DYN), which can contribute to excessive alcohol consumption and negative emotional symptoms of alcohol withdrawal by converging on GABA

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circuitry in the CeA (Roberto *et al.*, 2004; Roberto *et al.*, 2010; Gilpin *et al.*, 2015). For example, acute and chronic alcohol consumption increase GABA transmission in the CeA (Roberto *et al.*, 2004), while $GABA_A$ receptor antagonism in the CeA reduces alcohol self-administration in rats (Hyytia *et al.*, 1995).

The precise mechanisms by which yohimbine precipitates reinstatement of operant alcohol seeking are not fully understood. Yohimbine is an α_2-adrenergic receptor antagonist that increases noradrenaline release in several brain areas (Tanaka *et al.*, 2000), elevates glucocorticoid levels in both rats (Marinelli *et al.*, 2007) and humans (Vythilingam *et al.*, 2000), and elicits anxiety in both rodents (Pellow *et al.*, 1987) and humans (McDougle *et al.*, 1995). A recent study reported that yohimbine reinstates operant responding in rats previously trained with lever pressing resulting in cue presentation with or without reward delivery, suggesting yohimbine may not necessarily act as a stressor (Chen *et al.*, 2015). Indeed, low doses of yohimbine that reinstate operant responding are not anxiogenic in alcohol naïve rats (Arrant *et al.*, 2013). However, alcohol (and other drugs of abuse) induces adaptations in brain stress systems (Koob, 2009a; Zhou *et al.*, 2014; Walker *et al.*, 2016), which may influence the impact of yohimbine in alcohol-experienced rats. In healthy human subjects, alcohol and yohimbine have additive effects on subjective reports of intoxication and anxiety (McDougle *et al.*, 1995), while yohimbine increases anxiety and cortisol levels in recently detoxified alcoholic subjects (Krystal *et al.*, 1994) and increases subjective craving in human alcoholics (Umhau *et al.*, 2011). Furthermore, yohimbine has been used extensively in animal models of stress-alcohol interaction, including stress-induced reinstatement of alcohol seeking paradigms (Marinelli *et al.*, 2007; Richards *et al.*, 2008; Le *et al.*, 2013; Ryan *et al.*, 2013; Funk *et al.*, 2016; Kastman *et al.*, 2016; Walker *et al.*, 2016). To our knowledge, previous investigations of the effects of yohimbine on anxiety-like behaviour and

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neuronal activation have examined alcohol naïve rats, and employed yohimbine doses typically higher than required to induce reinstatement of drug seeking (Singewald *et al.*, 2003; Funk *et al.*, 2006a).

Here we demonstrate for the first time that low doses of yohimbine are anxiogenic in male iP rats with previous alcohol / abstinence experience. Furthermore, we demonstrate that CRF, dynorphin (DYN) and GABA neuronal activation increases in the CeA following yohimbine-induced reinstatement of alcohol seeking and identify the CeA as another node within the 'stress-relapse' circuitry in which the relaxin-3/RXFP3 system acts to mediate stress (yohimbine)-induced reinstatement of alcohol seeking.

Methods

Animals

Adult male alcohol-preferring (iP) rats were obtained from the breeding colony of The Florey Institute of Neuroscience and Mental Health. Parental stock was obtained from Professor T.K. Li (while at Indiana University, IN, USA). This strain of rat was chosen as they voluntarily consume high levels of ethanol resulting in relevant blood ethanol concentrations (BECs) (Ryan et al., 2013; Walker et al., 2016). Rats were housed 2 per cage with a littermate in a 12 hour (h) light/dark cycle (0700 - 1900), with access to food (laboratory chow) and water *ad libitum*. After surgery rats were single housed. All behavioural studies were performed in accordance with the Prevention of Cruelty to Animals Act (2004), under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia (2013) and approved by The Florey Animal Ethics Committee. All efforts were made to minimize animal suffering and reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines.

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Alcohol self-administration

Male iP rats were trained to self-administer alcohol (10 percent (v/v)) under operant conditions using a fixed ratio of 3 (FR3, three lever presses for one reward delivery) during 20-minute sessions (Ryan *et al.*, 2013). Two levers were available during the session, one that delivered a 100 µL alcohol reward (active lever), and the other that delivered the same volume of water (inactive lever). 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 soft- ware (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 positioned below a stimulus light and adjacent to a fluid receptacle. Availability of alcohol was conditioned by the presence of an olfactory cue (S+; one drop of vanilla essence (Queen Foods, Alderley, QLD, Australia)) placed directly underneath the alcohol-paired lever of the operant conditioning chamber. A 1-second light stimulus (CS+) occurred when the FR3 requirement was obtained with both the alcohol-paired and water-paired lever. Rats underwent an extended period of alcohol self-administration (>7 weeks, five consecutive days/week).

Light-dark box paradigm

Following alcohol self-administration male iP rats (n = 24) underwent 10 days of home cage abstinence and were subsequently tested for anxiety-like behaviour in a 10 min light-dark (L/D) box test. Experiments were conducted between 1000 and 1300 h. Rats were placed in the 42 cm (length) × 42 cm (width) × 40 cm (height) automated locomotor cell (Med Associates). Half of the locomotor cell was covered by the ‘dark’ box made of plastic opaque to visible light, but transparent to photo beams. A small opening (7 cm × 7 cm) enabled rats to enter or leave the ‘dark’ side. The light side was lit by an array

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of light-emitting diodes (475 LUX in the center), creating an aversive stimulus. Movements of rats were tracked using activity monitor software (Med Associates). Rats were administered with either the α_2-adrenergic receptor antagonist yohimbine, dissolved in distilled water (1mg/kg, i.p., Tocris Bioscience, Bristol, UK), or vehicle (1 ml/kg, i.p.) 30 min prior to being placed in the dark side of the box to commence the 10 min session. Boxes were cleaned and dried between each test.

Extinction & yohimbine-induced reinstatement of alcohol seeking

In order to model relapse of alcohol seeking, rats underwent extinction-reinstatement testing (Ryan *et al.*, 2013). During extinction training, no cues were present and there was no programmed response following instrumental responses. Extinction sessions continued until mean responding on the alcohol-paired lever were <15 lever presses for three consecutive days (~10 days in total). Rats were habituated to i.p. injections for the final 4-5 days of extinction training. To examine reinstatement, lever responding was measured in rats during a 20 min operant session, 30 min following administration of yohimbine (1 mg/kg, i.p.) under the same conditions used during self-administration with the exception that there were no deliveries of ethanol or water.

Immunohistochemistry

Another cohort of alcohol-experienced rats were randomly divided into three groups; group 1 (n = 5) were administered yohimbine (1 mg/kg, i.p.), and 30 min later underwent reinstatement testing. Group 2 (n = 5) were administered yohimbine (1 mg/kg, i.p.) but returned to their home cage and not subjected to reinstatement. Group 3 (n = 5) were administered vehicle (1 mL/kg, i.p.), while another group of age-matched alcohol naïve rats (n = 5) did not receive any intervention before perfusion. Rats were anaesthetised (pentobarbitone 100 mg/kg, i.p., Virbac, Milperra, NSW, Australia) 60 min after the end of

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reinstatement testing (or equivalent time) and transcardially perfused. Rats were perfused with 400 mL phosphate buffered saline (PBS, 0.1 M, pH 7.4) followed by 400 mL 1% paraformaldehyde (PFA, Sigma-Aldrich, Sydney, NSW, Australia) in PBS with 15% (v/v) picric acid (Sigma-Aldrich), decapitated, brains removed and post-fixed (1 h) in 50 mL perfusion solution. Brains were incubated in 20% sucrose in PBS (50 mL) at 4°C overnight, before being frozen over liquid nitrogen and stored at -80^{o}C. Coronal sections (50 µm) were cut on a cryostat at -18°C (Cryocut, 1800; Leica Microsystems, Heerbrugg, Switzerland) and collected into PBS in a 1/3 series. Sections were pre-blocked in 10% normal donkey serum (NDS, Millipore, Billerica, MA, USA) and 0.5% Triton-X (BDH Chemicals, QLD, Australia) in PBS, for 1 h at room temperature (RT). Sections were then incubated in a primary antibody mix of either rabbit anti-CRF (1:1000, PBL#C70) (Sawchenko et al., 1984), guinea pig anti-prodynorphin (pDYN, 1:1000; Neuromics, Edina, MN, USA, GP10109) or rabbit anti-GABA (1:1000; Sigma Aldrich, A2052), with 1:500 goat anti-c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA, #SC-52-G), 2% NDS in PBS containing 0.1% TritonX-100 (48-72 h at 4°C). Sections were washed 3 × 5 min in PBS and incubated for 2 h with donkey anti-goat-Alexa 647 (1:400; Life Technologies, Carlsbad, CA, USA, A21447) and donkey anti-rabbit-Alexa 488 (1:400; Life Technologies, A21206). For pDYN sections were incubated for 2 h in Biotin-SP affinity pure donkey anti-guinea pig (1:500; Jackson ImmunoResearch, West Grove, PA, USA, JI706065148), washed 3 × 5 min in PBS and then incubated for 2 h at room temperature with donkey anti-goat-Alexa 647 (1:400; Life Technologies, A21447) and streptavidin-Alexa 488 (1:400; Life Technologies, S11223). Sections were washed 3 × 5 min, mounted on microscope slides and coverslipped with fluorescence mounting medium (DAKO, Carpentaria, CA, USA). Images were acquired on a confocal microscope (Leica DM LB2, Leica Microsystems, Wetzlar, Germany) and quantified using Image J (NIH). The number of CRF, pDYN, GABA, Fos-positive neurons and % CRF, %pDYN or %GABA neurons that

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expressed Fos were determined unilaterally in a 1-in-3 series throughout the CeA from bregma 2.1 mm (Paxinos *et al.*, 1986) by an investigator blinded to treatment allocation.

Stereotaxic implantation of central nucleus of the amygdala cannulae

After stabilization of alcohol self-administration a separate cohort of rats were anaesthetised and underwent bilateral stereotaxic implantation of cannulae into the CeA. Rats were deeply anaesthetised with isoflurane (5% (v/v) induction, 2% maintenance), positioned in a stereotaxic frame (Stoelting Co. Wood Dale, IL; USA) and the scalp shaved and cleaned with 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 stainless steel 26 gauge bilateral cannula cut 8 mm below the pedestal (PlasticsOne, Roanoke, VA, USA) was implanted relative to bregma: anteroposterior, -2.2 mm; mediolateral, ±4.4 mm and dorsoventral, -9.5 mm (Paxinos *et al.*, 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) and analgesia (Meloxicam; 3 mg/kg, i.p., Troy Laboratories, Glendenning, NSW) plus antibiotic (Baytril, enrofloxacin, 3 mg/kg, i.p, Bayer Health Care, Pymble, NSW, Australia) were administered.

Central nucleus of the amygdala infusions

After recovery from surgery, rats re-acquired alcohol responding to pre-surgical levels before extinction training as described. Once extinction criteria were met, rats underwent a yohimbine-induced reinstatement session, whereby yohimbine (1 mg/kg, i.p.) was administered 30 min prior to test (Ryan *et al.*, 2013). During this session, rats (n = 19) received bilateral intra-CeA

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infusions of either the selective RXFP3 receptor antagonist R3(B1-22)R (1 µg in 0.5 µL aCSF) (Ryan *et al.*, 2013) or vehicle, directly prior to reinstatement testing in a randomized manner. This dose was chosen as it reduced yohimbine-induced reinstatement of alcohol seeking within the BNST (Ryan et al., 2013). Subsequently, rats received 2 days of alcohol reacquisition to re-stabilize alcohol consumption and were then re-extinguished. A second reinstatement test was performed in which rats received the alternate intra-CeA treatment in a counterbalanced manner. Rats in which the injections were outside the CeA were used as anatomical controls (n = 7). Bilateral CeA infusions were made using 40 cm polyethylene connectors (PlasticsOne) attached to 1 µl microsyringes (SGE Analytical Science, Ringwood, VIC, Australia). R3(B1-22)R or vehicle was infused bilaterally (0.25 µl/min) by an automated syringe pump (Harvard Apparatus, Holliston, MA, USA) and injectors were left in place for 2 min after infusion. After completion of behavioural testing, rats were anesthetized with pentobarbitone (100 mg/kg, i.p.). Correct cannula positioning was verified in each rat by infusing methylene blue (0.25 µl/side). After decapitation under anaesthesia, brains were collected, frozen over liquid nitrogen and sectioned for injection site validation, which was performed by an investigator blinded to the identity of the treatment.

Data and Statistical analysis

All data analysis and generation of histograms were performed using GraphPad Prism Version 6 for Windows (GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com). L/D box data was examined with an unpaired Student's t-test. Differences in the number of CRF-IR, pDYN-IR, GABA-IR, Fos-IR, co-localisation of CRF, pDYn or GABA with Fos-IR and % of activated CRF, pDYN or GABA cells were analyzed using one-way ANOVA with post-hoc Tukey's Multiple Comparison Tests. Reinstatement and time course data were analyzed using repeated measures (RM) two-way ANOVA with post-hoc

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Tukey's Multiple Comparison Tests. Latency to reward was analyzed using a Student's t-test. Differences were considered significant at $p < 0.05$. Data are presented as mean ± SEM. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

Results

Low dose yohimbine provokes anxiety in alcohol experienced rats

We assessed the effect of yohimbine administration on anxiety-like behaviour in adult male iP rats with prior alcohol self-administration experience. During alcohol self-administration, rats averaged 92 ± 9 active lever responses (0.59 ± 0.05 g/kg/session alcohol intake). Following 10 days of home cage abstinence rats were tested for anxiety-like behaviour in the light-dark box 30 min following yohimbine (1 mg/kg, i.p.) or vehicle (1 ml/kg, i.p.) administration. Yohimbine-treated rats spent significantly less time in the light side of the locomotor cell (unpaired Student's t-test, $p < 0.05$, Fig. 1).

GABA, CRF and pDYN neurons in CeA are activated by yohimbine-induced reinstatement of alcohol seeking

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Next we examined neuronal activation in the CeA in alcohol naïve rats or rats which underwent extended alcohol self-administration/extinction followed by vehicle administration, yohimbine administration or yohimbine-induced reinstatement of alcohol seeking. During the last 5 days of alcohol self-administration, rats averaged 90 ± 7 active lever responses (0.59 ± 0.04 g/kg/session alcohol intake. No differences were observed in ethanol self-administration (Fig. 2A) or extinction (Fig. 2B) across groups. For rats which underwent reinstatement, two way ANOVA revealed a main effect of treatment ($F_{(2, 10)} = 67.72$, $p < 0.05$), lever ($F_{(1, 5)} = 146.3$, $p < 0.05$), and a treatment × lever interaction ($F_{(2, 10)} = 72.05$, $p < 0.05$). Post-hoc analysis revealed that active lever responding significantly decreased during extinction training ($p < 0.05$) and yohimbine precipitated reinstatement of active lever ($p < 0.05$), but not inactive lever ($p > 0.05$), responding (Fig 2C).

Subsequently, we examined neuronal activation in the CeA as reflected by Fos-IR following yohimbine-induced reinstatement of alcohol seeking. One-way ANOVA revealed an overall main effect of treatment on the number of Fos-positive neurons in 3 separate series of adjacent sections (lowest $F = 10.38$, $p < 0.05$). Post-hoc analysis showed a significant increase in the number of Fos-positive neurons in the yohimbine-treated and yohimbine-induced reinstatement rats, compared to vehicle or naïve (p values < 0.05, Table 1). No differences were observed between naïve and vehicle-treated rats ($p > 0.05$). Furthermore, no significant differences were observed in the number of GABA-IR ($F_{(3, 16)} = 0.946$, $p > 0.05$), CRF-IR ($F_{(3, 16)} = 1.61$, $p > 0.05$) or pDYN-IR ($F_{(3, 16)} = 0.75$, $p > 0.05$) across treatment groups (Table 1). One-way ANOVA revealed an overall main effect of treatment on the number of GABA/Fos-IR cells ($F_{(3, 16)} = 28.61$, $p < 0.05$) and % GABA neurons co-expressing Fos-IR ($F_{(3, 16)} = 54.75$, $p < 0.05$). Post-hoc analysis revealed a significant increase in the number GABA/Fos- IR and %GABA/Fos-positive neurons in the yohimbine-treated and yohimbine-induced reinstatement rats,

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compared to all other groups (p values < 0.05, Fig. 2J/K). No differences were observed between naïve and vehicle-treated rats (p > 0.05; Table 1).

The % CRF neurons co-expressing Fos-IR ($F_{(3, 16)} = 5.32$, $p < 0.05$) also displayed a significant difference. Post-hoc analysis revealed a significant increase in %CRF/Fos-positive neurons in the yohimbine-induced reinstatement rats, compared to naïve and vehicle-treated groups (p values < 0.05, Fig. 2F/G). No differences were observed between naïve and vehicle-treated rats (p > 0.05; Table 1). One-way ANOVA also revealed a significant increase in % pDYN neurons co-expressing Fos-IR ($F_{(3, 16)} = 16.15$, $p < 0.05$), with post-hoc analysis showing a significant increase in the % pDYN/Fos-positive neurons in the yohimbine-induced reinstatement rats, compared to naïve, vehicle- and yohimbine-treated groups (p values < 0.05, Fig. 2H/I). Once again, no differences were observed between naïve and vehicle-treated rats (p > 0.05; Table 1).

Bilateral administration of R3(B1-22)R into CeA attenuates yohimbine-induced reinstatement of alcohol-seeking in iP rats

Lastly, we investigated the effect of bilateral intra-CeA injections of R3(B1-22)R on yohimbine-induced reinstatement of alcohol seeking. Rats made 122 ± 9 active lever responses, consuming 0.76 ± 0.04 g/kg/session of alcohol during self-administration training prior to, and following surgery. RM two-way ANOVA revealed a main effect of lever ($F_{(1, 18)} = 203.1$, $p < 0.05$), treatment ($F_{(2, 36)} = 39.16$, $p < 0.05$) and a treatment × lever interaction ($F_{(2, 36)} = 53.31$, $p < 0.05$). Tukey's post- hoc analysis indicated that yohimbine precipitated alcohol seeking in vehicle-treated rats (EXT vs. VEH, $p < 0.05$), while R3(B1-22)R injected bilaterally into the CeA significantly attenuated yohimbine-induced reinstatement (VEH vs. R3(B1-22)R, $p < 0.05$; Fig. 3A), with ~42% reduction in active lever responding compared to vehicle treated rats. No differences were observed on the inactive lever (p values > 0.05). Analysis of

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time course data indicated an effect of R3(B1-22)R on active lever responding over time with a main effect of treatment ($F_{(1, 34)} = 11.26$, $p < 0.05$) and time ($F_{(3,102)} = 29.27$, $p < 0.05$). Post-hoc analysis revealed a significant reduction at the early stage of the reinstatement session when responding is at its highest ($p < 0.05$; Fig. 3D), although no difference in latency to active lever response was observed (Student's t-test $p < 0.05$; Fig. 3C). Injection sites were validated histologically for all R3(B1-22)R-treated rats (Fig. 3E). Rats in which the injection sites were adjacent to the CeA were analysed separately as anatomical controls. RM two-way ANOVA revealed a main effect of lever ($F_{(1,6)} = 36.19$, $p < 0.05$), treatment ($F_{(2, 12)} = 5.61$, $p < 0.05$) and a treatment × lever interaction ($F_{(2, 12)} = 5.61$, $p < 0.05$). Tukey's post-hoc analysis indicated that yohimbine precipitated alcohol seeking in vehicle-treated rats (EXT vs. VEH, $p < 0.05$); however, R3(B1-22)R injected immediately adjacent to the CeA failed to significantly attenuate yohimbine-induced reinstatement (VEH vs. R3(B1-22)R, $p = 0.467$, Fig. 3B), with a non-significant ~22% reduction in active lever responding compared to vehicle treated rats. No differences were observed on the inactive lever (p values > 0.05).

Discussion

These experiments provide evidence that low dose yohimbine (1 mg/kg) produces anxiety-like behaviour in male iP rats with the same history of alcohol self-administration followed by abstinence as used in our model of relapse to alcohol seeking. Furthermore, in these alcohol-experienced rats, Fos immunohistochemistry revealed that CRF, pDYN and GABA neurons within the CeA are activated following yohimbine-induced reinstatement of alcohol seeking. We also provide evidence for the involvement of relaxin-3/RXFP3 signalling within the CeA in yohimbine-induced reinstatement of alcohol seeking in iP rats.

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Effects of yohimbine on anxiety-like behaviour have been previously observed in alcohol naïve rats at doses ranging from 2.5 - 10 mg/kg (Pellow *et al.*, 1985; Baldwin *et al.*, 1989), but these effects were not observed at a lower 1 mg/kg dose (Pellow *et al.*, 1985; Arrant *et al.*, 2013), except in rats that have been housed in isolation (Cai *et al.*, 2012). Given these data and recent evidence suggesting yohimbine reinstates lever pressing in rats that were previously trained with lever pressing resulting in cue presentation with or without reward delivery (Chen *et al.*, 2015), yohimbine may, theoretically at least, act in a similar manner to reinstate alcohol seeking. In this study we tested male iP rats after 10 days of abstinence from alcohol, to align with the duration of extinction training when rats do not have access to alcohol. Under these conditions, yohimbine (1 mg/kg) was anxiogenic. Notably, as withdrawal from alcohol alters stress systems (Zorrilla *et al.*, 2001), it is possible that long-term alcohol experience plus subsequent abstinence contribute to the anxiogenic properties of low dose yohimbine. Accordingly, we suggest that although yohimbine may increase cue reactivity, in our model of alcohol seeking, yohimbine is also an anxiogenic acute stressor that apparently contributes to reinstatement of alcohol seeking.

Low dose yohimbine also increased neuronal activation in the CeA of alcohol experienced / abstinent rats. These data are in line with studies in alcohol naïve rats, whereby higher doses of yohimbine also result in activation of the CeA (Singewald *et al.*, 2003; Funk *et al.*, 2006a; Funk *et al.*, 2016). We also observed an increase in CeA Fos-IR following yohimbine-induced reinstatement, similar to that observed following footshock-induced reinstatement of alcohol seeking (Zhao *et al.*, 2006; Schank *et al.*, 2015). Both footshock stress and yohimbine activate similar brain regions, increase CRF mRNA and induce relapse to drug (including alcohol) seeking driven via a CRF-dependent mechanism (Le *et al.*, 2000; Funk *et al.*, 2006a; Funk *et al.*, 2006b; Marinelli *et al.*, 2007). Therefore, findings that both yohimbine and

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yohimbine-induced reinstatement of alcohol seeking increase Fos-IR within the CeA implicates the CeA as a key structure in the precipitation of 'relapse' after exposure to multiple stressors, including yohimbine.

Notably, the majority of Fos-IR was localised within the lateral and capsular divisions of the CeA, while sparse within the medial division of the CeA. The lateral and capsular divisions of the CeA receive concentrated dopaminergic innervation (Freedman *et al.*, 1994), and express a variety of neuropeptides implicated in addictive disorders. These include CRF, DYN, cocaine and amphetamine regulated transcript, neuropeptide Y and nociceptin (Koylu *et al.*, 1997; Roberto *et al.*, 2012). Neuropeptides have been attributed a prominent role in negative affective aspects of drug and alcohol abuse (Koob, 2009b). The relationship between stress-related disorders and addiction-related behaviours has been a focus of research in recent years, driven in part by high comorbidity in the clinical setting (Volkow, 2004). The CRF/CRF_1 system has been strongly implicated in these behaviours (see (Koob, 2013) for review). CRF neurons in the CeA are a combination of GABAergic and glutamatergic, projection and interneurons (Pomrenze *et al.*, 2015) and alcohol and CRF signalling in the CeA increases GABAergic *and* glutamatergic transmission (Roberto *et al.*, 2004).

More than half of the neurons activated following yohimbine-induced reinstatement of alcohol seeking were GABA positive, representing activation of ~30% of all GABAergic neurons in the CeA. Due to the topography of Fos-IR we further assessed the involvement of two neuropeptides strongly implicated in stress-induced relapse, CRF and DYN. Significant increases in % dual CRF/Fos-IR and DYN/Fos-IR were observed following yohimbine-induced reinstatement of alcohol seeking. These data are potentially an under estimate caused by limitations in the detection of cytoplasmic CRF peptide without colchicine pretreatment, which we deliberately avoided due to

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confounds with behavioural and Fos expression studies. Nevertheless, our findings clearly establish that subpopulations of both CRF and DYN neurons within the CeA are activated during yohimbine-induced reinstatement of alcohol seeking.

An endogenous ligand for the Kappa opioid receptor (KOR), DYN is implicated in alcohol abuse and dependence, as well as the reinforcing and rewarding properties of other drugs of abuse. Further, the DYN/KOR system induces dysphoric/anhedonic properties, which may contribute to alcohol seeking and consumption (Walker *et al.*, 2012). In line with this, we observed an increase in activation of DYN positive neurons within the CeA following yohimbine-induced reinstatement of alcohol seeking. Further, the CRF and DYN systems within the CeA are also closely linked, with 1/3 of CRF neurons also expressing DYN (Marchant *et al.*, 2007).

Emerging evidence suggests that CRF signalling interacts with the relaxin-3/RXFP3 system to modulate both stress responses and alcohol seeking (Tanaka *et al.*, 2005; Banerjee *et al.*, 2010; Ma *et al.*, 2013; Ma *et al.*, 2015; Walker *et al.*, 2016; Walker *et al.*, 2017). Given that the CeA is a stress-sensitive node in reward circuitry (Funk *et al.*, 2006b; Koob, 2009a; Koob, 2009b; Gilpin *et al.*, 2015), receives relaxin-3 innervation from the NI (Goto *et al.*, 2001; Olucha-Bordonau *et al.*, 2012; Santos *et al.*, 2016) and expresses dense RXFP3 mRNA (Ma *et al.*, 2007), we tested the effects of the selective RXFP3 receptor antagonist, R3(B1-22)R, on yohimbine-induced reinstatement of alcohol seeking. Intra-CeA microinjection of R3(B1-22)R (1 µg) attenuated yohimbine-induced reinstatement of alcohol seeking in iP rats. In contrast, R3(B1-22)R injected immediately adjacent to the CeA had no effect, suggesting the action is specifically associated within the CeA. Antagonism of CeA RXFP3 decreased responding at the beginning of the test session, when responding is at its highest; however it did not alter latency,

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suggesting the effect of treatment is not due to sedation or a deficit in procedural memory. Furthermore, central administration of a 10-fold higher dose of R3(B1-22)R has no effect on locomotor activity in iP rats, suggesting the antagonist did not impact general activity (Ryan *et al.*, 2013).

The CeA therefore represents an additional locus where RXFP3 signalling is involved in yohimbine-induced reinstatement to alcohol seeking, with previous reports implicating the BNST (Ryan *et al.*, 2013). The degree of attenuation of reinstatement observed after bilateral intra-CeA R3(B1-22)R administration is similar to that observed when the peptide antagonist was delivered within the BNST. The CeA and BNST have extensive interconnectivity and both regions are involved in controlling fear and anxiety responses to environmental stimuli (see (Tovote *et al.*, 2015) for review). Relaxin-3/RXFP3 signalling may act jointly in these regions to facilitate yohimbine-induced reinstatement of alcohol seeking, however, the involvement of other brain regions cannot be ruled out. Our immunohistochemical data suggest that GABA, CRF and DYN signalling in the CeA is activated following yohimbine-induced reinstatement of alcohol seeking which may be influenced by intra-CeA relaxin-3/RXFP3 signalling to drive relapse to alcohol seeking. Given RXFP3 is $G_{i/o}$ coupled G protein receptor, and the CeA has a complex network of intrinsic connectivity, relaxin-3/RXFP3 signalling in the CeA may facilitate relapse to alcohol seeking via disinhibition of downstream CeA signalling. This and other anatomical and molecular questions require further investigation.

Collectively our results emphasize the role of the CeA as an interface of stress and relapse to alcohol seeking. We provide evidence that low doses of yohimbine increase anxiety-like behaviour in iP rats with previous alcohol experience. Furthermore, in alcohol-experienced rats low dose yohimbine causes robust activation of CeA GABAergic neurons, (plus CRF and DYN) and reinstatement of alcohol seeking. Relaxin-3/RXFP3 signalling within the

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CeA contributes to yohimbine-induced reinstatement of alcohol seeking. We therefore suggest that the relaxin-3/RXFP3 system represents a legitimate potential target for the treatment of both anxiety and substance use disorders.

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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 conducted experiments and performed related analysis; LCW and AJL wrote the manuscript. All authors reviewed the content and approved the final version of the manuscript.

Conflict of interest

LCW, HEK, EVK, ALG, AJL declare no competing conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure legends

Figure 1. Yohimbine (YOH) administration in alcohol experienced iP rats decreased the % time spent in the light compartment of the light/dark box test (*p < 0.05). Data shown as mean ± SEM (n = 12/group).

Figure 2. Yohimbine-induced reinstatement of alcohol seeking activates CRF, pDYN and GABA neurons in the central nucleus of the amygdala (CeA) of iP rats. Active lever responding during (**A**) the last 5 days of self-administration and (**B**) extinction training across treatment groups. (**C**) Yohimbine administration induced reinstatement of active lever responding (*p < 0.05), but not inactive lever responding. (**D**) Rat brain schematic outlining CeA region in which CRF, pDYN, GABA and Fos-IR was quantified. (**E_{1-3}**) Representative low magnification micrographs of CRF-, Fos- and merged-IR in the CeA. Histogram illustrating (**F**) % CRF cells, (**H**) % DYN cells, (**J**) % GABA cells activated in naïve (NAV), vehicle (VEH) treated, yohimbine (YOH)-treated and rats that underwent yohimbine-induced reinstatement of alcohol seeking (REIN). (**G_{1-3}**) Representative high magnification micrograph of CRF (green), Fos (magenta) and co-localisation in a REIN rat. (**I_{1-3}**) Representative image of pDYN (green), Fos (magenta) and co-localisation in a REIN rat. (**K_{1-3}**) Representative high magnification images of GABA (green), Fos (magenta) and co-localisation in a REIN rat. Rats that underwent reinstatement displayed significantly increased %CRF neurons expressing Fos, compared to VEH (*p < 0.05) and a significant increase in % pDYN neurons expressing Fos, compared to VEH and YOH-treated animals (#p < 0.05). YOH-treated and REIN rats displayed significantly increased Fos-IR and %GABA neurons expressing Fos, compared to VEH (*p < 0.05). Scale bar: low magnification, 150 μm; high magnification, 20 μm. All data expressed as mean ± SEM, n = 5/group.

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Figure 3. R3(B1-22)R injected bilaterally into the central nucleus of the amygdala (CeA) attenuates yohimbine-induced reinstatement for alcohol seeking in IP rats. Active lever and inactive lever responding following within subject counterbalanced (**A**) bilateral intra-CeA (n = 19) or (**B**) anatomical control (n = 7) injections of either vehicle (VEH) or R3(B1-22)R. (**C**) Latency to active lever responding for vehicle (VEH)- and R3(B1-22)R-treated rats and (**D**) time course of lever responding throughout reinstatement session. (**E**) Neuroanatomical representation of injection sites for R3(B1-22)R-treated rats. Green circles represent CeA injections, red circles represent anatomical controls. Data were analyzed by RM two-way ANOVA with post-hoc Tukey's multiple comparison test and expressed as mean ± SEM. Extinguished rats (EXT), VEH-, and R3(B1-22)R-treated rats. Yohimbine induced reinstatement of alcohol seeking in VEH-treated rats (EXT vs. VEH #$p < 0.05$), which was attenuated by R3(B1-22)R microinjection within the CeA (VEH vs. R3(B1-22)R *$p < 0.05$). Furthermore, time course data revealed a reduction in responding by R3(B1-22)R treated rats during the first 5 min (VEH vs. R3(B1-22)R *$p < 0.05$), but did not significantly increase latency to active lever responding. All data expressed as mean ± SEM.

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Tables

Table 1. Mean (SEM) counts of Fos-IR, CRF-IR, pDYN-IR, GABA-IR and number and percentages of dual peptide/Fos-IR cells in the CeA of iP rats

	pDYN-IR	Fos-IR	pDYN/Fos-IR	% pDYN/Fos-IR
NAV	157.2 (9)	9.7 (2)	1.5 (1)	1.0 (1)
VEH	162.8 (6)	25.8 (6)	4.6 (1)	2.8 (1)
YOH	149.8 (3)	133.4 (13) *	7.6 (1)	5.1 (1)
REIN	151.8 (6)	213 (21) *#	18 (4) *#	11.9 (2) *#

	CRF-IR	Fos-IR	CRF/Fos-IR	% CRF/Fos-IR
NAV	157.6 (5)	6.1 (1)	1.6 (1)	1.0 (1)
VEH	163.9 (14)	20.4 (6)	2.0 (1)	1.2 (1)
YOH	152.2 (6)	137.8 (4) *	3.6 (1)	2.4 (1)
REIN	135.9 (10)	217.2 (63) *	4.9 (1)*	3.6 (1) *

	GABA-IR	Fos-IR	GABA/Fos- IR	% GABA/Fos-IR
NAV	560.2 (17)	28 (3)	12.8 (3)	2.3 (1)
VEH	508.2 (28)	60.2 (14)	25.6 (6)	5.0 (3)
YOH	405.2 (45)	230.3 (28) *	119.5 (24) *	29.5 (10) *
REIN	507.4 (30)	274.4 (11) *	158.2 (13) *	31.2 (7) *

The number of CRF-IR, pDYN-IR, GABA-IR, Fos-IR and co-localisation of cells were counted in adjacent sections in a 1/3 series throughout the CeA from bregma 2.1 – 2.4 mm of alcohol naïve (NAV); vehicle-treated (VEH); yohimbine-treated (YOH) and yohimbine-induced reinstatement (REIN) treated iP rats. One-way ANOVA showed no significant difference in number of CRF-IR, pDYN-IR and GABA-IR cells across treatment groups ($p > 0.05$), while one-way ANOVA with Tukey's multiple comparisons revealed a significant difference between NAV/VEH animals and YOH/REIN animals with

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number of Fos-IR cells, number of GABA/Fos-IR cells and % GABA/Fos-IR cells. Number of CRF/Fos cells and % CRF/Fos-IR cells were also different in REIN rats compared to NAV/VEH groups, while the number of pDYN/Fos cells and %pDYN/Fos cells were significantly different to NAV/VEH/YOH-treated rats. *p < 0.05 compared to VEH-treated animals, #p< 0.05 compared to YOH-treated. n = 5/group. Data are presented as group means ± SEM.

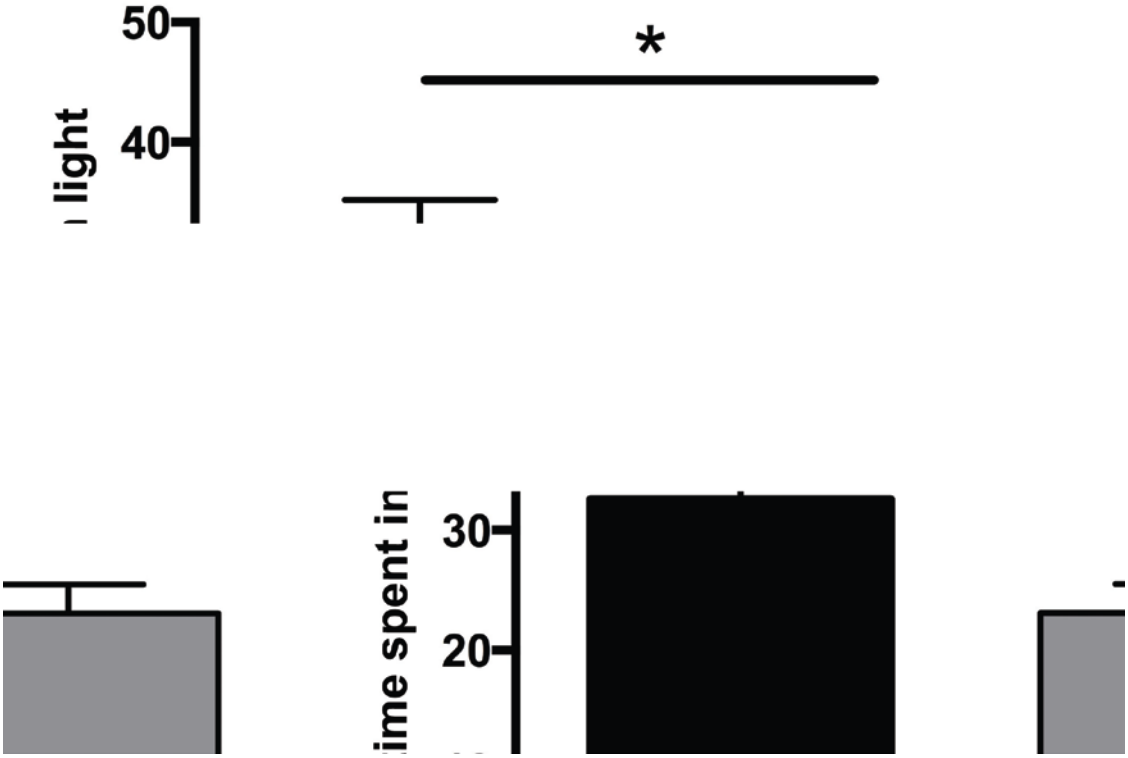

Figure 1

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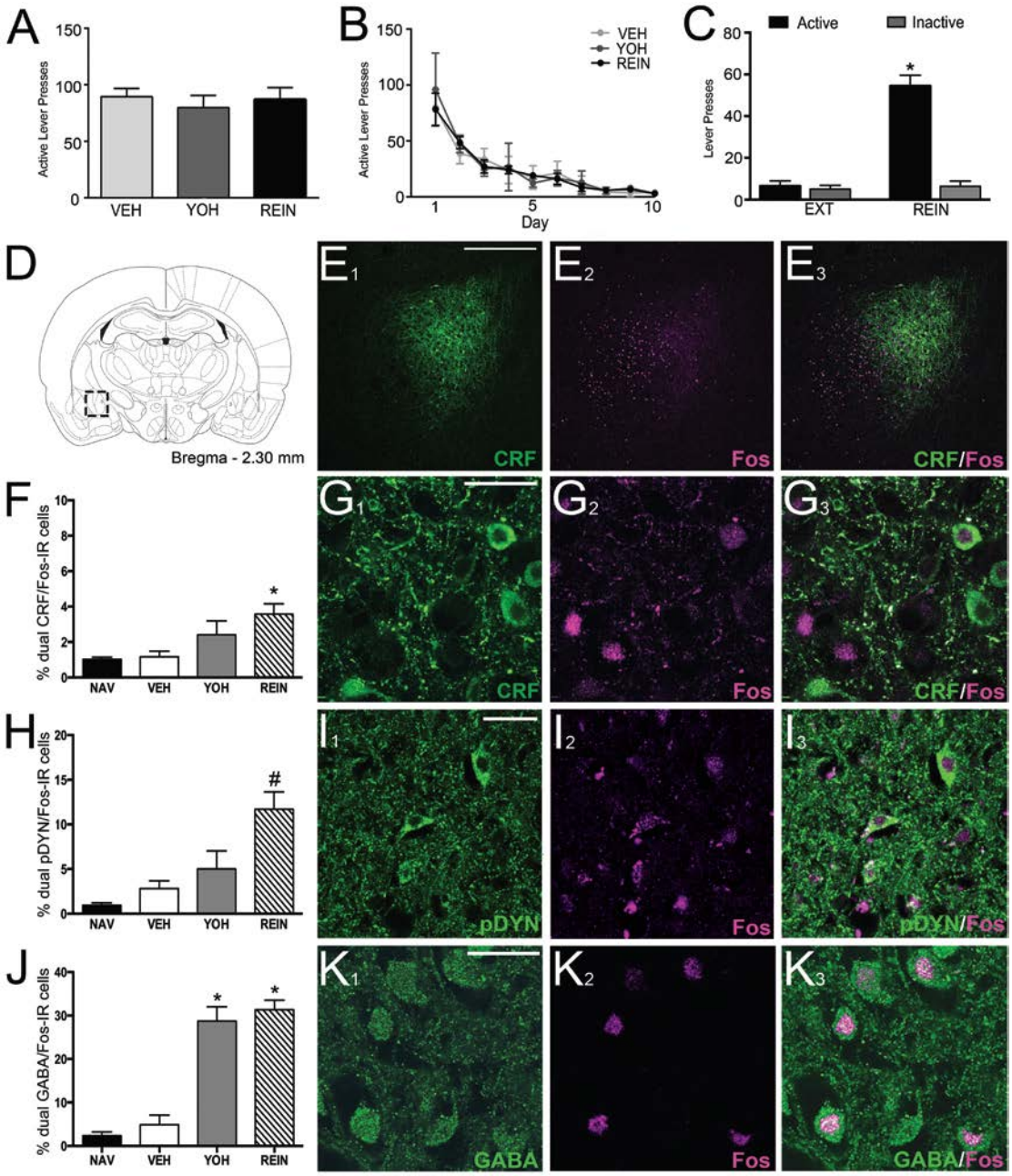

Figure 2

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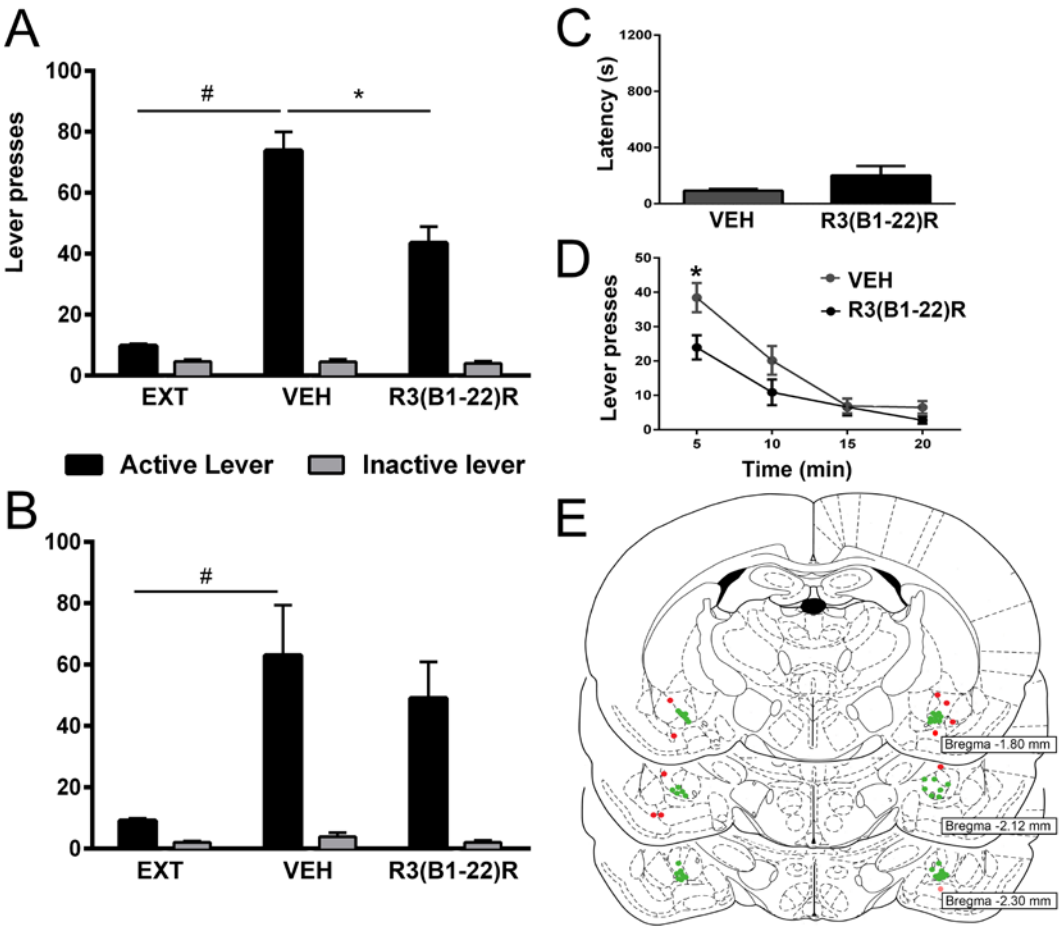

Figure 3

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