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Pattern of neural activation following yohimbine-induced reinstatement of alcohol seeking in rats

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Abstract

Alcohol use disorders represent an extensive socioeconomic burden, yet effective treatment options are suboptimal. A major hurdle in treating alcohol use disorders is the high rate of relapse. Stress is a major factor that promotes relapse in abstinent drug users; therefore, understanding neural mechanisms that underpin the effects of stress on alcohol seeking is critical. In rodent models of stress-induced relapse, the α_2-adrenoceptor antagonist, yohimbine, is a widely used chemical stressor to elicit reinstatement of drug/alcohol seeking. However, the exact mechanism how yohimbine precipitates reinstatement of alcohol seeking and the pattern of neural activation associated with yohimbine-induced reinstatement is poorly understood. Therefore, we counted Fos-protein positive nuclei across 42 brain regions in alcohol-experienced alcohol preferring rats that received either yohimbine in the home-cage (1 mg/kg i.p.) or following yohimbine-induced reinstatement of alcohol seeking. The number of Fos-protein positive nuclei was increased in the prefrontal cortex and extended amygdala after home-cage yohimbine compared to naïve and vehicle treated rats. Yohimbine-induced reinstatement increased the number of Fos-protein expressing nuclei in multiple other regions including the thalamus, hypothalamus and hippocampus. We then examined inter-regional correlations in Fos-protein expression for all 42 brain regions, which showed Fos expression was more strongly positively correlated following yohimbine-induced reinstatement of alcohol seeking, compared to home-cage yohimbine. These data suggest low dose yohimbine in a non-drug associated context activates stress/impulsivity

centres within the brain, while yohimbine in the drug associated context recruits additional brain regions to drive alcohol seeking.

Introduction

Despite the extensive disease burden of alcohol use disorders (AUD) effective pharmacotherapies are still lacking. While FDA approved medications are efficacious in certain subpopulations with AUD, they are all inadequate at a population level (Jupp & Lawrence, 2010; Lyon, 2017; Walker & Lawrence, 2018). A major obstacle in treating addiction is the high rate of relapse, with up to 90% of addicts relapsing within 12 months of abstinence (Dejong, 1994). While many factors can promote relapse in abstinent drug users, stress is a major contributor, with relapse often precipitated by exposure to stressors (Hefner & Curtin, 2012). Thus, understanding the neurocircuitry that underlies the influence of stress on alcohol seeking is critical for guiding future treatment strategies.

A common procedure used to study relapse-like behaviour in rodents is the extinction-reinstatement model (Shaham *et al.*, 2003). Stress-induced reinstatement of drug seeking can be induced by multiple stimuli, with intermittent foot shock and the pharmacological stressor yohimbine being most commonly employed (Mantsch *et al.*, 2016). Yohimbine is a frequently used stress manipulation in animal studies of reinstatement, due to its robust and reliable nature (Nair *et al.*, 2011; Bossert *et al.*, 2013; Walker *et al.*, 2017b) and translational relevance (See & Waters, 2011; Mantsch *et al.*, 2016). Yohimbine is an α_2-adrenoceptor antagonist that preferentially antagonises autoreceptors, increasing noradrenergic cell firing and enhancing noradrenaline release in terminal areas (Abercrombie *et al.*, 1988). Yohimbine induces anxiety-like responses in humans (Charney *et al.*, 1989; McDougle *et al.*, 1995) and rodents (Pellow *et al.*, 1985; Baldwin *et al.*, 1989) and elicits craving in human alcoholics (Umhau *et al.*, 2011). However, evidence suggests possible differences between yohimbine and intermittent foot-shock

on reward seeking (Le *et al.*, 2005; Cifani *et al.*, 2012; Mantsch *et al.*, 2016).

Little is known about the pattern of Fos-protein induction produced by yohimbine-induced reinstatement of alcohol seeking. Previous studies have examined the expression of *c-fos* mRNA or Fos-protein following both footshock-induced reinstatement of alcohol seeking (Zhao *et al.*, 2006; Schank *et al.*, 2015) and yohimbine administration in alcohol naïve rodents (Tsujino *et al.*, 1992; Singewald & Sharp, 2000; Singewald *et al.*, 2003; Funk *et al.*, 2006; Funk *et al.*, 2016). However, this is the first detailed examination of the pattern of Fos-protein expression induced in the rat brain following low dose (1 mg/kg) yohimbine administration in the home-cage of alcohol-experienced rats and following yohimbine-induced reinstatement of alcohol seeking.

Methods

Animals

Adult male Indiana alcohol preferring (iP) rats were obtained from the breeding colony of The Florey Institute of Neuroscience and Mental Health. Parental stock was obtained from the late Professor T.K. Li (while at Indiana University, USA). Rats were housed 2 per cage with a littermate in a 12-hour (h) light/dark cycle (lights on 7a.m - 7p.m), with access to food (laboratory chow) and water *ad libitum*. 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. Ethanol preferring rats were utilised as they consistently obtain high levels of voluntary alcohol self-administration and preference, and drink pharmacologically relevant quantities of alcohol (Walker *et al.*, 2017a; Walker *et al.*, 2017b).

Self-administration

Male iP rats (250-300g) were trained to self-administer alcohol [10% (v·/v)] under operant conditions using a fixed ratio of 3 (FR3) schedule during 20 min sessions as previously described in (Kastman *et al.*, 2016; Walker *et al.*, 2017b). Two levers were available during the session, one that delivered a 100 µL alcohol reward (active lever), and the other that delivered 100 µL of water (water lever). Operant conditioning chambers (Med Associates, St Albans, VT, USA) were accommodated 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 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. A 1 s 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 per week).

Extinction and yohimbine-induced reinstatement

To model stress-induced relapse of alcohol seeking, rats underwent extinction-reinstatement testing (Walker *et al.*, 2017a). During extinction training, no cues were present and there was no programmed response following instrumental responses. Extinction sessions continued until rats met criteria (<15 lever presses for three consecutive days, ~10 days in total). Rats were habituated to i.p. injections for the final 3-4 days of extinction training. Alcohol experienced rats were then randomly divided into three groups; group 1 (n = 6) where reinstatement of alcohol seeking was measured in rats during a 20 min operant session, 30 min following administration of yohimbine (1 mg/kg, i.p.) as previously described (Walker et al., 2017b). Group 2 (n = 6) were administered yohimbine (1 mg/kg, i.p.) and placed back into their home-cage. Group 3 (n = 7) were administered vehicle (1 ml/kg, i.p.) and placed into their home-cage, while group 4 (alcohol naïve, n = 6) did not receive any intervention (Kastman *et al.*, 2016; Walker *et al.*, 2017b).

Tissue harvesting and processing

Rats were anaesthetised (pentobarbitone 100 mg/kg, i.p.) 60 min after the end of reinstatement (or equivalent time) and transcardially perfused with 400 mL phosphate buffered saline (PBS, 0.1 M, pH 7.4) followed by 400 mL 4% w/v paraformaldehyde (PFA, Sigma-Aldrich) in PBS. Rats were decapitated, brains removed and post-fixed (1 h) in perfusion solution before being transferred into 50 ml 20% w/v sucrose in PBS at 4°C overnight and freezing over liquid nitrogen. Coronal brain sections (40 µm) were cut on a cryostat at -18°C (Cryocut, 1800; Leica Microsystems) and collected into PBS in a 1/3 series.

Immunohistochemistry

Sections were pre-blocked in 10% normal donkey serum (NDS; Millipore) and 0.5% Triton-X (TX100; BDH Chemicals) in PBS (1 h at room temperature; RT). Sections were incubated at RT overnight in anti-goat Fos primary antibody (1:1000, Santa Cruz Biotechnology, SC-52- G) with 2% NHS, 0.1% TX-100 in PBS. The following day, sections were washed in PBS (3 x 5 min) before incubating in biotinylated horse anti-goat IgG (Vector laboratories, BA-9500) in PBS for 2 h at RT (Walker *et al.*, 2018; Campbell *et al.*, 2019). Following further washing in PBS, sections were incubated for 1 hour at RT in 1:100 avidin– biotin complex solution (VectaStain ABC kit; Vector Laboratories) before washing again in PBS. Immunostaining was visualized using a standard peroxidase– chromogen reaction by placing the sections in 0.05% 3,3-diaminobenzidine (DAB) in PBS (Sigma-Aldrich, Castle Hill, NSW, Australia) followed by addition of 0.03% H_2O_2 for a further 5 min. Sections were washed for 3x5 min and mounted onto gelatinised glass microscope slides (Menzel-glaser, LOMB scientific PL), and left to dry overnight. Sections were then dehydrated and cleared through a series of ethanol (50%, 70%, 90% and 100%) and xylene and coverslipped using SafetyMount mounting media (Fronine Laboratory Supplies, Riverstone, NSW, Australia)

Image acquisition

Images were acquired using the Mirax digital slide scanner at 20X

magnification (Ziess, Oberkochen, Germany) and quantified semi-automatically using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). The number of Fos positive nuclei were counted unilaterally in a 1 in 3 series at 3 matched levels 120 µm apart in the prelimbic cortex (PrL), infralimbic cortex (IL), orbitofrontal cortex (OFC), nucleus accumbens (Acb) core (AcbC), Acb shell (AcbSh), anterior cingulate cortex (Cg1 and Cg2), dorsal bed nucleus of the stria terminalis (dBNST), ventral BNST (vBNST), ventral palladium (VP), medial and lateral posterior limb of the anterior commissure (IPACM, IPACL), lateral preoptic area (LPO), claustrum (CL), dorsal entorhinal cortex (dEN), paraventricular nucleus of the hypothalamus (PVN), anterior paraventricular nucleus of the thalamus (aPVT), PVT and posterior PVT (pPVT), Cornu Ammonis area 1 and 3 (CA1, CA3), dentate gyrus (DG), basolateral nucleus of the amygdala (BLA), central nucleus of the amygdala (CeA), dorsomedial hypothalamus (DMH), perifornical region of the hypothalamus (PeF), lateral hypothalamus (LH), arcuate nucleus (Arc), medial habenula (MHb), lateral Hb (LHb), ventral tegmental area (VTA), dorsal raphe (DR), median raphe (MnR), paramedian raphe nuclei (PMnR), medial and lateral parabrachial nuclei (MPB, LPB), nucleus incertus (NI), Barrington's nucleus (Bar), pontine central gray (PCG), locus coeruleus (LC) and lateral reticular nucleus (LRt) (Paxinos & Watson, 2006). See Table 1 for bregma levels where counts were conducted.

Data and statistical analysis

All data analysis and generation of histograms were performed using GraphPad Prism Version 7 for windows (GraphPad software Inc, San Diego, CA, USA). Operant self-administration and reinstatement testing were analysed by two-way ANOVA followed by Tukey's Multiple Comparisons *post hoc* analysis, while extinction data were examined using a repeated measures (RM) one-way ANOVA to examine lever pressing across days. Fos-immunoreactivity was analysed separately for each brain region using one-way ANOVA with *post hoc* Tukey's multiple comparisons analysis to determine differences in treatment groups. For the yohimbine and reinstatement groups, all possible pairwise correlations of Fos signal between

each of the 42 brain regions were determined using Pearson correlations and displayed as colour coded correlation matrices (Wheeler *et al.*, 2013). Final group sizes for Fos-immunohistochemistry may differ between brain regions due to tissue loss in specific regions during sectioning or staining. Groups were processed simultaneously by an investigator blinded to treatment allocation. Data are expressed as mean ± SEM. Data considered significant at $p < 0.05$.

Results

Operant self-administration and reinstatement of alcohol seeking

During the last three weeks of alcohol self-administration, rats averaged 86.2 ± 5.0 active lever responses (0.51 ± 0.03 g/kg/session alcohol intake) and 4.1 ± 0.3 water lever responses. No difference in alcohol responding across treatment group were observed on either active ($F_{(2, 16)} = 1.103$, $p = 0.3557$) or inactive ($F_{(2,16)} = 0.4008$, $p = 0.6763$) lever (Fig. 1B). Further, no difference in treatment group was observed across extinction training on active ($F_{(2,16)} = 0.2025$, $p = 0.8188$) or inactive ($F_{(2,16)} = 0.0084$, $p = 0.9916$) lever (Fig. 1C). For the reinstatement group, two-way ANOVA revealed a main effect of lever ($F_{(1, 5)} = 61.31$, $p < 0.0001$), treatment ($F_{(2,10)} = 34.96$, $p < 0.0001$), and a treatment x lever interaction ($F_{(2,10)} = 27.42$, $p < 0.0001$). Tukey's *post hoc* analysis showed active lever responding was significantly decreased in the last 3 days of extinction training (baseline vs. extinction, $p < 0.0001$), without any effect on water lever responding ($p > 0.9999$, Fig. 1D). Additionally, yohimbine precipitated reinstatement of active lever (extinction vs. reinstatement, $p < 0.001$) without affecting water lever responding ($p > 0.9999$, Fig. 1D).

2.3.2 Pattern of Fos expression following home-cage yohimbine administration and yohimbine-induced reinstatement of alcohol seeking

To determine brain regions activated by low dose yohimbine administration, Fos-immunohistochemistry was employed. No differences were observed between naïve and vehicle treated animals in any region, suggesting no

significant effect of prior alcohol exposure or animal handling on Fos induction in any of the regions examined in this study. Table 1 summarises analyses and mean total number of Fos cells for individual regions.

One-way ANOVA with Tukey's *post hoc* comparisons revealed that 1 mg/kg yohimbine administered in the home-cage and yohimbine-induced reinstatement of alcohol seeking both increased the number of Fos-positive cells in the PrL ($p < 0.0076$; Fig. 2A), IL ($p < 0.0231$; Fig. 2B), OFC ($p < 0.0087$; Fig. 2C), AcbC ($p < 0.0058$), AcbSh ($p < 0.0017$; Fig. 2D), dBNST ($p < 0.0084$; Fig. 2E) and CeA ($p < 0.0024$; Fig 2F), with a trend observed in the LC ($p = 0.0619$) compared to vehicle controls (Table 1). Yohimbine-induced reinstatement of alcohol seeking also increased the number of Fos-positive cells in the Cg1 ($p < 0.0037$), Cg2 ($p < 0.0434$), vBNST ($p < 0.017$), IPACL ($p < 0.0244$), LPB ($p < 0.0283$), LC ($p < 0.0011$), with strong trends in the NI ($p = 0.0520$), pPVT ($p = 0.0503$) and BLA ($p = 0.0624$) compared to vehicle. Additionally, Fos expression was increased in the VP ($p < 0.0056$), PVN ($p < 0.0187$), aPVT ($p < 0.0231$), PVT ($p < 0.0082$), Arc ($p < 0.0210$), DMH ($p < 0.0279$), PeF ($p < 0.0001$), LH ($p < 0.0002$), DG ($p < 0.0156$), CA1 ($p < 0.0025$), CA3 ($p < 0.0001$), DRV ($p < 0.0001$) and LRt ($p < 0.0137$) compared to both vehicle-treated and home-cage yohimbine-treated rats (Table 1).

Pearson correlations for each animal across all brain regions revealed that Fos expression was more strongly positively correlated following yohimbine-induced reinstatement of alcohol seeking compared to home-cage yohimbine (Fig 3A & 3B). This was particularly evident within forebrain regions, where expression was more tightly coupled following yohimbine-induced reinstatement of alcohol seeking. The prefrontal cortex showed a marked increase in positive correlations, especially with the AcbC and AcbSh (Fig. 3C). The dBNST and CeA were significantly positively correlated following both home-cage yohimbine and yohimbine-induced reinstatement of alcohol seeking. An increased correlation was observed between the AcbSh and CeA following reinstatement (Fig 3D).

Discussion

Home-cage yohimbine and yohimbine-induced reinstatement of alcohol seeking increase Fos-immunoreactivity in discrete regions throughout the neuraxis. These data largely agree with Fos expression following footshock-induced reinstatement of alcohol seeking and yohimbine administration in alcohol naïve rats; however, differences exist related to yohimbine dose (See Table 2). Notably, inter-regional correlations show a change in the pattern of correlation following yohimbine-induced reinstatement of alcohol seeking compared to home-cage yohimbine.

2.4.1 Fos-protein induction following home-cage yohimbine and yohimbine-induced reinstatement

Low dose yohimbine administration robustly and specifically increased Fos-positive cells in components of the prefrontal cortex (PrL, IL and OFC) and extended amygdala (AcbSh, dBNST and CeA). The AcbC also showed increased Fos-immunoreactivity following yohimbine. These data are in line with previous studies of yohimbine-induced *c-fos* mRNA and Fos-protein expression following several doses of yohimbine (1.25-5 mg/kg) in alcohol naïve rats (Tsujino *et al.*, 1992; Singewald & Sharp, 2000; Singewald *et al.*, 2003; Funk *et al.*, 2006; Cippitelli *et al.*, 2010; Funk *et al.*, 2016). The general concordance suggests that operant alcohol consumption for >7 weeks does not substantially moderate the effects of yohimbine on neural activation; however, some small discrepancies exist (Table 2). Home-cage effects of yohimbine appear to be dose-dependent. Considerably higher doses of yohimbine (2.5-5mg/kg) induce Fos in the Cg1, Cg2, LPO, LH, DMH, DR and LC (Tsujino *et al.*, 1992; Singewald & Sharp, 2000; Singewald *et al.*, 2003; Funk *et al.*, 2006), but not at 1 mg/kg. However, rat strain, prior ethanol experience and mode or timing of Fos/*c-fos* detection may contribute to discrepancies between studies (McReynolds *et al.*, 2018).

Low dose yohimbine (1-1.25 mg/kg) causes robust reinstatement of drug and alcohol seeking (Le *et al.*, 1998; Le *et al.*, 2005; Walker *et al.*, 2017a; Walker *et al.*, 2017b), suggesting the brain regions activated at this low dose may be involved in triggering relapse behaviour. Indeed, examination of neural 'activation' following yohimbine-induced reinstatement revealed increased Fos

in the PFC and extended amygdala.

Both these regions are key components of the putative stress-induced relapse neurocircuitry (Koob & Volkow, 2016). Further, alcohol related dysregulation of PFC function can adversely affect executive functions including decision making and inhibitory control, weakening self-control and increasing the vulnerability to relapse (Goldstein & Volkow, 2011). It has also been suggested that under stress, habitual and emotional responses within the extended amygdala and striatum direct behavioural responses due to a decrease in the regulatory function of the PFC (Sinha, 2001). Further, noradrenaline released in response to yohimbine can impair inhibitory functions of the PFC (Arnsten & Li, 2005; Fitzgerald, 2011) and increase rapid-response impulsivity in rats (Arnsten & Li, 2005; Sun et al., 2010) and humans (Swann et al., 2005). Following home-cage yohimbine there was little inter-regional correlation of Fos-expression within the PFC; however, following yohimbine-induced reinstatement of alcohol seeking these regions were positively correlated (Fig 3B). This suggests the act of reinstatement co-ordinates activity between interconnected prefrontal regions.

Within the extended amygdala, the BNST and CeA have an extensive system of primarily GABAergic interneurons that express a variety of neuropeptides; several of which are linked to yohimbine-induced relapse to alcohol seeking. Home-cage yohimbine administration (1 mg/kg i.p.) and yohimbine-induced reinstatement of alcohol seeking activate GABAergic (~30%), corticotrophin releasing factor (CRF; ~4%) and preprodynorphin (~12%) cells within the CeA (Walker et al., 2017), suggesting yohimbine may act in part via modulation of dynorphin/Kappa opioid receptors (KOR) and CRF signaling in the CeA to precipitate relapse. Other systems are also involved, including glucocorticoid signaling within the CeA (Simms et al., 2012) and relaxin-3/RXFP3 signalling in the BNST and CeA (Ryan et al., 2013; Walker et al., 2017b). Inter-regional correlations showed strong positive correlation between the CeA and BNST following both home-cage yohimbine and yohimbine-induced reinstatement. Further, yohimbine-induced reinstatement of alcohol seeking increased the positive correlation between the CeA and AcbSh (Fig 3D). The BNST and

CeA have strong bi-directional interconnections implicated in stress and alcohol related behaviours (Erb *et al.*, 2001; Normandeau *et al.*, 2018; de Guglielmo *et al.*, 2019).

Together, these data suggest that following alcohol consumption, yohimbine 'activates' the PFC and extended amygdala, potentially increasing impulsivity and stress responsiveness. During reinstatement yohimbine in combination with the previously alcohol associated context then 'activates' cells widely throughout the neuraxis in a more coordinated manner driving reinstatement of alcohol seeking. However, the mechanisms underpinning these actions require elucidation.

Fos-protein induction elicited by yohimbine-induced reinstatement, but not home-cage yohimbine alone

While the actions of yohimbine in the home-cage were confined to several key networks, yohimbine-induced reinstatement of alcohol seeking increased Fos-immunoreactivity widely throughout the neuraxis (See Table 2 & Figure 4). Inter-regional correlations showed a shift, whereby, forebrain regions were more strongly positively correlated together following yohimbine-induced reinstatement (Fig 3). A key difference between the home-cage yohimbine and yohimbine-induced reinstatement group is the placement of rodents back into the previously drug-paired context. The IL, PrL, OFC, AcbC, AcbSh, dBNST, CeA all show increased Fos expression following cue/context-induced reinstatement of alcohol seeking (Zhao *et al.*, 2006; Dayas *et al.*, 2007; Hamlin *et al.*, 2007; Jupp *et al.*, 2011), suggesting overlapping networks through which stress (including yohimbine) and drug predictive cues may act to drive/facilitate relapse. The BLA, VP, hypothalamus and hippocampus are also activated following cue/context induced relapse suggesting they may play a role in integrating drug associated cues/contexts during relapse (Zhao *et al.*, 2006; Dayas *et al.*, 2007; Hamlin *et al.*, 2007; Jupp *et al.*, 2011).

Following yohimbine-induced reinstatement of alcohol seeking an overall increase in positively correlated regions within the forebrain was observed (Fig 3). This suggests that while low dose yohimbine can increase Fos

expression in stress responsive regions, and elicit relapse behavior, the drug associated cues and contexts are also vital. Indeed, the importance of stress and context are established for relapse, as footshock given outside of the drug-taking context does not reinstate drug seeking (Buczek *et al.*, 1999; Shalev *et al.*, 2000). This is in line with the pattern of correlations observed in our study, which suggest yohimbine in the previously drug associated context precipitates interconnected neuronal activity to drive relapse. An important caveat exists - correlation is not causation. A limitation of Fos studies is the lack of temporal data in individual subjects. Therefore, although brain regions may be strongly correlated, one cannot confirm functional interplay between regions with the current data set.

Several components of the hypothalamus (PVN, DMH, PeF, LH and Arc) were specifically activated during yohimbine-induced reinstatement. The PVN is integral in mediating HPA mediated stress responses (Herman and Cullinan, 1997). However, the lack of activation in the PVN following low dose yohimbine alone suggests the effects of yohimbine in precipitating relapse are likely independent of the HPA axis, but it, along with other hypothalamic nuclei are 'activated' during reinstatement. The PVN has been implicated as a region in which yohimbine acts to induce a greater degree of ethanol consumption and seeking in female rats (Bertholomey *et al.*, 2016). While females were not examined in the current study, further research into how yohimbine precipitates relapse in females may aid the development of sex-specific 'anti-relapse' medications.

Our data largely align with previous literature examining yohimbine administration in alcohol naïve animals or following footshock-induced reinstatement of alcohol seeking (Table 2). The lack of robust Fos-induction within the LC following home-cage yohimbine was unexpected. The LC is abundant in α_2-adrenoceptors and doses of 2.5–5 mg/kg yohimbine robustly increase Fos mRNA and protein within this region (Tsujino *et al.*, 1992; Singewald & Sharp, 2000; Funk *et al.*, 2006).This is the first description of LC examination following administration of a low (but reinstatement eliciting) dose of yohimbine. While yohimbine's anxiogenic activity is thought to be driven via

activation of the adrenergic system, the exact mechanism by which yohimbine induces relapse is unknown. Yohimbine also acts as a 5-HT_{1A} receptor agonist, but only at doses greater than 1 mg/kg (Zaretsky *et al.*, 2015). Further, yohimbine's effect on reinstatement of food and cocaine seeking is not blocked by the α_2-adrenoreceptor agonist clonidine (Nair *et al.*, 2011), suggesting yohimbine is possibly acts via multiple systems to exert its effects. Indeed, yohimbine reinstates food seeking independent of the availability of the reward, suggesting potential effects of yohimbine on cue reactivity (Chen *et al.*, 2015). However the low 1 mg/kg yohimbine dose induced anxiety-like behaviour in iP rats with prior alcohol/abstinence experience (Walker *et al.*, 2017b), suggesting its action may be altered by alcohol consumption. The α_1-adrenoreceptor antagonist, prazosin, also reduces yohimbine-induced Fos expression in the PFC, AcbSh, vBNST, and BLA while i.c.v. and systemic prazosin administration attenuated yohimbine-induced reinstatement (Funk *et al.*, 2016), suggesting yohimbine may activate neurons in these regions which precipitate relapse, and prazosin can counteract this. Further examination of the mechanism in which yohimbine precipitates relapse at this low dose is required.

Conclusions

Together our results show the PFC and extended amygdala are robustly activated by yohimbine, but inter-regional activation does not necessarily correlate strongly. During yohimbine-induced reinstatement of alcohol seeking, these regions plus others, including the hypothalamus, thalamus and hippocampus are also activated. The pattern of inter-regional correlation shifts following yohimbine-induced reinstatement of alcohol seeking, whereby forebrain regions are strongly and positively correlated, likely due to the combination of yohimbine and previously drug associated cues/contexts. This detailed examination of the neural pattern of activation following yohimbine induced reinstatement is important considering the widespread use of yohimbine in experimental procedures and considerable limitations in our knowledge of the mechanism of action of yohimbine. This 'map' may help guide future experiments to deconstruct the circuitry of alcohol seeking.

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Competing interests:

L.C.W., H.E.K. and A.J.L. declare no competing conflicts of interest.

Author contributions:

L.C.W, H.E.K and A.J.L contributed to the design and analysis of the study. L.C.W and H.E.K conducted experiments. L.C.W performed analysis. L.C.W and A.J.L wrote the manuscript. All authors reviewed the content and approved the final version of the manuscript.

Data accessibility:

Raw data files are available by contacting the corresponding author

Abbreviations:

AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Arc, arcuate nucleus; AUD, alcohol use disorders; Bar, Barrington's nucleus; BLA, basolateral nucleus of the amygdala; CA1, Cornu Ammonis area 1; CA3, Cornu Ammonis area 3; CeA, central nucleus of the amygdala; Cg, anterior cingulate cortex; CL, claustrum; CRF, corticotrophin releasing factor; D1, dopamine 1 receptor; DAB, 3, 3'-diaminobenzidine tetrahydrochloride hydrate; dBNST, dorsal bed nucleus of the stria terminalis; dEN, dorsal entorhinal cortex; DG, dentate gyrus; DMH, dorsomedial hypothalamus; DR, dorsal raphe; FR3, fixed ratio of 3; H_2O_2, hydrogen peroxide; IL, infralimbic cortex; iP, Indiana alcohol preferring; i.p., intraperitoneal; IPACL, lateral posterior limb of the anterior commissure; IPACM, medial posterior limb of the anterior commissure; KOR, kappa opioid receptor; LC, locus coeruleus; LE, Long

Evans; LH, lateral hypothalamus; LHb, lateral Hb; LPB, lateral parabrachial nuclei; LPO, lateral preoptic area; LRt, lateral reticular nucleus; MHb, medial habenula; MnR, median raphe; MPB, medial parabrachial nuclei; NDS, normal donkey serum; NI, nucleus incertus; OFC, orbitofrontal cortex; PBS, phosphate buffered saline; PeF, perifornical region of the hypothalamus; PCG, pontine central gray; PFA, paraformaldehyde; PMnR, paramedian raphe nuclei; PrL, prelimbic cortex; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; RT, room temperature; SD, Sprague Dawley; vBNST, ventral bed nucleus of the stria terminalis; VP, ventral pallidum; VTA, ventral tegmental area.

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Region	Bregma	F value	Naïve	Vehicle	Yohimbine	Reinstatement
IL	**+3.7**	$F_{(3,18)}$ = 12.63 p < 0.0001	357.1 (61)	529 (92)	1035 (86) *	1184 (158) **
OFC	**+3.7**	$F_{(3,18)}$ = 15.67 p < 0.0001	1143 (178)	1250 (189)	2545 (177) **	3117 (356) ***
PrL	**+3.7**	$F_{(3,18)}$ = 16.34 p < 0.0001	320.6 (69)	423.2 (68)	1220 (71) **	1552 (252) ***
Cg1	**+2.2**	$F_{(3,16)}$ = 9.10 p < 0.0010	104.8 (25)	147.4 (23)	428.6 (121)	628.9 (105)**
Cg2	**+2.2**	$F_{(3,16)}$ = 5.25 p < 0.0103	113.8 (18)	159.5 (22)	554.6 (144)	657.4 (190)*
ACbC	**+2.2**	$F_{(3,20)}$ = 14.44 p < 0.0001	114.7 (25)	183.2 (44)	656.3 (124)**	785.1 (114) ***
ACbSh	**+2.2**	$F_{(3,19)}$ = 16.46 p < 0.0001	172.4 (42)	236.2 (35)	884.0 (164)**	1016 (110) ***
VP	**-0.3**	$F_{(3,17)}$ = 11.80 p < 0.0004	12.0 (2)	12.2 (1)	16.2 (4)	38.7 (6)*** ††
dBNST	**-0.3**	$F_{(3,18)}$ = 11.66 p < 0.0002	67.4 (8)	82.6 (12)	228.7 (45) **	249.1 (32) **
vBNST	**-0.3**	$F_{(3,18)}$ = 11.8 p < 0.0002	107.1 (9)	172.9 (15)	190.8 (9)	251.1 (25) *
LPO	**-1.0**	$F_{(3,17)}$ = 10.22 p < 0.0293	32.8 (5)	53.9 (7)	49.2 (4)	76.8 (14)
IPACM	**-1.0**	$F_{(3,18)}$ = 6.51 p < 0.0036	11.3 (1)	29.7 (4)	32.5 (7)	47.9 (8)
IPACL	**-1.0**	$F_{(3,18)}$ = 7.20 p < 0.0022	9.6 (1)	26.1 (6)	48.8 (16)	65.8 (8) *
DEn	**-1.8**	$F_{(3,19)}$ = 0.84 p = 0.4106	50.4 (25)	53.2 (20)	41.7 (7)	78.6 (14)
CL	**-1.8**	$F_{(3,19)}$ = 3.21 p < 0.0462	26.0 (5)	35.9 (5)	30.7 (6)	51.7 (8)
aPVT	**-1.8**	$F_{(3,20)}$ = 8.12 p < 0.0010	113.2 (24)	183.0 (51)	141.1 (22)	349.2 (42) * ††
PVN	**-1.8**	$F_{(3,19)}$ = 9.12 p < 0.0006	74.2 (11)	100.8 (11)	107.5 (14)	169.2 (16) * †
BLA	**-2.6**	$F_{(3,19)}$ = 6.95 p < 0.0024	36.8 (9)	127.2 (36)	133.4 (30)	260.2 (51)
PVT	**-2.6**	$F_{(3,19)}$ = 10.1 p < 0.0003	84.1 (17)	131.3 (22)	95.57 (17)	267.7 (41) ** ††
CeA	**-2.6**	$F_{(3,19)}$ = 16.09 p < 0.0001	55.3 (14)	101.5 (19)	306.6 (55) **	286.3 (32) **
DG	**-3.3**	$F_{(3,18)}$ = 6.57 p < 0.0034	75.8 (12)	76.5 (11)	69.8 (19)	151.7 (18) * ††
DMH	**-3.3**	$F_{(3,19)}$ = 7.91 p < 0.0008	53.6 (6)	92.9 (12)	74.3 (13)	180.3 (33) * ††
Arc	**-3.3**	$F_{(3,19)}$ = 9.35 p < 0.0005	35.8 (8)	62.3 (9)	48.1 (11)	122.5 (19) * ††
CA1	**-3.3**	$F_{(3,18)}$ = 11.30 p < 0.0002	1.9 (1)	7.7 (3)	7.8 (3)	39.4 (9) ** ††

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Table 1. Mean (SEM) total No. Fos-positive cells following home-cage yohimbine- and yohimbine-induced reinstatement of alcohol seeking.

CA3	**-3.3**	$F_{(3,18)} = 21.46$ p < 0.0001	6.7 (1)	9.9 (2)	10.9 (2)	39.3 (6) **** ††††
LH	**-3.3**	$F_{(3,19)} = 21.72$ p < 0.0001	43.9 (8)	86.79 (7)	64.6 (10)	220.0 (30) *** †††
PeF	**-3.3**	$F_{(3,19)} = 26.69$ p < 0.0001	78.2 (13)	141.4 (19)	128.2 (11)	357.8 (39) **** ††††
MHb	**-3.8**	$F_{(3,17)} = 2.05$ p = 0.1453	1.4 (1)	4.1 (2)	6.1 (2)	12.3 (5)
LHb	**-3.8**	$F_{(3,17)} = 4.83$ p < 0.0126	7.9 (2)	38.6 (12)	26.5 (7)	49.7 (8)
pPVT	**-3.8**	$F_{(3,17)} = 5.27$ p < 0.0094	157.2 (42)	214.5 (49)	220.4 (29)	416.1 (67)
VTA	**-5.2**	$F_{(3,15)} = 3.62$ p < 0.0380	28.9 (3)	40.6 (5)	33.5 (6)	51.9 (5)
PMnR	**-7.3**	$F_{(3,15)} = 0.45$ p = 0.7180	28.8 (5)	25.3 (6)	21.6 (3)	27.5 (5)
MnR	**-7.3**	$F_{(3,15)} = 1.01$ p = 0.4157	33.2 (4)	31.0 (7)	32.2 (6)	43.2 (6)
DRD	**-7.3**	$F_{(3,15)} = 2.12$ p = 0.1409	25.7 (4)	39.9 (3)	33.8 (5)	40.5 (7)
DRV	**-7.3**	$F_{(3,15)} = 69.68$ p < 0.0001	24.8 (3)	38.2 (4)	38.8 (4)	118.1 (8) **** ††††
MPB	**-9.3**	$F_{(3,19)} = 2.13$ p = 0.1308	42.7 (3)	44.7 (7)	31.8 (5)	55.4 (10)
LPB	**-9.3**	$F_{(3,19)} = 6.93$ p < 0.0024	117.8 (19)	178.8 (34)	277.7 (56)	352.9 (31) *
Bar	**-9.6**	$F_{(3,19)} = 1.81$ p = 0.1790	21.8 (4)	37.8 (9)	33.8 (6)	43.2 (7)
NI	**-9.6**	$F_{(3,19)} = 3.08$ p = 0.0520	76.8 (16)	81.4 (16)	122.2 (32)	176.2 (36)
PCG	**-9.6**	$F_{(3,19)} = 3.27$ p < 0.0441	34.5 (4)	48.3 (7)	43. 0 (10)	70.4 (11)
LC	**-10**	$F_{(3,18)} = 10.2$ p < 0.0004	41.7 (10)	43 (4)	128.6 (21)	188.9 (43) **
LRt	**-13.6**	$F_{(3,13)} = 22.08$ p < 0.0001	20.6 (2)	57.0 (15)	94.9 (13)	154.9 (10) *** †

Table 2. Comparison of findings with previous literature for Fos/*c-fos* induced by yohimbine or stress-induced reinstatement.

Panel	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Citation	Current study	Funk *et al.*, 2006	Cippitelli *et al.*, 2010	Sheth *et al.*, 2017	Funk *et al.*, 2006	Singewald *et al.*, 2003	Singewald *et al.*, 2000	Tsujino *et al.*, 1992	Current study	Zhao *et al.*, 2006	Schank *et al.*, 2016
Paradigm	HC	HC	HC	HC	HC	HC	HC	HC	REIN	REIN	REIN
Rat strain	iP	SD	Wistar	LE	Wistar	SD	SD	Wistar	iP	Wistar	Wistar
Dose (mg/kg)	1	1.25	1.25	2	2.5	5	5	5	1	FS	FS
Detection	IHC	IHC	ISH	IHC	ISH	IHC	IHC	IHC	IHC	IHC	IHC
IL	+				+				+	-	+
PL	+	+			+				+	-	+
OFC	+	+							+		
AcbC	+	+			+	+			+	+	+
AcbSh	+	+	+		+	+			+	+	+
Cg1	-				+	+			+		
Cg2	-				+	+			+		
dBNST	+	+			+	+		+	+	+	+
vBNST	-	+			+	+		+	+		+
VP	-			-					+		
LPO	-							+	+		
IPACm	-								+		
IPACl	+								+		

PVN	-				+	+		+	+	+		
aPVT	-					+			+			
DeN	-								-			
CL	-								+			
CeA	+	+	+		+	+		+	+	+	+	
BLA	-	+	+		+	+		+	trend	+	-	
PVT	-					+			+			
CA1	-				-	-			+	-		
CA3	-				-	-			+	-		
DG	-				-	+			+	-		
DMH	-					+		+	+			
PeF	-								+			
LH	-			+		+		-	+			
ARC	-								+			
pPVT	-								trend			
LHb	-								+			
MHb	-								-			
VTA	-				-			-	+	+		
DRD	-								-		+	
DRV	-				+		+		+		+	
MnR	-								-			
PMnR	-								-			
LPB	-								+			
MBP	-								-			
PCG	-								-			
BAR	-								-			
NI	-								trend			
LC	-				+		+	+	+			
LRt	-								+			

Table captions

Table 1. Mean (SEM) total No. Fos-positive cells following yohimbine- and yohimbine-induced reinstatement of alcohol seeking. The anterior-posterior location of each brain region is given in millimeter (mm) relative to bregma (Paxinos and Watson, 1988). One-way ANOVA results shown for individual brain regions and total Fos-positive cell counts in naïve, vehicle, yohimbine-treated animals, or animals that underwent yohimbine-induced

reinstatement of alcohol seeking. One-way ANOVA with Tukey's *post hoc* comparison reveal yohimbine activates the PrL, IL, OFC, AcbC, AcbSh, dBNST and CeA, while yohimbine-induced reinstatement also activates the Cg1, Cg2, VP, vBNST, IPACL, PVT, PVN, DG, CA1, CA3, Arc, DMH, PeF, LH, DRV, LPB, LC and LRt. Abbreviations: prelimbic cortex (PrL), infralimbic cortex (IL), orbitofrontal cortex (OFC), nucleus accumbens core (AcbC) and shell (AcbSh), anterior cingulate cortex (Cg1 and Cg2), dorsal and ventral bed nucleus of the stria terminalis (dBNST, vBNST), ventral palladium (VP), medial and lateral posterior limb of the anterior commissure (IPACM, IPACL), lateral preoptic area (LPO), claustrum (CL), dorsal entorhinal cortex (dEN), paraventricular nucleus (PVN), anterior, medial and posterior paraventricular thalamus (aPVT, PVT, pPVT), Cornu Ammonis area 1 and 3 (CA1, CA3), dentate gyrus (DG), basolateral nucleus of the amygdala (BLA), central nucleus of the amygdala (CeA), dorsomedial hypothalamus (DMH), perifornical area of the hypothalamus (PeF), lateral hypothalamus (LH), arcuate nucleus (Arc), medial and lateral habenula (MHb, LHb), ventral tegmental area (VTA), dorsal, median and paramedian raphe nuclei (DR, MnR, PMnR), medial and lateral parabrachial nuclei (MPB, LPB), nucleus incertus (NI), Barrington's nucleus (Bar), pontine central gray (PCG), locus coeruleus (LC) and lateral reticular nucleus (LRt). *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (compared to VEH-treated animals); †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$,††††$p < 0.0001$ (compared to YOH-treated animals). N = 5-6/group.

Table 2. Comparison of findings with previous literature for Fos/*c-fos* induced by yohimbine or stress-induced reinstatement. Panel A summarizes Fos-protein expression from the current study following home-cage yohimbine administration (1 mg/kg) compared. Panel B-H summarizes Fos/c-fos expression from previous literature following yohimbine administration (1.25-5 mg/kg). Panel I summarises Fos-protein expression from the current study following yohimbine-induced reinstatement. Panel J-K summarizes Fos/c-fos expression from previous literature following footshock (FS)-induced reinstatement of alcohol seeking. (-) = no increase in Fos, (+) =

increase in Fos, blank = no current literature. Abbreviations are listed in Table 1 and the abbreviations list.

Figure captions:

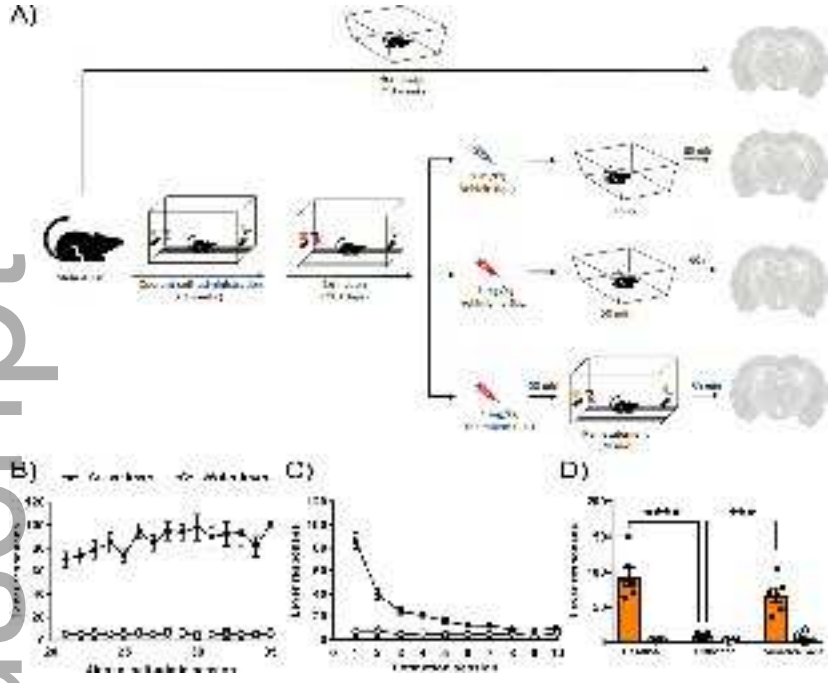
Figure 1. Operant self-administration, extinction and yohimbine-induced reinstatement of alcohol seeking. (A) Schematic of behavioural paradigm used in this study. Active and inactive lever responding during **(B)** the last 15 sessions of ethanol self-administration and **(C)** extinction. **(D)** In the reinstatement group, yohimbine administration reinstates active lever responding (EXT vs. REIN ****$p < 0.0001$), without affecting responding on the water lever (all p's > 0.9999). All data are expressed as mean ± SEM, n = 6-7/group.

Figure 2. Home-cage yohimbine and yohimbine-induced reinstatement of alcohol seeking increase Fos expression in the prefrontal cortex and extended amygdala. Representative low magnification micrograph, high magnification micrograph of treatment dependent Fos expression and mean ± SEM Fos cell counts in the **(A)** prelimbic cortex, **(B)** infralimbic cortex, **(C)** orbitofrontal cortex, **(D)** nucleus accumbens shell, **(E)** bed nucleus of the stria terminalis and **(F)** central nucleus of the amygdala of naïve (NAV), vehicle-treated (VEH), yohimbine-treated (YOH) and rats that underwent yohimbine-induced reinstatement of alcohol seeking (REIN). Yohimbine administration and yohimbine-induced reinstatement of alcohol seeking increased Fos-positive neurons in the prelimbic cortex, infralimbic cortex, orbitofrontal cortex, nucleus accumbens shell, bed nucleus of the stria terminalis and central nucleus of the amygdala. Scale bar = 100 µm, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All data are expressed as mean ± SEM, n = 5-6/group.

Figure 3. Inter-regional correlations of Fos expression. Matrices showing inter-regional correlations of Fos expression following **(A)** home-cage yohimbine administration, or **(B)** yohimbine-induced reinstatement to alcohol seeking. Comparison of inter-regional correlation of Fos expression in the **(C)**

prefrontal cortex and **(D)** extended amygdala of home-cage yohimbine (top) and yohimbine-induced reinstatement to alcohol seeking (bottom). Mean correlation coefficients were greater following yohimbine-induced reinstatement to alcohol seeking. Colours represent correlation strength (see scale). Abbreviations are listed in Table 1 and the abbreviations list.

Figure 4. Summary of differential Fos-protein expression following home-cage yohimbine administration and yohimbine-induced relapse to alcohol seeking. Rat brain schematic showing brain regions activated by home-cage yohimbine administration and yohimbine-induced relapse to alcohol seeking (red), yohimbine-induced reinstatement to alcohol seeking only (orange) or not activated by either yohimbine or yohimbine-induced reinstatement of alcohol seeking (blue). Abbreviations are listed in Table 1 and the abbreviations list.



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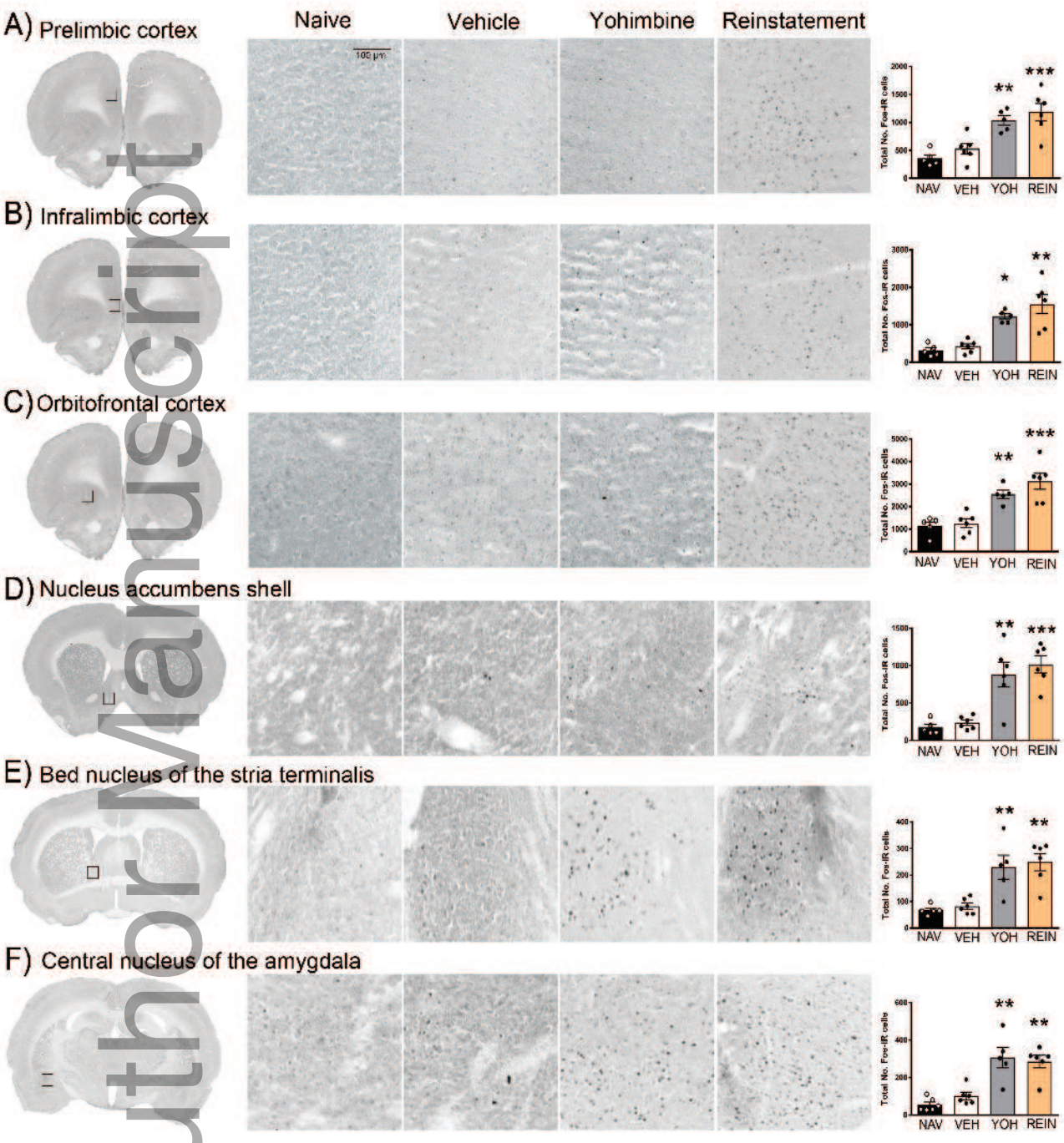

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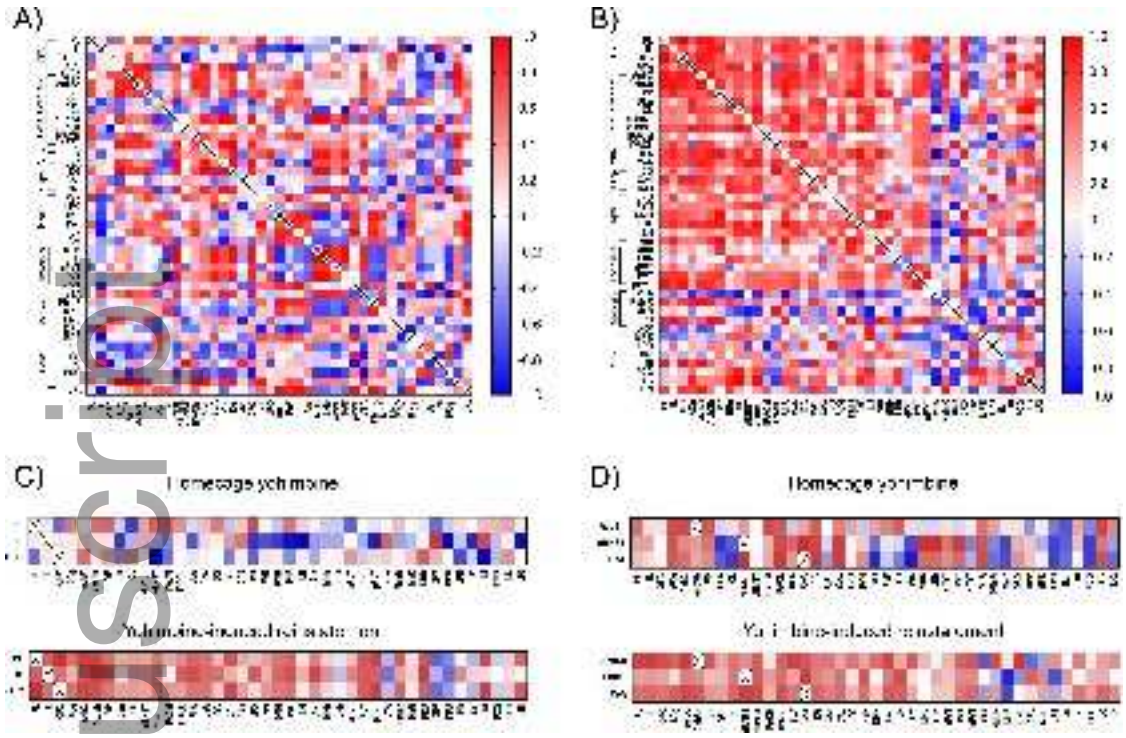

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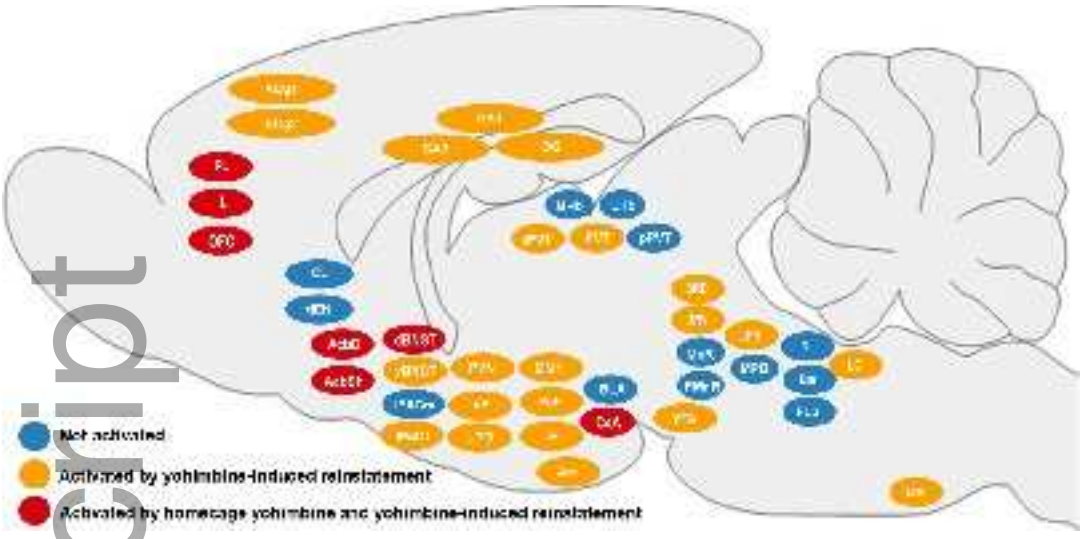

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