

Title: Muscarinic M₄ and M₅ receptor subtypes in the ventral subiculum differentially modulate alcohol seeking vs consumption in male alcohol preferring rats

Running title: vSub muscarinic receptors modulate alcohol-related behaviours

Leigh C. Walker^{1,2,5,6}, Kate L. Huckstep^{1,2,5}, Nicola A. Chen^{1,2}, Lexi J. Hand^{1,2}, Craig W. Lindsley⁴, Christopher J. Langmead⁴, Andrew J. Lawrence^{1,2,6}

1. Florey Institute of Neuroscience and Mental Health, Parkville, Australia, 3052.
2. Florey Department of Neuroscience and Mental Health, University of Melbourne, Parkville, Australia, 3052.
3. Warren Center for Neuroscience Drug Discovery, Vanderbilt University, Nashville, TN, USA, 37232,
4. Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia, 3052
5. Authors contributed equally
6. Corresponding authors

Corresponding authors

Leigh C Walker
leigh.walker@florey.edu.au
ORCID ID: 0000-0002-9282-2743

Andrew J Lawrence HonFBPhS
andrew.lawrence@florey.edu.au
ORCID ID: 0000-0001-6836-727X

Word counts

Abstract: 248/250
Discussion: 1346/1500
Total (excluding abstract, methods, references and figure legends): 3741/4000
Figures & Tables: 8/10
References: 60/60

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1111/bph.15513](https://doi.org/10.1111/bph.15513)

Abstract

Background and purpose:

Muscarinic acetylcholine receptors (mAChRs) mediate alcohol consumption and seeking in rats, and while M_4 and M_5 mAChRs have recently been implicated to mediate these behaviours in the striatum, their role in other regions remain unknown. The ventral tegmental area (VTA) and ventral subiculum (vSub) both densely express M_4 and M_5 mAChRs and modulate alcohol seeking via their projections to the nucleus accumbens shell (AcbSh).

Experimental approach: In Indiana alcohol preferring (iP) male rats we examined *Chrm4* (M_4) and *Chrm5* (M_5) expression in the VTA and vSub following long-term alcohol consumption and abstinence using RT-qPCR. Next, using a combination of retrograde tracing and RNAscope, we examined the localisation of *Chrm4* and *Chrm5* on vSub cells that project to the AcbSh. Using selective allosteric modulators, we examined the functional role of M_4 and M_5 mAChRs within the vSub in alcohol consumption, context-induced alcohol seeking, locomotor activity and food/water consumption.

Key results: Long-term alcohol and abstinence dysregulated mAChR gene expression in the vSub, not VTA. *Chrm4* was downregulated following long-term alcohol and abstinence, while *Chrm5* was upregulated following long-term alcohol consumption. In line with these data, intra-vSub M_4 positive allosteric modulator (VU0467154) reduced context-induced alcohol seeking, but not motivation for alcohol self-administration, while M_5 negative allosteric modulator (ML375) reduced initial motivation for alcohol self-administration, but not context-induced alcohol seeking.

Conclusion and implications: Collectively our data highlight alcohol-induced cholinergic dysregulation in the vSub and distinct roles for M_4 and M_5 mAChR allosteric modulators to reduce alcohol consumption or seeking.

Keywords: muscarinic receptor, alcohol, allosteric modulation, relapse, context, ventral subiculum, ventral tegmental area

Abbreviations:

AcbSh; nucleus accumbens shell
AUD; alcohol use disorders
Chrm4; muscarinic M_4 acetylcholine receptor
Chrm5; muscarinic M_5 acetylcholine receptor
CT β ; Cholera Toxin- β
FISH; Fluorescent in Situ Hybridisation
FR3; fixed ratio 3
GPCR; G-protein coupled receptor
Hsp90; heatshock protein 90
iP; Indiana alcohol preferring rat
LH; lateral hypothalamus
mAChR; muscarinic acetylcholine receptor
nAChR; nicotinic acetylcholine receptor
NAM; negative allosteric modulator
NRQ; normalised relative quantity
PAM; positive allosteric modulator

PFC; prefrontal cortex
PR; progressive ratio
RM; repeated measures
Tbp; TATA binding box protein
vSub; ventral subiculum
VTA; ventral tegmental area

Main text

Introduction

Despite the large social and economic burden of alcohol use disorders (AUD) on society (Rehm *et al.*, 2017; Bonomo *et al.*, 2019), current pharmacotherapeutic treatment options remain ineffective and insufficient at a population level (Walker & Lawrence, 2018; Witkiewitz *et al.*, 2019). Using selective allosteric modulation, we have recently identified a role for [M₄](#) and [M₅ muscarinic acetylcholine receptors](#) (mAChRs) [G-protein coupled receptors](#) (GPCRs) in alcohol consumption and seeking, in part via signalling in the dorsolateral striatum (Berizzi *et al.*, 2018; Walker *et al.*, 2020; Walker & Lawrence, 2020). However, while involvement of muscarinic receptors in the striatum was evident from those studies, the data also implicated mAChR signalling in other brain regions.

The ventral tegmental area (VTA) and the main ventral hippocampal output region, the ventral subiculum (vSub), receive cholinergic input (Mesulam *et al.*, 1983), express muscarinic and [nicotinic](#) AChRs including the M₄ and M₅ mAChRs (Vilaró *et al.*, 1990; Lebois *et al.*, 2018) and have critical roles in relapse to drug seeking, specifically following exposure to a context previously associated with drug availability (Bossert *et al.*, 2004; Crombag *et al.*, 2008; Bossert & Stern, 2014; Bossert *et al.*, 2016; Marchant *et al.*, 2016). The VTA and vSub both send dense projections to the nucleus accumbens shell (AcbSh) (Naber & Witter, 1998) another critical node implicated in context-induced reinstatement (Bossert *et al.*, 2006; Crombag *et al.*, 2008; Cruz *et al.*, 2014; Marchant *et al.*, 2015; Bossert *et al.*, 2016). Moreover, discrete functional inhibition of the vSub → AcbSh pathway decreases context-induced drug and alcohol seeking (Bossert *et al.*, 2016; Marchant *et al.*, 2016). In line with this, activation of VTA and vSub mAChRs modulate downstream accumbal [dopamine](#) release (Moss *et al.*, 2003; Solecki *et al.*, 2013), suggesting potential pathways in which cholinergic dysregulation may impact the mesocorticolimbic reward system. Given these data, the aim of the present study was to examine the roles of M₄ and M₅ mAChRs within the VTA and vSub in alcohol consumption and seeking behaviours.

First to determine whether the VTA and/or vSub show cholinergic dysregulation following long-term intermittent home cage alcohol consumption (with and without abstinence), we used RT-qPCR to assess *Chrm4* (M₄ mAChR) and *Chrm5* (M₅ mAChR) expression in male Indiana alcohol preferring (iP) rats. We found specific dysregulation of mAChR mRNA expression in the vSub, with a significant decrease in *Chrm4* expression following alcohol and 14 days abstinence and an increase in *Chrm5* expression following alcohol with 24 hours abstinence. Based on the specific dysregulation observed within the vSub, we concentrated solely on this region for subsequent experiments. We next mapped the distribution pattern of *Chrm4* and *Chrm5* expression within the vSub, focusing on vSub projections to the AcbSh, revealing both M₄ and M₅ mAChRs are expressed to a varying degree on vSub → AcbSh projections. Finally, we sought to modulate cholinergic signalling within the vSub via the M₄ positive allosteric modulator (PAM, VU0467154) (Bubser *et al.*, 2014) or M₅ negative allosteric modulator (NAM, ML375) (Gentry *et al.*, 2013) and assess

alcohol seeking (context-induced reinstatement) and motivation to obtain an alcohol reward (progressive ratio schedule, PR). Allosteric modulators modulate downstream signalling through actions at allosteric binding sites, either potentiating (PAM) or inhibiting (NAM) endogenous signalling in comparison to ligands that act at an orthosteric binding site (Langmead & Christopoulos, 2006; 2014). These small molecules may provide advantages including increased GPCR subtype selectivity and more nuanced modulation of downstream signalling (e.g. biased signalling and/or functional selectivity) (Conn *et al.*, 2009; Langmead & Christopoulos, 2014). We found vSub M₄ enhancement with VU0467154 (to counteract *Chrm4* downregulation in abstinence) specifically reduced context induced reinstatement of alcohol seeking, while M₅ disruption with ML375 (to counteract *Chrm5* upregulation after alcohol) specifically reduced alcohol self-administration in the first 15 min of the PR test. Together these data suggest distinct cholinergic dysregulation within the vSub may underpin different aspects of alcohol consumption and seeking, which can be reduced through mAChR allosteric modulation to regulate vSub output pathways.

Methods

Animals

Inbred male Indiana alcohol preferring (iP) rats (~8 weeks old at commencement, N = 86) were obtained from the breeding colony at The Florey Institute of Neuroscience and Mental Health, The University of Melbourne. Parental stock was previously obtained from the late Professor T.K. Li (while at Indiana University, USA). All rats were pair-housed in open top cages until surgery, after which they were single housed. Food (Barastoc rat and mouse) and water were available *ad libitum* and all rats were maintained on a normal 12-hour light/dark cycle (0700 lights on). Rats were provided woodchip and tissue bedding, which was replaced weekly. The iP rat strain was selected as they voluntarily consume high levels of alcohol (Walker *et al.*, 2019; Walker *et al.*, 2020). All experiments were performed in accordance with the Prevention of Cruelty to Animals Act (2004), under the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of animals for Experimental Purposes (2013) and approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics Committee. All efforts were made to minimise animal suffering and reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015).

Compounds

The selective M₄ PAM VU0467154 (5-amino-3,4-dimethyl-N-(4-((trifluoromethyl)sulfonyl) benzyl)thieno[2,3-c]-pyridazine-6-carboxamide) and selective M₅ NAM ML375 ((9bS)-9b-(4-chlorophenyl)-1-(3,4-difluorobenzoyl)-2,3-dihydroimidazo[2,1-a]isoindol-5-one) were synthesized at Vanderbilt University (Nashville, TN, USA) (Bubser *et al.*, 2014). VU0467154 (3µmol/hemisphere) was dissolved into 80% dimethyl sulfoxide (DMSO, v/v) in sterile saline and ML375 (105pmol/hemisphere) was formulated in 5% DMSO and 95% sterile saline (v/v). These doses have previously been shown to reduce alcohol consumption and seeking in iP rats when microinjected within the dorsolateral striatum (Berizzi *et al.*, 2018; Walker *et al.*, 2020).

Stereotaxic surgery

AcbSh retrograde tracer injections

To determine vSub projections to the AcbSh, for experiment 2, rats underwent intracranial retrograde tracer (Cholera Toxin- β -488; CT β -488) injection into the AcbSh. Rats were anaesthetized with isoflurane (5% v/v induction, 2% v/v maintenance) and positioned in a stereotaxic frame (Stoelting Co. Wood Dale, USA). The surgical site was shaved and cleaned with 10% povidone/iodine (Orion Laboratories, Arkles Bay, NZ) and 80% (v/v) ethanol solution. A small incision was made along the midline to expose the skull and the area was cleaned and dried. A small hole was drilled into the skull and a 2 μ L syringe (Hamilton, USA) was lowered into the AcbSh (anteroposterior, +1.6 mm; mediolateral, $\pm$ 2.3 mm; dorsoventral, - 7.5 mm), and 150nL of CT β -488 (1%, Thermofisher Scientific, CAT# C34775) was unilaterally infused at a rate of 75nL/min (Marchant *et al.*, 2016). To prevent backflow the syringe was left in place for 5 min. Rats received meloxicam (3 mg kg⁻¹, i.p.) for analgesia, the antibiotic, baytril (3 mg kg⁻¹, i.p.) and saline (10 ml kg⁻¹, s.c.) at the end of surgery, and were dosed with meloxicam and baytril (3mg kg⁻¹ i.p.) every 24 h for 3 days.

vSub cannulae implantation

For experiments 3-7 rats underwent stereotaxic cannula implantation to allow direct drug administration within the vSub. Rats were anaesthetized and prepared as per retrograde tracer injections. Next, four holes were drilled into the skull and screws (1.4mm diameter and 2 mm length; Mr Specs. Parkdale, Australia) were inserted to anchor the cannula to the skull. Two small holes were drilled into the skull, through which 26 G stainless-steel guide cannulas cut 8 mm below the pedestal (PlasticsOne) were implanted into the vSub (anteroposterior, -6.0 mm; mediolateral, $\pm$ 5.3 mm; dorsoventral, - 7.8 mm on 4-degree angle) as previously described (Marchant *et al.*, 2016; Walker *et al.*, 2017a; Walker *et al.*, 2017b; Campbell *et al.*, 2019). Cannula were fixed to the skull using dental cement (Vertex-Dental) and a dummy, cut projecting 1.5 mm beyond the cannula tip was inserted (PlasticsOne). Rats received meloxicam (3 mg kg⁻¹, i.p.) for analgesia, the antibiotic, baytril (3 mg kg⁻¹, i.p.) and saline (10 ml kg⁻¹, s.c.) at the end of surgery, and were dosed with meloxicam and baytril (3mg kg⁻¹ i.p.) every 24 h for 3 days.

Drug microinfusion

Bilateral intra-vSub infusions of VU0467154 or ML375 were made using 40 cm polyethylene connectors (PlasticsOne) attached to 1 μ L syringes (SGE Analytical Science, Ringwood). Rats were gently held while dummies were removed, and injectors inserted into cannulae. VU0467154 (0.5 μ L), ML375 (0.5 μ L) or their respective vehicle was infused bilaterally (0.25 μ L/min) by an automated syringe pump (Harvard Apparatus, Holliston, USA). The injectors were left in place for 1 min after infusion. Rats were habituated to the equipment prior to testing and each rat received both drug and vehicle treatment in a counterbalanced manner. Following behavioural testing to test cannula positioning, methylene blue (0.5 μ L) was infused bilaterally, and animals were anaesthetized with pentobarbitone (100 mg kg⁻¹, i.p. Virbac, Milperra, Australia) before decapitation. Brains were collected, frozen over super-cooled isopentane, sectioned (40 μ m) on a cryostat (Leica, Leica Microsystems) and mounted onto SuperFrost Plus slides (Menzel-Glaser). After drying, slides were counterstained with Neutral Red solution (Sigma-Aldrich) for 2 min and cleared through a series of ethanol (50%, 70%, 90%, 100%) and X-3B (Olichem Pty Ltd.) before cover-slipping with

SafetyMount mounting media (Sigma-Aldrich). An investigator blinded to treatment groups and behavioural outcomes performed injection site validations (Kastman *et al.*, 2016; Walker *et al.*, 2017b). In total 15 rats were excluded from testing and/or analysis due to illness (n = 4) or misplaced guide cannula (n = 11) across pharmacological experiments. No extensive damage or lesioning was observed in injection sites.

RT-qPCR

For experiment 1, rats were euthanised via Lethobarb (pentobarbital sodium) overdose (2 ml kg⁻¹; i.p.) 24 hours or 14 days following final home cage intermittent alcohol access. Brains were immediately removed and quickly frozen over liquid nitrogen until sectioning. The VTA and vSub of each animal were dissected using needles with inner diameters 1.5 mm. Remaining brain sections were used to validate the accuracy of the micro-punches under a dissection microscope by an experimenter blinded to treatment group. RNA extraction and analysis were performed as described (Chen *et al.*, 2014; Walker *et al.*, 2020). Briefly, RNA from brain tissue was extracted using the QIAGEN Qiazol + RNeasy Plus Micro Kit according to the manufacturer's protocol. All RNA samples had 260/280 ratios between 1.8 - 2.1 and RNA samples were analyzed with an Agilent 2100 Bioanalyzer (RINs all > 8). Total RNA (1000 ng) extracted from rodent vSub and VTA tissues were then reverse-transcribed into cDNA using SuperScriptII qRT-PCR kit (Invitrogen, USA). SYBR Powerup PCR kit (Qiagen) was used in 10 µL reactions containing 4 µL cDNA, and 1 µL 20 nmol/µL primer mixture. qPCR was performed with ViiA7(Applied Biosystems, USA), followed by melt-curve analysis. The qRT-PCR program was set to 2 min at 50 °C, 2 min at 95 °C, and 15 s at 95 °C and 1 min at 60 °C (40 cycles). Reactions were performed in 3 technical replicates along with a no template and water control. Two reference genes (*Hsp90*; heatshock protein 90 and *Tbp*; TATA binding box protein) were selected from 3 candidate genes from geNorm (geNORM, RRID:SCR_006763) analysis using qbase+ tool according to the instructions given in the qbase+ manual (www.qbaseplus.com). Forward and reverse primers (Table 1) for rat housekeeping genes and genes of interest were designed using Primer3 v. 0.4.0 software (RRID:SCR_003139; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Whitehead Institute for Biomedical Research, USA) based on coding DNA sequences acquired from GenBank (National Center for Biotechnology Information (NCBI)). Primer specificity was verified (BLAST Interface, NCBI; RRID:SCR_001653)).

Fluorescent in Situ Hybridisation (FISH)

To examine *Chrm4* and *Chrm5* expression on vSub → AcbSh projecting cells in experiment 2, brains were rapidly extracted and frozen for 20 secs on dry ice-cooled 2-methylbutane (Bacto Laboratories, NSW, Australia). Brains were stored at -80°C until use. Coronal sections of AcbSh (40 µm, for CTβ-488 injection site validation) and vSub (16 µm) were cut in a 1/3 series using a cryostat (Leica Biosystems, NSW, Australia) and mounted directly onto Super Frost Plus slides (Fisher Scientific, NH, USA). Slides were stored at -80°C until use. The RNAscope Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics) was used to detect *Chrm4*, and *Chrm5* in the vSub, CTβ-488 was detected endogenously (Wang *et al.*, 2012; Gibson *et al.*, 2018; Ch'ng *et al.*, 2019). Slides were fixed in 4% w/v paraformaldehyde for 15 mins at 4°C, before rinsing in 0.1 M PBS (pH 7.4) followed by dehydration in increasing concentrations of ethanol (50%, 70%, 100% and 100% v/v) for 5 mins each at RT. Slides were then air-dried for 10min at RT and a hydrophobic barrier was drawn around the brain sections. Sections were then protease treated (pre-treatment 4) at RT for 5 min. Next, sections were rinsed in distilled water followed by probe application. The

target probes used were, *Chrm4* (456671-C2) and *Chrm5* (479161-C3) (Walker *et al.*, 2020). Slides were first incubated with probes at 40°C for 1 hr. Following this, slides were incubated with preamplifier and amplifier probes (AMP1, 40°C for 30min; AMP2, 40°C for 15min; AMP3, 40°C for 30 min), followed by incubation in fluorescently labelled probes to select a specific combination of reporters associated with each channel (AMP4 Alt A to detect *Chrm4* in Atto 550 channel and *Chrm5* in Atto 647, while CT β -488 was detected endogenously). Finally, sections were incubated for 20 s with DAPI and coverslipped with DAKO Fluorescent Mounting Medium (North Sydney, NSW, Australia). One rat was excluded from processing and analysis as CT β -488 spread was observed outside of the AcbSh. A LSM 780 Zeiss Axio Imager 2 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) using a 20 X objective was used to take overview images of the endogenous CT β -488 fluorescence in the injection site, and placements were confirmed by two independent researchers. For FISH imaging, a 40 X objective was used to take contralateral overview images of the vSub (pixel size 0.10um), which were quantified from 3 sections per rat (~Bregma -6.0 mm; Paxinos and Watson, 2006) using image J (National Institutes of Health; RRID:SCR_003070). For a cell to be deemed positive for the expression of a given receptor, five or more mRNA puncta needed to be present (Ch'ng *et al.*, 2019; Walker *et al.*, 2020).

Intermittent access home cage drinking procedure

As per our previous studies, rats were given access to one bottle of 20% ethanol (v/v) and one bottle of tap water over three 24 h-sessions per week (Walker *et al.*, 2020; Walker *et al.*, 2021; Campbell *et al.*, 2019). Alcohol solutions were prepared in tap water from 100% (v/v) ethanol. Daily sessions began at 10.00 am. After 24h, the alcohol bottle was replaced with a second water bottle for the subsequent 24-48h alcohol-free period. The following day, the second water bottle was replaced with the 20% alcohol bottle, and the location of the alcohol bottle was alternated from the previous session. Total alcohol consumption in grams was calculated for each session, using the weight difference between the beginning and end of the session multiplied by 0.97 (density of 20% ethanol) and divided by 2 (number of rats per cage) (Walker *et al.*, 2020).

Operant self-administration apparatus

Standard operant chambers (Med Associates, St Albans, VT, USA) enclosed in a ventilated sound-attenuating cubicle were used for self-administration (Ryan *et al.*, 2013; Kastman *et al.*, 2016; Walker *et al.*, 2021). Each chamber was equipped with two retractable levers, on opposite sides of the chamber. An active lever press resulted in the delivery of 10% v/v ethanol (0.1ml/delivery) into the receptacle via an 18-gauge blunt needle connected to a 60ml syringe controlled by a 220v syringe pump. An inactive lever press had no programmed consequence. Chambers were connected to a computer running Med-PC IV software (Med Associates, RRID:SCR_012156) to record data.

Locomotor activity apparatus

Locomotor activity was recorded in a 43.2cm x 43.2cm x 30.5cm chamber (Med Associates). Distance travelled (m) was recorded using photobeam detectors.

Experimental Design

Experiment 1: M₄ and M₅ mRNA expression in the VTA and vSub following chronic intermittent alcohol consumption

To determine whether chronic alcohol consumption and/or abstinence induces cholinergic dysregulation in the VTA or vSub, rats underwent long-term voluntary intermittent alcohol access. Following 57 sessions of home cage intermittent alcohol access rats were divided into two groups; alcohol-experienced (n = 7) and alcohol-abstinence (n = 7). Alcohol-experienced rats were then killed 24 h after the last ethanol access, while alcohol-abstinence rats were killed 14 days after their last alcohol session. The 24 h timepoint was selected to ensure no acute effects of alcohol were present, while abstinence was examined 14 days after final alcohol exposure to examine the persistence or emergence of changes induced by long-term alcohol consumption/abstinence. Another group of age-matched alcohol-naïve rats (n = 7) were maintained on water throughout the experiment before brain collection and RT-qPCR (Walker *et al.*, 2020).

Experiment 2: Phenotyping M₄ and M₅ mAChR expression on vSub → AcbSh projections

Given the vSub → AcbSh pathway is critical for context-induced relapse to alcohol seeking, we sought to examine the distribution of M₄ and M₅ mAChRs on vSub → AcbSh projection cells by combining a retrograde tracer injection into the AcbSh with FISH (RNAscope). Alcohol naïve rats (n = 5) underwent stereotaxic surgery to inject the retrograde tracer (CTβ-488) into the AcbSh. Following 10 days of recovery, rats were culled, and brains taken for injection site confirmation (AcbSh) and RNAscope (vSub). RNAscope for *Chrm4*, *Chrm5* and endogenous CTβ-488 expression in the vSub was conducted, imaged and analysed.

Experiment 3 and 4: Examining M₄ or M₅ mAChRs in context-induced alcohol seeking

Based on our observations that *Chrm4* expression was decreased, and *Chrm5* expression was increased in the vSub following chronic alcohol consumption and/or abstinence and the expression of the *Chrm4* and *Chrm5* receptors on vSub → AcbSh projections, we next examined whether enhancing M₄ receptor activity (via positive allosteric modulation) or reducing M₅ receptor activity (via negative allosteric modulation) could prevent context-induced reinstatement of alcohol seeking. Following 9-12 sessions of intermittent home cage alcohol access, rats underwent >30 operant self-administration sessions, each lasting 20 min, in context A on a fixed ratio 3 schedule (FR3, 3 lever presses result in 1 alcohol delivery). Rats were next implanted with canulae targeting the vSub and following 5-7 days recovery allowed to reacquire operant alcohol self-administration to baseline levels in context A. Lever pressing was then extinguished in context B (the opposite context to which they received training). In these sessions, lever presses were not rewarded, and no cues were presented. Animals completed daily extinction sessions until they met a pre-set criterion (<15 active lever presses for 3 consecutive days). Rats then underwent testing for alcohol seeking in context A (ABA) and context B (ABB), in a randomised and counterbalanced order 24 hours apart (Hamlin *et al.*, 2007). Test sessions were conducted as per training, with the difference that lever presses were not rewarded with alcohol. In addition, for experiment 3 either the M₄ PAM, VU0467154 (n = 8) or vehicle (n = 6), or for experiment 4: M₅ NAM, ML375 (n = 7) or vehicle (n = 5) were microinfused into the vSub immediately prior to test.

Experiment 5 & 6: Examining M₄ or M₅ mAChRs in the motivation to consume alcohol

To determine whether vSub M₄ and M₅ receptors regulate motivation to obtain alcohol, we next tested under a progressive ratio schedule. As per Experiments 3 & 4, rats underwent 9-12 sessions of intermittent home cage alcohol access followed by >30 operant self-administration sessions, each lasting 20 min, on a FR3 schedule. An additional olfactory cue (Vanilla essence, Queen Foods) was placed under the active lever during training (and testing). Rats were then implanted with canulae targeting the vSub and following 5-7 days recovery allowed to reacquire operant alcohol self-administration to baseline levels on FR3 before subsequent testing on a progressive ratio (PR3-4) schedule for a 2 h session. For the PR3-4 schedule 32 active lever responses are required for the 10th delivery of alcohol (Walker *et al.*, 2020; Walker *et al.*, 2021). Breakpoint was defined as the final ratio completed within the 2 h session. Rats then underwent a second PR3-4 schedule and received the alternate treatment in a randomised and counterbalanced manner after at least 3 days of baseline FR3 operant alcohol self-administration. Compounds VU0467154/vehicle (n = 13) or ML375/vehicle (n = 6) were microinfused into the vSub immediately prior to test.

Experiment 7: Examining M₄ and M₅ mAChRs in locomotor and consummatory behaviours

A subset of randomly selected rats (from experiments 1-4) that had previously undergone operant testing (at least 3 days prior) received either VU0467154 (n = 7) or vehicle (n = 7); or ML375 (n = 6) or vehicle (n = 6) infusions into the vSub. Immediately following intra-vSub infusion rats underwent locomotor testing (1 hour) and subsequently food (standard chow) and water consumption was measured in the home cage 2 and 24 hours after locomotor activity to assess any off-target effect on consummatory behaviour. Please note that rats received the same treatment for locomotor testing as that received during their final operant test.

Statistical analysis

GraphPad Prism (RRID:SCR_002798) statistical software was employed for all analysis. For context-induced reinstatement (context x treatment), progressive ratio time course (treatment x time), food and water intake (intake x time) data were analysed by repeated measures (RM) two-way ANOVA with Bonferroni post hoc analysis where appropriate. Progressive ratio breakpoint and total distance in the locomotor test were analysed by Students t-test. Data are represented as mean ± SEM and significance set at p < 0.05. qbase⁺ software version 3.2, which is based on a generalized model of the 2^{-ΔΔC_q} approach, was used for qPCR data evaluation (Biogazelle, Zwijnaarde, Belgium; www.qbaseplus.com). In qbase⁺ C_q values were converted into normalized relative quantities (NRQs) based on the expression of the reference genes. One-way ANOVA was performed on NRQ's in GraphPad Prism for each brain region. Sample sizes used in each behavioural test were determined based on findings from our previous studies examining similar behaviour (Kastman *et al.*, 2016; Walker *et al.*, 2017a; Campbell *et al.*, 2019; Walker *et al.*, 2019; Walker *et al.*, 2021). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018).

Nomenclature of Targets and Ligands

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 (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

Results

Long-term alcohol consumption alters *Chrm4* and *Chrm5* mAChR expression in the vSub.

We first examined the expression of *Chrm4* and *Chrm5* within the vSub and VTA in three groups of iP rats, 1) alcohol naïve controls (naïve); 2) rats which underwent long-term intermittent two-bottle choice and were culled 24 hours after their last alcohol session (alcohol) and 3) rats which underwent long-term intermittent two-bottle choice and were culled 14 days after their last alcohol session (abstinence). Rats consumed high levels of alcohol, averaging ~8 g/kg/24 h session (Fig. 1A). Following dissection RT-qPCR was conducted to determine whether alcohol or abstinence from alcohol alters *Chrm4* or *Chrm5* expression in the vSub or VTA. In the vSub, one-way ANOVA of *Chrm4* expression revealed a significant difference between groups ($F_{2,18} = 9.975$, $p = 0.0012$). Bonferroni *post-hoc* comparison revealed a significant decrease in *Chrm4* expression after abstinence compared to alcohol ($p = 0.0236$) and naïve groups ($p = 0.0011$, Fig. 1C). Further one-way ANOVA of *Chrm5* expression also revealed a significant difference between treatment groups ($F_{2,18} = 4.685$, $p = 0.0230$), with post-hoc analysis revealing a significant increase in *Chrm5* expression in alcohol vs. naïve groups ($p = 0.0295$, Fig 1 C). In contrast, in the VTA one-way ANOVA showed no significant difference in either *Chrm4* ($F_{2,18} = 0.145$, $p = 0.8657$) or *Chrm5* ($F_{2,18} = 0.8712$, $p = 0.4354$) expression (Fig. 1D).

Chrm4 and *Chrm5* mRNA are expressed on vSub projections to the AcbSh.

Next the distribution of *Chrm4* and *Chrm5* and their co-localisation on vSub → AcbSh projections was examined using RNAscope in combination with an intra-AcbSh injection of the retrograde tracer CTβ-488 (Fig 2A). Density calculations revealed 551 ± 40 *Chrm4* expressing cells/mm² and 173 ± 6 *Chrm5* expressing cells/mm² within the vSub (Fig 2B). Within the vSub, $25 \pm 1\%$ of *Chrm4* expressing cells also expressed *Chrm5*, while $78 \pm 2\%$ of *Chrm5*-positive cells also expressed *Chrm4* (Figure 2C-D). Quantification of the percentage of CTβ-positive cells expressing mAChRs revealed $70 \pm 5\%$ of CTβ-positive cells also expressed *Chrm4*, $18 \pm 2\%$ expressed *Chrm5*, and $13 \pm 2\%$ expressed both *Chrm4* and *Chrm5*, while $25 \pm 5\%$ of CTβ-positive cells expressed neither *Chrm4* nor *Chrm5* (Fig. 2F). However, *Chrm4* and *Chrm5* mRNA was also detected on CTβ-negative cells. Therefore, the total percentage of *Chrm4*-positive and *Chrm5*-positive cell populations projecting to the AcbSh was also examined. This revealed $32 \pm 4\%$ of *Chrm4*-positive, $26 \pm 1\%$ of *Chrm5*-positive cells expressed CTβ-488, suggesting they project to the AcbSh (Fig 2G-2H).

M₄ mAChRs in the vSub mediate context-induced reinstatement of alcohol seeking.

Based on the decreased expression of *Chrm4* within the vSub following alcohol abstinence and its dense expression on vSub → AcbSh projections, we next examined whether vSub M₄ mAChRs have a functional role in context-induced alcohol seeking. As *Chrm4* expression was decreased following abstinence we microinjected the selective M₄ PAM VU0467154 directly into the vSub to enhance signalling. Rats reached a stable level of responding in context A, averaging 85 ± 9 active lever responses (0.60 ± 0.06 g/kg/session) and 2 ± 0.3 inactive lever responses over the 7 days preceding extinction training (Fig. 3A). Rats extinguished responding in context B, averaging 9 ± 1 active lever presses and 5 ± 1 inactive over their

final 3 extinction sessions (Fig. 3B). After random assignment to treatment group, no significant differences were observed in responding for alcohol in context A prior to extinction (unpaired t test; $t_{13} = 1.179$, $p = 0.2597$, *data not shown*). On test day, repeated measures (RM) two-way ANOVA analysis revealed a main effect of context ($F_{1,12} = 24.85$, $p = 0.0003$), treatment ($F_{1,12} = 33.12$, $p < 0.0001$), and context x treatment interaction ($F_{1,12} = 27.03$, $p = 0.0002$). Bonferroni's *post-hoc* analysis revealed that VU0467154 reduced the number of active lever presses in context A (ABA; VU vs VEH, $p < 0.0001$, Fig. 3C) but not context B (ABB; VU vs VEH, $p > 0.9999$, Fig. 3D). Analysis of latency to first active lever press in context A revealed a main effect of lever ($F_{1,12} = 6.110$, $p = 0.0294$) but no significant effect of treatment ($F_{1,12} = 0.5845$, $p = 0.4593$) or lever x treatment interaction ($F_{1,12} = 2.300$, $p = 0.1552$, *data not shown*).

M₅ mAChRs in the vSub do not mediate context-induced reinstatement of alcohol seeking.

We next examined the functional role of the M₅ mAChR in context-induced alcohol seeking. Based on the observation that *Chrm5* was upregulated following alcohol we microinjected the selective M₅ NAM ML375 within the vSub to reduce signalling. Rats successfully acquired alcohol self-administration in context A and returned to pre-surgical levels of responding prior to extinction training, averaging 131 ± 11 active lever responses (0.76 ± 0.07 g/kg/session alcohol intake) and 3.2 ± 1 inactive lever responses over the final 7 self-administration sessions in context A (Fig. 4A). Rats extinguished responding in context B (Fig. 4B), averaging 7 ± 1 active lever responses and 5 ± 1 inactive lever responses over their final 3 days of extinction training. There was no difference in average active lever responding between treatment groups in context A (unpaired t-test; $t_{11} = 0.09610$, $p = 0.9252$). Bilateral intra-vSub microinjection of ML375 had no significant effect on context-induced alcohol seeking in context A (ABA, Fig. 4C) or active lever pressing in context B (ABB, Fig. 4D). RM two-way ANOVA revealed a main effect of context ($F_{1,10} = 29.02$, $p = 0.0003$), but no significant effect of treatment ($F_{1,10} = 1.431$, $p = 0.259$), or context x treatment interaction ($F_{1,10} = 0.200$, $p = 0.664$) ($n = 5-7$ /group). Analysis of latency to first active lever press in context A revealed a main effect of lever ($F_{1,10} = 10.70$, $p = 0.008$), lever x treatment interaction ($F_{1,10} = 5.291$, $p = 0.044$), but no significant effect of treatment ($F_{1,10} = 1.773$, $p = 0.213$). Bonferroni's *post-hoc* analysis showed a significant increase in latency to inactive lever response ($p = 0.040$), but not active lever latency ($p = 0.844$, *data not shown*).

M₄ mAChRs in the vSub do not mediate motivation for alcohol self-administration.

To determine whether the actions of vSub M₄ mAChRs are specific to context-induced reinstatement, or generally decrease the motivation to obtain and consume alcohol we examined the role of intra-vSub VU0467154 under a PR schedule. During training, rats reached a steady level of responding for alcohol, averaging 97 ± 8 active lever responses (0.64 ± 0.04 g/kg/session) and 2 ± 1 inactive lever responses across the 7 operant training sessions prior to testing (Fig. 5A). There was no significant difference between groups in breakpoint for alcohol (paired t-test; $t_{12} = 0.437$, $p = 0.669$; $n = 13$; Fig. 5B), or latency to first active lever press ($t_{12} = 0.788$, $p = 0.448$; *data not shown*). RM two-way ANOVA of time-course data revealed a main effect of time ($F_{3,69} = 57.47$, $p < 0.0001$), but no significant effect of treatment ($F_{1,23} = 0.124$, $p = 0.616$) nor time x treatment interaction ($F_{3,69} = 0.625$, $p = 0.739$, Fig. 5C).

M₅ mAChRs in the vSub mediate motivation for alcohol self-administration.

Based on our observation that vSub *Chrm5* mRNA was upregulated following alcohol but not abstinence we next sought to examine whether the M₅ mAChR mediates motivation to obtain and consume alcohol. During training, rats reached a steady level of responding for alcohol, averaging 100 ± 10 active lever

responses (0.59 ± 0.05 g/kg/session) and 2 ± 1 inactive lever responses across the 7 operant training sessions prior to testing (Fig. 6A). There was no significant difference between groups in breakpoint for alcohol seeking (paired t-test; $t_5 = 1.612$, $p = 0.168$; Fig. 6B), nor latency to first active lever press ($t_5 = 0.725$, $p = 501$; *data not shown*). However, RM two-way ANOVA of time-course data revealed an effect of time ($F_{3,15} = 70.72$, $p < 0.0001$) and time x treatment interaction ($F_{3,15} = 5.446$, $p = 0.042$), but not treatment ($F_{1,5} = 0.2507$, $p = 0.279$). Bonferroni's *post hoc* analysis revealed a significant difference between vehicle and ML375 treated rats in the first 15 min ($p = 0.0161$) suggesting a small, but significant, reduction of alcohol responding in the early stages of the PR3-4 test when the drive to consume is highest (Fig. 6C).

M₄ and M₅ mAChRs in the vSub do not mediate locomotor activity, food or water consumption.

Finally, to examine any potential off- or on-target adverse actions we examined spontaneous locomotor activity, food and water intake following VU0467154, ML375 (or their respective vehicle) administration into the vSub.

For VU0467154 there were no differences between treatment groups in total distance travelled ($t_{13} = 0.459$, $p = 0.654$, Fig. 7A) or across the 60 min period [RM two-way ANOVA; main effect of time, $F_{11,143} = 37.00$, $p < 0.0001$; no significant effect of treatment, $F_{1,13} = 0.211$, $p = 0.654$; nor interaction, $F_{11,143} = 0.171$, $p = 0.998$; Fig. 7B]. Further, we assessed home-cage chow intake, VU0467154 had no significant effect on consumption [RM two-way ANOVA; main effect of time ($F_{1,13} = 617.4$, $p < 0.0001$) but no significant effect of treatment ($F_{1,13} = 0.009$, $p = 0.927$) or time x treatment interaction ($F_{1,13} = 0.182$, $p = 0.677$), Fig. 7C]. Additionally, VU0467154 did not alter water intake [RM two-way ANOVA; main effect of time ($F_{1,13} = 362.2$, $p < 0.0001$) but no significant effect of treatment ($F_{1,13} = 0.325$, $p = 0.578$) nor time x treatment interaction ($F_{1,13} = 0.492$, $p = 0.495$), Fig 7D].

For ML375 there were no significant differences between treatment groups in total distance travelled ($t_{10} = 0.522$, $p = 0.613$, Fig. 7E) or across the 60 min period [RM two-way ANOVA; main effect of time, $F_{11,110} = 44.28$, $p < 0.0001$; no significant effect of treatment, $F_{1,10} = 1.888$, $p = 0.199$; nor interaction, $F_{11,110} = 0.966$, $p = 0.481$; Fig. 7F]. Further, ML375 had no significant effect on food intake [RM two-way ANOVA; main effect of time ($F_{1,10} = 271.9$, $p < 0.0001$) but no significant effect of treatment ($F_{1,10} = 0.183$, $p = 0.677$) or time x treatment interaction ($F_{1,10} = 0.001$, $p = 0.991$), Fig. 7G], or water intake [RM two-way ANOVA; main effect of time ($F_{1,10} = 362.7$, $p < 0.0001$) but no significant effect of treatment ($F_{1,10} = 1.455$, $p = 0.255$) nor time x treatment interaction ($F_{1,10} = 1.080$, $p = 0.328$), Fig 7H].

Discussion

Our present study highlights new evidence for dissociable roles of vSub M₄ and M₅ mAChRs in alcohol seeking versus consumption. Following long-term alcohol consumption, we observed striking mAChR dysregulation within the vSub, but not the VTA, demonstrating anatomic specificity. Specifically, after 14 days abstinence from alcohol *Chrm4* expression was decreased, while after alcohol consumption, *Chrm5* expression was increased within the vSub. M₄ and M₅ mAChR are GPCRs, with the M₄ mAChR G_{i/o} coupled (inhibitory), while the M₅ G_{q/11} coupled (stimulatory) (Caulfield, 1993; Langmead *et al.*, 2008). Therefore, while alcohol or abstinence dysregulated M₄ and M₅ receptor expression in opposing directions, both actions would likely increase glutamatergic output of the vSub. Indeed, stimulation of the vSub has

previously been shown to reinstate drug seeking (Vorel *et al.*, 2001; Taepavarapruk *et al.*, 2015), while reversible inactivation decreases reinstatement of drug and alcohol seeking (Sun & Rebec, 2003; Bossert & Stern, 2014), suggesting increased vSub activity may enhance the propensity to relapse. This is in line with our findings that long-term alcohol consumption / abstinence reduces inhibitory *Chrm4* expression and enhances excitatory *Chrm5* expression, presumably enhancing vSub activity.

Based on this vSub mAChR dysregulation, and the known role of the vSub → AcbSh projection in alcohol seeking (Marchant *et al.*, 2016), we used RNAscope to characterise M₄ and M₅ mAChR mRNA expression within the vSub and specifically on vSub cells projecting to the AcbSh. In line with previous studies, we found discrete expression of the *Chrm4* and *Chrm5* within the vSub (Vilaró *et al.*, 1990; Lebois *et al.*, 2018; Walker & Lawrence, 2020). We further quantified density and receptor overlap, observing greater density of *Chrm4* > *Chrm5*, and that *Chrm4* (70%) and to a lesser extent *Chrm5* (18%) are expressed on vSub → AcbSh projecting cells. However, while *Chrm4* expression was observed on the majority of vSub → AcbSh projections, this only accounted for ~30% of all vSub *Chrm4*-positive cells. Previous studies have shown complete vSub inactivation reduces alcohol seeking to a greater extent than discrete vSub → AcbSh inhibition (Marchant *et al.*, 2016), suggesting other vSub populations could also contribute to this behaviour. Aside from the AcbSh, the vSub sends distinct glutamatergic projections to the prefrontal cortex (PFC) and lateral hypothalamus (LH) (Wee & MacAskill, 2020), and context-induced reinstatement of heroin seeking is associated with activation of vSub → PFC projecting cells (Bossert *et al.*, 2016). However, anatomical disconnection of this pathway does not decrease heroin seeking (Bossert *et al.*, 2016), nor is the vSub → PFC pathway “activated” following context-induced relapse to alcohol seeking (Marchant *et al.*, 2016). Therefore, future studies are required to determine whether M₄ receptors on vSub → AcbSh projections are critical for alcohol seeking behaviour, or whether this may more broadly rely on M₄ receptors within the vSub. Further, whether discrete outputs regulate different aspects of alcohol (or drug) seeking may provide an interesting avenue of research. While heavily implicated in context-induced alcohol seeking, the vSub also regulates relapse to drug seeking and taking precipitated by drug primes, cues (Sun & Rebec, 2003; Hiranita *et al.*, 2006) and stress (Yu & Sharp, 2015), suggesting a somewhat conserved role of the vSub in promoting relapse. While the vSub → PFC pathway does not regulate context-induced heroin seeking, whether discrete vSub outputs differentially regulate other relapse triggers remains to be determined. Given that women are more vulnerable to relapse to alcohol (Heffner *et al.*, 2011), a major limitation of this study is the lack of female subjects. Little is known about sex differences in muscarinic receptor activity (Walker and Lawrence, 2020), or whether sex differences in vSub activity exist and contribute to alcohol seeking behaviours. Recently the ventral hippocampus (including, but not confined to the vSub) to AcbSh pathway has been shown to underpin sex-specific stress susceptibility in an androgen dependent manner (Williams *et al.*, 2020). Future studies are required to determine the contribution of muscarinic signalling within the vSub in alcohol seeking in females.

Recently, several subtype selective mAChR allosteric modulators have been developed, enhancing the ability to examine specific behavioural roles of discrete mAChRs in affective disorders (Gentry *et al.*, 2013; Langmead & Christopoulos, 2014; Berizzi *et al.*, 2016; Bender *et al.*, 2018; Gould *et al.*, 2019; Walker & Lawrence, 2020). The selective M₄ PAM VU0467154 possesses both cognitive enhancing and antipsychotic-like effects (Bubser *et al.*, 2014; Gould *et al.*, 2018), and reduces alcohol consumption and seeking when administered systemically or within the dorsolateral striatum (Walker *et al.*, 2020). Based on our observations that vSub *Chrm4* is down-regulated following chronic alcohol/abstinence, in combination

with the dense expression of *Chrm4* on the vSub → AcbSh pathway and known role of the vSub in context-induced alcohol seeking, we examined whether enhancement of M₄ mAChRs (via the selective M₄ PAM VU0467154) functionally altered context-induced alcohol seeking or alcohol self-administration under a PR schedule. In line with our qPCR observations, microinjection of VU0467154 within the vSub reduced context-induced reinstatement. These data suggest VU0467154 may act to restore an alcohol/abstinence induced reduction in acetylcholine signalling within the vSub. Further, no alterations in *Chrm4* expression were observed after alcohol without a period of abstinence, suggesting M₄ signalling is predominantly impacted during periods of abstinence when relapse risk is highest, and may also explain why enhancing M₄ receptor signalling had no significant effect on ongoing alcohol self-administration in the PR test.

In contrast, reduction of M₅ receptor signalling (via the M₅ NAM, ML375) showed a small, but significant reduction in alcohol self-administration in the first 15 min under a PR schedule, but not context-induced reinstatement of alcohol seeking during abstinence. Again, this aligns with dysregulation observed in gene expression with *Chrm5* upregulated following alcohol, but not after alcohol/abstinence. However, the functional results showed a modest effect size, possibly due to the relatively lower density of M₅ receptor expression, and/or the degree of *Chrm5* dysregulation observed after long-term alcohol. In addition, the M₅ NAM ML375 has less than optimal physicochemical and pharmacokinetic properties, limiting solubility and dosing for intracerebral microinjections. Nevertheless, it is important to note that the same dose of ML375 has robust effects in similar procedures when injected directly into the dorsal striatum (Berizzi *et al.*, 2018). New generation M₅ NAMs with improved aqueous solubility and pharmacokinetics may show greater utility in the future. Importantly, neither VU0467154 nor ML375 altered procedural memory, locomotor activity or natural consummatory behaviour when administered within the vSub, suggesting our observations are specific to aberrant alcohol seeking behaviours, and align with previous studies examining food self-administration following vSub inactivation (Sun & Rebec, 2003).

Much of the research into the therapeutic potential of mAChR modulation has surrounded their ability to act as cognitive enhancers and alleviate cognitive symptoms of schizophrenia (Carruthers *et al.*, 2015). One potential mechanism in which cholinergic signalling may reduce alcohol seeking is via reversing alcohol-induced cognitive deficits that impair decision making and enhance relapse (Perry, 2016; Charlton *et al.*, 2019). Indeed, *in vivo* microdialysis revealed that chronic alcohol consumption significantly reduces [acetylcholine](#) release in the hippocampus (Melis *et al.*, 1996), while repeated doses of VU0467154 improved memory acquisition in mice (Gould *et al.*, 2018). Together, these data suggest restoration of cholinergic signalling may help remediate alcohol-induced cognitive deficits to counteract relapse risk; however, this theory remains untested at present. Several pharmaceutical companies have muscarinic compounds in clinical trial for neuropsychiatric disorders (Walker and Lawrence, 2020). For example, The M₄ PAM (CVL-231, Cerevel) is currently undergoing phase 1 clinical trials to assess safety, tolerability and pharmacokinetics (clinicaltrials.gov ID: NCT04136873), while the M₄ agonist, HTL0016878 (Sosei Heptares), has completed phase 1 clinical trials to assess safety, however, results are yet to be reported (clinicaltrials.gov ID: NCT03244228). Additionally, KarXT (Karuna Therapeutics) a non-selective M₁/M₄ agonist combined with a peripheral antagonist (to block peripheral adverse effects) has begun a randomized, double-blind, placebo-controlled, multicentre phase 3 clinical trial for the treatment of schizophrenia (clinicaltrials.gov ID: NCT04659161; NCT04659174). Together, our promising preclinical data presented here and previously (Berizzi *et al.*, 2018; Walker *et al.*, 2020) and the possibility of repurposing muscarinic compounds approved for other indications may provide an efficient way to fast-

track a novel treatment for AUD.

References (55/60)

- Alexander, S. P., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peters, J. A., ... & Yao, C. (2019). The Concise Guide to PHARMACOLOGY 2019/20: G protein-coupled receptors. *British Journal of Pharmacology*, **176**, S21-S141.
- Bender, A.M., Garrison, A.T. & Lindsley, C.W. (2018) The Muscarinic Acetylcholine Receptor M5: Therapeutic Implications and Allosteric Modulation. *ACS Chemical Neuroscience*, **10**, 1025-1034.
- Berizzi, A.E., Gentry, P.R., Rueda, P., Den Hoedt, S., Sexton, P.M., Langmead, C.J. & Christopoulos, A. (2016) Molecular mechanisms of action of M5 muscarinic acetylcholine receptor allosteric modulators. *Molecular Pharmacology*, **90**, 427-436.
- Berizzi, A.E., Perry, C.J., Shackleford, D.M., Lindsley, C.W., Jones, C.K., Chen, N.A., Sexton, P.M., Christopoulos, A., Langmead, C.J. & Lawrence, A.J. (2018) Muscarinic M 5 receptors modulate ethanol seeking in rats. *Neuropsychopharmacology*, **43**, 1510.
- Bonomo, Y., Norman, A., Biondo, S., Bruno, R., Daglish, M., Dawe, S., Egerton-Warburton, D., Karro, J., Kim, C. & Lenton, S. (2019) The Australian drug harms ranking study. *Journal of Psychopharmacology*, **33**, 759-768.
- Bossert, J.M., Adhikary, S., Laurent, R.S., Marchant, N.J., Wang, H.-L., Morales, M. & Shaham, Y. (2016) Role of projections from ventral subiculum to nucleus accumbens shell in context-induced reinstatement of heroin seeking in rats. *Psychopharmacology*, **233**, 1991-2004.
- Bossert, J.M., Gray, S.M., Lu, L. & Shaham, Y. (2006) Activation of group II metabotropic glutamate receptors in the nucleus accumbens shell attenuates context-induced relapse to heroin seeking. *Neuropsychopharmacology*, **31**, 2197-2209.
- Bossert, J.M., Liu, S.Y., Lu, L. & Shaham, Y. (2004) A role of ventral tegmental area glutamate in contextual cue-induced relapse to heroin seeking. *Journal of Neuroscience*, **24**, 10726-10730.
- Bossert, J.M. & Stern, A.L. (2014) Role of ventral subiculum in context-induced reinstatement of heroin seeking in rats. *Addiction Biology*, **19**, 338-342.
- Bubser, M., Bridges, T.M., Dencker, D., Gould, R.W., Grannan, M., Noetzel, M.J., Lamsal, A., Niswender, C.M., Daniels, J.S. & Poslusney, M.S. (2014) Selective activation of M4 muscarinic acetylcholine receptors reverses MK-801-induced behavioral impairments and enhances associative learning in rodents. *ACS Chemical Neuroscience*, **5**, 920-942.
- Campbell, E.J., Flanagan, J.P., Walker, L.C., Hill, M.K., Marchant, N.J. & Lawrence, A.J. (2019) Anterior Insular Cortex is Critical for the Propensity to Relapse Following Punishment-Imposed Abstinence of Alcohol Seeking. *Journal of Neuroscience*, **39**, 1077-1087.
- Carruthers, S.P., Gurvich, C.T. & Rossell, S.L. (2015) The muscarinic system, cognition and schizophrenia. *Neuroscience & Biobehavioral Reviews*, **55**, 393-402.
- Caulfield, M.P. (1993) Muscarinic receptors—characterization, coupling and function. *Pharmacology & Therapeutics*, **58**, 319-379.

- Ch'ng, S.S., Fu, J., Brown, R.M., Smith, C.M., Hossain, M.A., McDougall, S.J. & Lawrence, A.J. (2019) Characterization of the relaxin family peptide receptor 3 system in the mouse bed nucleus of the stria terminalis. *Journal of Comparative Neurology*, **527**, 2615-2633.
- Charlton, A.J., May, C., Luikinga, S.J., Burrows, E.L., Kim, J.H., Lawrence, A.J. & Perry, C.J. (2019) Chronic voluntary alcohol consumption causes persistent cognitive deficits and cortical cell loss in a rodent model. *Scientific Reports*, **9**, 1-19.
- Chen, N.A., Jupp, B., Sztainberg, Y., Lebow, M., Brown, R.M., Kim, J.H., Chen, A. & Lawrence, A.J. (2014) Knockdown of CRF1 receptors in the ventral tegmental area attenuates cue-and acute food deprivation stress-induced cocaine seeking in mice. *Journal of Neuroscience*, **34**, 11560-11570.
- Conn, J.P., Christopoulos, A. & Lindsley, C. (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nature Reviews Drug Discovery* **8**, 41–54.
- Crombag, H.S., Bossert, J.M., Koya, E. & Shaham, Y. (2008) Context-induced relapse to drug seeking: a review. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363**, 3233-3243.
- Cruz, F.C., Babin, K.R., Leao, R.M., Goldart, E.M., Bossert, J.M., Shaham, Y. & Hope, B.T. (2014) Role of nucleus accumbens shell neuronal ensembles in context-induced reinstatement of cocaine-seeking. *Journal of Neuroscience*, **34**, 7437-7446.
- Curtis, M.J., Alexander, S., Cirino, G., Docherty, J.R., George, C.H., Giembycz, M.A., Hoyer, D., Insel, P.A., Izzo, A.A. & Ji, Y. (2018) Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. Wiley Online Library.
- Gentry, P.R., Kokubo, M., Bridges, T.M., Kett, N.R., Harp, J.M., Cho, H.P., Smith, E., Chase, P., Hodder, P.S. & Niswender, C.M. (2013) Discovery of the First M5-Selective and CNS Penetrant Negative Allosteric Modulator (NAM) of a Muscarinic Acetylcholine Receptor:(S)-9b-(4-Chlorophenyl)-1-(3, 4-difluorobenzoyl)-2, 3-dihydro-1 H-imidazo [2, 1-a] isoindol-5 (9b H)-one (ML375). *Journal of Medicinal Chemistry*, **56**, 9351-9355.
- Gibson, G.D., Prasad, A.A., Jean-Richard-dit-Bressel, P., Yau, J.O., Millan, E.Z., Liu, Y., Campbell, E.J., Lim, J., Marchant, N.J. & Power, J.M. (2018) Distinct accumbens shell output pathways promote versus prevent relapse to alcohol seeking. *Neuron*, **98**, 512-520. e516.
- Gould, R.W., Grannan, M.D., Gunter, B.W., Ball, J., Bubser, M., Bridges, T.M., Wess, J., Wood, M.W., Brandon, N.J. & Duggan, M.E. (2018) Cognitive enhancement and antipsychotic-like activity following repeated dosing with the selective M4 PAM VU0467154. *Neuropharmacology*, **128**, 492-502.
- Gould, R.W., Gunter, B.W., Bubser, M., Matthews, R.T., Teal, L.B., Ragland, M.G., Bridges, T.M., Garrison, A.T., Winder, D.G. & Lindsley, C.W. (2019) Acute Negative Allosteric Modulation of M5 Muscarinic Acetylcholine Receptors Inhibits Oxycodone Self-Administration and Cue-Induced Reactivity with No Effect on Antinociception. *ACS Chemical Neuroscience*, **10**, 3740-3750.
- Hamlin, A., Newby, J. & McNally, G. (2007) The neural correlates and role of D1 dopamine receptors in renewal of extinguished alcohol-seeking. *Neuroscience*, **146**, 525-536.

- Harding, S.D., Sharman, J.L., Faccenda, E., Southan, C., Pawson AJ, Ireland S, Gray AJG, Bruce L, Alexander SPH, Anderton S, Bryant C, Davenport AP, Doerig C, Fabbro D, Levi-Schaffer F, Spedding M, Davies JA, NC-IUPHAR (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucleic Acids Res.* **46**, D1091-1106.
- Heffner, J. L., Blom, T. J., & Anthenelli, R. M. (2011). Gender differences in trauma history and symptoms as predictors of relapse to alcohol and drug use. *The American Journal on Addictions*, **20**, 307–311.
- Hiranita, T., Nawata, Y., Sakimura, K., Anggadiredja, K. & Yamamoto, T. (2006) Suppression of methamphetamine-seeking behavior by nicotinic agonists. *Proceedings of the National Academy of Sciences*, **103**, 8523-8527.
- Kastman, H.E., Blasiak, A., Walker, L., Siwiec, M., Krstew, E.V., Gundlach, A.L. & Lawrence, A.J. (2016) Nucleus incertus Orexin2 receptors mediate alcohol seeking in rats. *Neuropharmacology*, **110**, 82-91.
- Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M. & Altman, D.G. (2010) Animal research: reporting in vivo experiments: the ARRIVE guidelines. *British Journal of Pharmacology*, **160**, 1577-1579.
- Langmead, C. J. & Christopoulos, A. Allosteric agonists of 7TM receptors: expanding the pharmacological toolbox. *Trends in Pharmacological Sciences* **27**, 475–481 (2006).
- Langmead, C.J. & Christopoulos, A. (2014) Functional and structural perspectives on allosteric modulation of GPCRs. *Current Opinion in Cell Biology*, **27**, 94-101.
- Langmead, C.J., Watson, J. & Reavill, C. (2008) Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacology & Therapeutics*, **117**, 232-243.
- Lebois, E., Thorn, C., Edgerton, J., Popielek, M. & Xi, S. (2018) Muscarinic receptor subtype distribution in the central nervous system and relevance to aging and Alzheimer's disease. *Neuropharmacology*, **136**, 362-373.
- Marchant, N.J., Campbell, E.J., Whitaker, L.R., Harvey, B.K., Kaganovsky, K., Adhikary, S., Hope, B.T., Heins, R.C., Prinszano, T.E. & Vardy, E. (2016) Role of ventral subiculum in context-induced relapse to alcohol seeking after punishment-imposed abstinence. *Journal of Neuroscience*, **36**, 3281-3294.
- Marchant, N.J., Kaganovsky, K., Shaham, Y. & Bossert, J.M. (2015) Role of corticostriatal circuits in context-induced reinstatement of drug seeking. *Brain Research*, **1628**, 219-232.
- McGrath, J.C. & Lilley, E. (2015) Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Wiley Online Library.
- Melis, F., Stancampiano, R., Imperato, A., Carta, G. & Fadda, F. (1996) Chronic ethanol consumption in rats: correlation between memory performance and hippocampal acetylcholine release in vivo. *Neuroscience*, **74**, 155-159.
- Mesulam, M., Mufson, E., Wainer, B. & Levey, A. (1983) Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6). *Neuroscience*, **10**, 1185-1201.

- Moss, S., Sharott, A., Goodhead, L.H. & Mitchell, S.N. (2003) Role of muscarinic receptors in the activation of the ventral subiculum and the consequences for dopamine release in the nucleus accumbens. *European Journal of Pharmacology*, **460**, 117-125.
- Naber, P. & Witter, M. (1998) Subicular efferents are organized mostly as parallel projections: a double-labeling, retrograde-tracing study in the rat. *Journal of Comparative Neurology*, **393**, 284-297.
- Perry, C.J. (2016) Cognitive decline and recovery in alcohol abuse. *Journal of Molecular Neuroscience*, **60**, 383-389.
- Rehm, J., Gmel Sr, G.E., Gmel, G., Hasan, O.S., Imtiaz, S., Popova, S., Probst, C., Roerecke, M., Room, R. & Samokhvalov, A.V. (2017) The relationship between different dimensions of alcohol use and the burden of disease—an update. *Addiction*, **112**, 968-1001.
- Ryan, P.J., Kastman, H.E., Krstew, E.V., Rosengren, K.J., Hossain, M.A., Churilov, L., Wade, J.D., Gundlach, A.L. & Lawrence, A.J. (2013) Relaxin-3/RXFP3 system regulates alcohol-seeking. *Proceedings of the National Academy of Sciences*, **110**, 20789-20794.
- Solecki, W., Wickham, R.J., Behrens, S., Wang, J., Zwerling, B., Mason, G.F. & Addy, N.A. (2013) Differential role of ventral tegmental area acetylcholine and N-methyl-D-aspartate receptors in cocaine-seeking. *Neuropharmacology*, **75**, 9-18.
- Sun, W. & Rebec, G.V. (2003) Lidocaine inactivation of ventral subiculum attenuates cocaine-seeking behavior in rats. *Journal of Neuroscience*, **23**, 10258-10264.
- Taepavarapruk, P., Butts, K.A. & Phillips, A.G. (2015) Dopamine and glutamate interaction mediates reinstatement of drug-seeking behavior by stimulation of the ventral subiculum. *International Journal of Neuropsychopharmacology*, **18**, pyu008.
- Vilaró, M.T., Palacios, J. & Mengod, G. (1990) Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. *Neuroscience Letters*, **114**, 154-159.
- Vorel, S.R., Liu, X., Hayes, R.J., Spector, J.A. & Gardner, E.L. (2001) Relapse to cocaine-seeking after hippocampal theta burst stimulation. *Science*, **292**, 1175-1178.
- Walker, L.C., Berizzi, A.E., Chen, N.A., Rueda, P., Perreau, V.M., Huckstep, K., Srisontiyakul, J., Govitrapong, P., Xiaojian, J. & Lindsley, C.W. (2020) Acetylcholine muscarinic M4 receptors as a therapeutic target for alcohol use disorder: converging evidence from humans and rodents. *Biological Psychiatry*, **88**, 898-909.
- Walker, L.C., Hand, L.J., Letherby, B., Huckstep, K.L., Campbell, E.J. & Lawrence, A.J. (2021) Cocaine and amphetamine regulated transcript (CART) signalling in the central nucleus of the amygdala modulates stress-induced alcohol seeking. *Neuropsychopharmacology*, **46**, 325-333.
- Walker, L.C., Kastman, H.E., Koeleman, J.A., Smith, C.M., Perry, C.J., Krstew, E.V., Gundlach, A.L. & Lawrence, A.J. (2017a) Nucleus incertus corticotrophin-releasing factor 1 receptor signalling regulates alcohol seeking in rats. *Addiction Biology*, **22**, 1641-1654.
- Walker, L.C., Kastman, H.E., Krstew, E.V., Gundlach, A.L. & Lawrence, A.J. (2017b) Central amygdala relaxin-3/relaxin family peptide receptor 3 signalling modulates alcohol seeking in rats. *British Journal of Pharmacology*, **174**, 3359-3369.

- Walker, L.C., Kastman, H.E. & Lawrence, A.J. (2019) Pattern of neural activation following yohimbine-induced reinstatement of alcohol seeking in rats. *European Journal of Neuroscience*.
- Walker, L.C. & Lawrence, A.J. (2018) Investigational drug therapies in phase I and phase II clinical trials for alcohol use disorders. *Expert Opinion on Investigational Drugs*, **27**, 677-690.
- Walker, L.C. & Lawrence, A.J. (2020) Allosteric modulation of muscarinic receptors in alcohol and substance use disorders *Advances in Pharmacology*. Elsevier, pp. 233-275.
- Wang, F., Flanagan, J., Su, N., Wang, L.-C., Bui, S., Nielson, A., Wu, X., Vo, H.-T., Ma, X.-J. & Luo, Y. (2012) RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *The Journal of Molecular Diagnostics*, **14**, 22-29.
- Wee, R.W. & MacAskill, A.F. (2020) Biased connectivity of brain-wide inputs to ventral subiculum output neurons. *Cell Reports*, **30**, 3644-3654. e3646.
- Williams, E. S., Manning, C. E., Eagle, A. L., Swift-Gallant, A., Duque-Wilckens, N., Chinnusamy, S., ... & Robison, A. J. (2020). Androgen-dependent excitability of mouse ventral hippocampal afferents to nucleus accumbens underlies sex-specific susceptibility to stress. *Biological Psychiatry*, **87**(6), 492-501.
- Witkiewitz, K., Litten, R. & Leggio, L. (2019) Advances in the science and treatment of alcohol use disorder. *Science Advances*, **5**, eaax4043.
- Yu, G. & Sharp, B.M. (2015) Basolateral amygdala and ventral hippocampus in stress-induced amplification of nicotine self-administration during reacquisition in rat. *Psychopharmacology*, **232**, 2741-2749.

Figure legends

Figure 1. *Chrm4* and *Chrm5* mRNA expression is altered by chronic alcohol consumption in the vSub, but not VTA. (A) Schematic of experimental procedures. (B) Rats consumed high level of alcohol for >57 24-hour sessions of intermittent two-bottle choice. (C) Chronic intermittent alcohol consumption and 14 days abstinence significantly decreased normalised relative quantities (NRQ) of *Chrm4* compared to naïve and alcohol groups, while chronic intermittent alcohol consumption and 24 h abstinence increased *Chrm5* expression in the vSub. (D) No changes in *Chrm4* or *Chrm5* expression were observed within the VTA. Data are expressed as mean $\pm$ SEM, $n = 7/\text{group}$, $*p < 0.05$.

Figure 2. *Chrm4* and *Chrm5* expressing cells in the vSub projection to the AcbSh. (A) Schematic of CT β -488 injection into the AcbSh and representative injection site. (B) Cell density of *Chrm4* and *Chrm5* expression within vSub. (C) Proportion of *Chrm4*-positive cells that express *Chrm5* within the vSub. (D) Proportion of *Chrm5*-positive cells that express *Chrm4* within the vSub. (E) Overview and high magnification representative microphotograph of CT β -488, *Chrm4*, *Chrm5* and overlay within the vSub. (F) Donut graph depicting proportions of CT β -488 positive, dual CT β -488/*Chrm4*-positive, CT β -488/*Chrm5*-positive and triple labelled CT β -488/*Chrm4*/*Chrm5*-positive cells that project to the AcbSh. (G) 70% of vSub to AcbSh projections express *Chrm4*, representing 32% of all *Chrm4*-positive cells expressed in the vSub. (H) In contrast only 18% of vSub to AcbSh projections express *Chrm5*, representing 25% of all *Chrm5*-positive cells expressed in the vSub. Data are expressed as mean, or mean $\pm$ SEM. Scale bars = 200 μm (overview) and 50 μm (high magnification), $n = 4$.

Figure 3. Intra-vSub administration of the M4 mAChR PAM VU0467154 reduces context-induced alcohol seeking. (A) Schematic of training procedures. (B) Lever responding during the 7 operant self-administration training days prior to test in context A and (C) extinction in context B. (D) Schematic of testing procedures. (E) VU0467154 (VU154) administered within the vSub reduced context-induced reinstatement in context A (ABA), but not (F) active lever responding in context B (ABB). (G) Schematic of vSub infusions, vehicle = black, VU154 = orange, images from (Paxinos and Watson, 2006), position expressed as mm from bregma. Data are expressed as mean $\pm$ SEM, $n = 6$ vehicle-treated and $n = 8$ VU154-treated rats. $*p < 0.05$.

Figure 4. Intra-vSub administration of the M5 mAChR NAM ML375 does not alter context-induced alcohol seeking. (A) Schematic of training procedures. (B) Lever responding during the 7 operant self-administration training days prior to extinction in context A and (C) extinction in context B. (D) Schematic of testing procedures. (E) ML375 administered within the vSub did not alter context-induced reinstatement in context A (ABA), or (F) active lever responding in context B (ABB). (G) Schematic of vSub infusions, vehicle = black, ML375 = green, images from (Paxinos and Watson, 2006), position expressed as mm from bregma. Data are expressed as mean $\pm$ SEM, $n = 5$ vehicle-treated and $n = 7$ ML375-treated rats.

Figure 5. Intra-vSub administration of the M4 mAChR PAM VU0467154 does not alter motivation to self-administer alcohol. (A) Schematic of training procedures. (B) Lever responding during the 7 operant self-administration training days prior to test. (C) Schematic of testing procedures. (D) VU0467154 (VU154) administered within the vSub did not alter breakpoint during the progressive ratio (PR3-4) test. (E) Further, VU154 did not alter active lever responding across the timecourse of the PR test. (F) Schematic of vSub

infusions, intra-vSub = black. Images from (Paxinos and Watson, 2006), position expressed as mm from bregma. Data are expressed as mean $\pm$ SEM, n = 13 tested in a randomised and counterbalanced manner.

Figure 6. Intra-vSub administration of the M5 mAChR NAM ML375 transiently alters motivation to self-administer alcohol. (A) Schematic of training procedures. (B) Lever responding during the 7 operant self-administration training days prior to test. (C) Schematic of testing procedures. (D) ML375 administered within the vSub did not alter breakpoint during the progressive ratio (PR3-4) test. (E) However, a reduction in lever pressing was observed in the first 15 min of the PR3-4 test following ML375 administration. (F) Schematic of vSub infusions, intra-vSub = black. Images from (Paxinos and Watson, 2006), position expressed as mm from bregma. Data are expressed as mean $\pm$ SEM, n = 6 tested in a randomised and counterbalanced manner. *p < 0.05

Figure 7. Intra-vSub administration of the M4 mAChR PAM VU0467154 or the M5 NAM ML375 does not alter locomotor activity, food or water consumption. (A) Schematic of testing procedures. (B) Following vSub VU0467154 (VU154) administration, no difference was observed in locomotor activity across time, nor (C) total locomotor activity. (D) No difference was observed in food consumption, nor (E) Water intake. Data are expressed as mean $\pm$ SEM, n = 7 vehicle-treated and 8 VU154-treated rats. (F) Following vSub ML375 administration, no difference was observed in locomotor activity across time, nor (G) total locomotor activity. (H) No difference was observed in food consumption, nor (I) water intake. Data are expressed as mean $\pm$ SEM, (A-D) n = 8 VU154-treated rats and n = 7 VU154 vehicle-treated controls. (E-G) n = 6 ML375 treated rats and n = 6 ML375 vehicle treated controls.

Author contributions

The study was conceived and planned by AJL, LCW and CJL. LCW and AJL jointly supervised students. LCW, KLH and NAC performed qPCR and fluorescent in situ hybridization; LCW, KLH, NAC and LJH performed behavioural studies. LCW and KLH analysed data. CKJ supplied VU0467154 and ML375. The manuscript was written and revised by LCW, KLH and AJL, with input from CJL and CKJ. All other authors approved the manuscript prior to submission.

Acknowledgements

This research was supported by a National Health and Medical Research Council project grant (Grant No. 1120576 [to AJL and CJL]), of which AJL is a Principal Research Fellow (Grant No. 1116930). We acknowledge the Victorian State Government Operational Infrastructure Program.

Conflict of interest statement

CWL is an inventor on patents that protect different classes of muscarinic receptor allosteric modulators. All other authors report no biomedical financial interests or potential conflicts of interest.

Declaration of transparency and scientific rigor

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for [Design and Analysis](#) and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Data availability statement

The data from this study are available from the corresponding authors upon reasonable request.

Bulletin summary

What is already known:

- Allosteric modulation of M₄ and M₅ muscarinic receptors reduce alcohol consumption/seeking.
- The vSub and VTA provide loci where muscarinic receptors may mediate alcohol consumption/seeking.

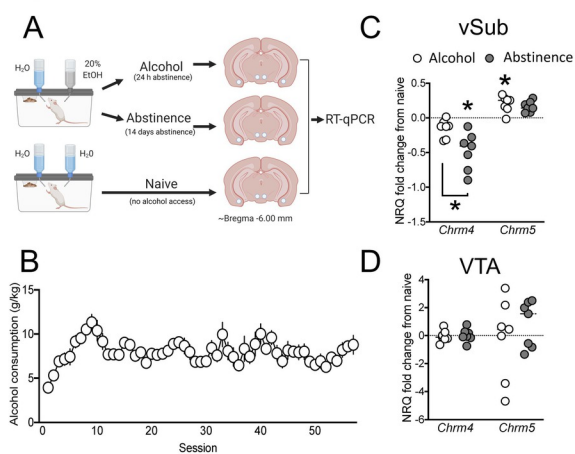
What this study adds:

- Long-term alcohol consumption/abstinence alters muscarinic receptor expression in the vSub, but not VTA.
- vSub M₄ and M₅ allosteric modulation have divergent actions on alcohol consumption and seeking.

What is the clinical significance:

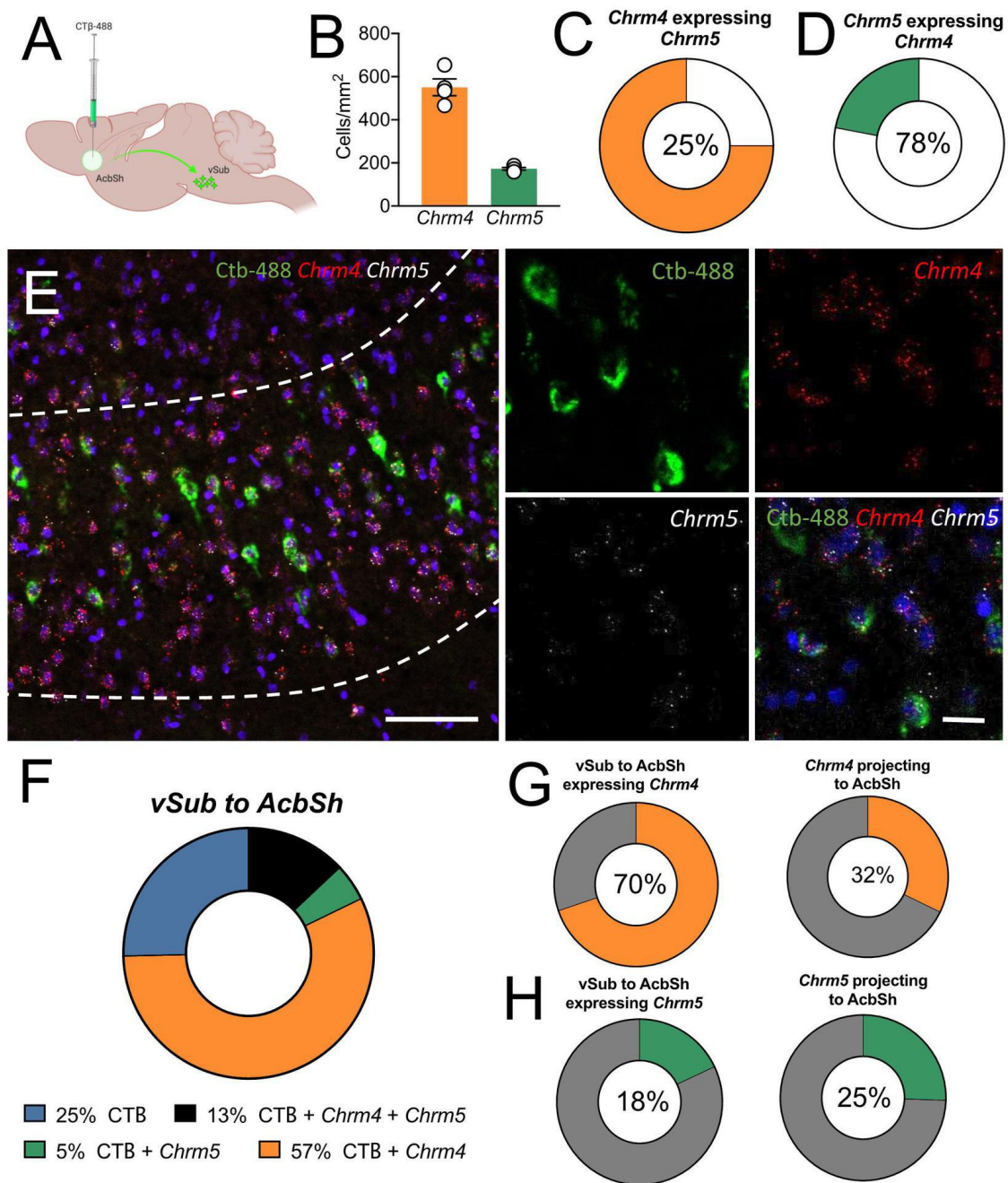
- Our results provide greater understanding of cholinergic dysregulation in AUD.
- This study further highlights the potential of compounds targeting muscarinic receptors to treat AUD.

Figure 1



BPH_15513_Figure 1.jpg

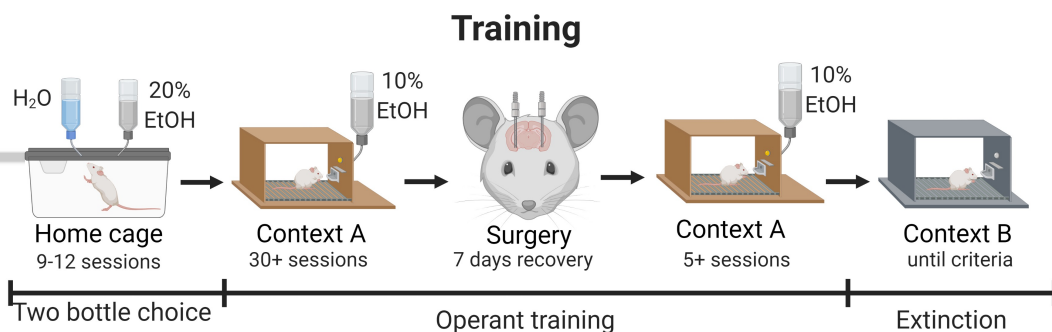
Figure 2



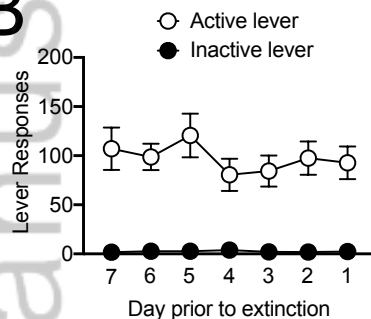
BPH_15513_Figure 2.jpeg

Figure 3

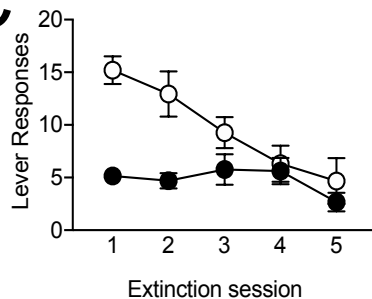
A



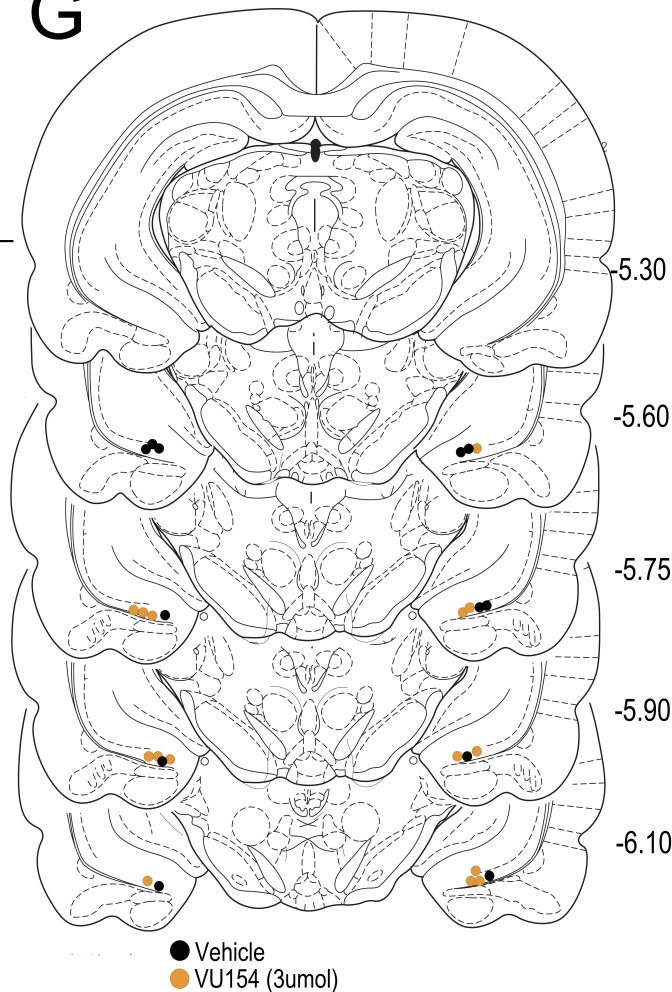
B



C

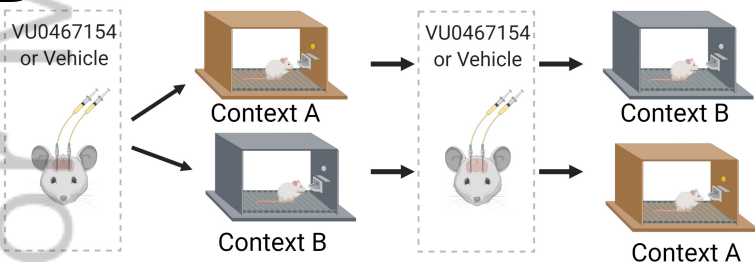


G

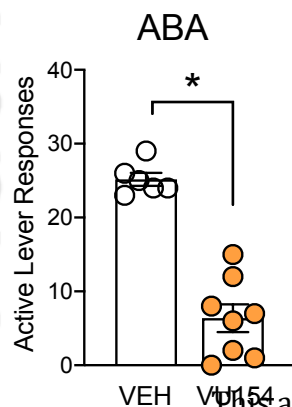


D

Test



E



F

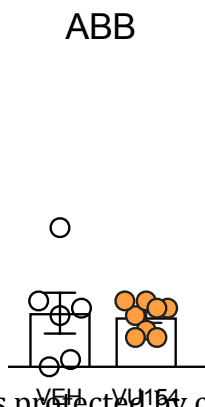


Figure 4

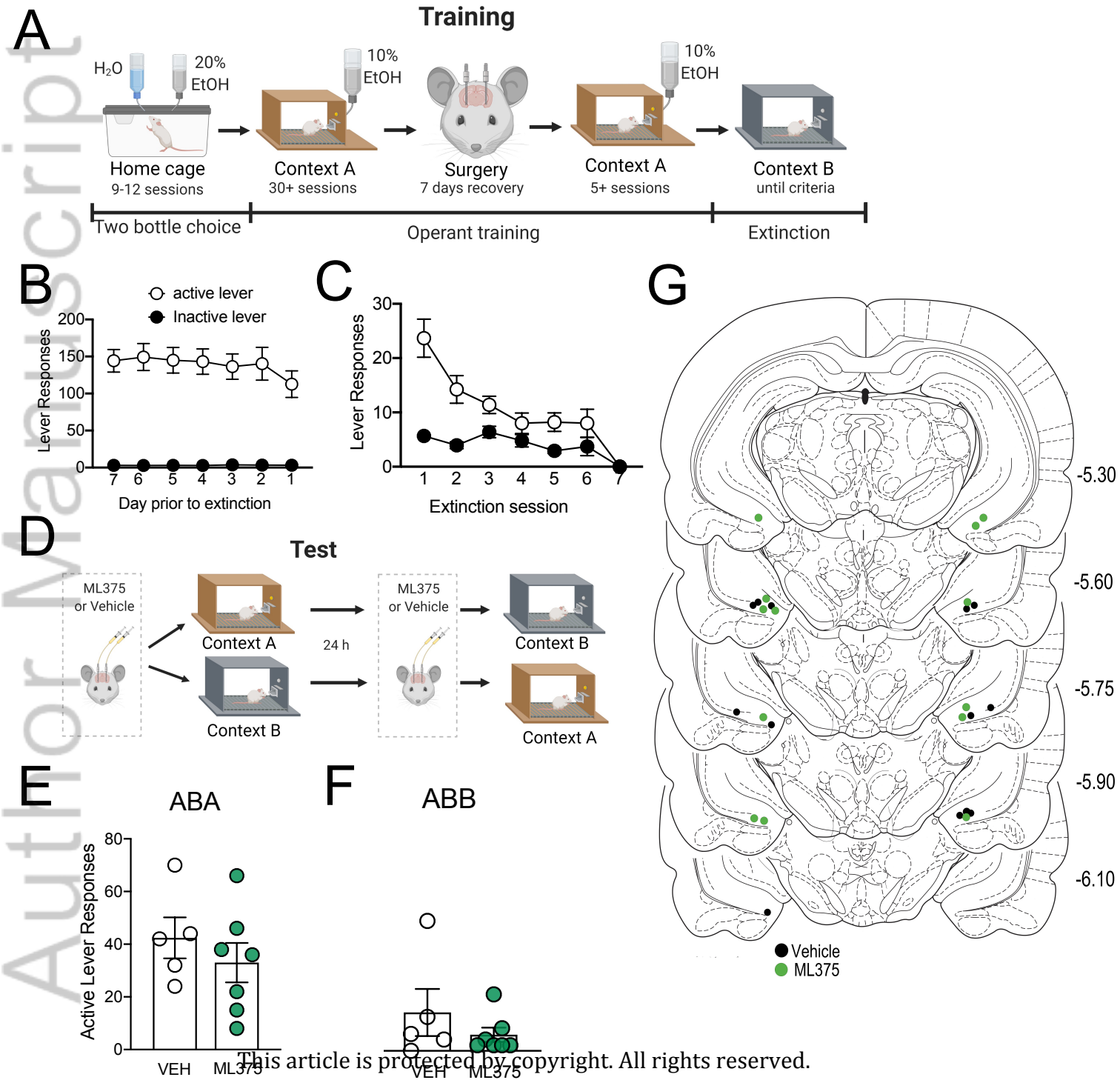


Figure 5

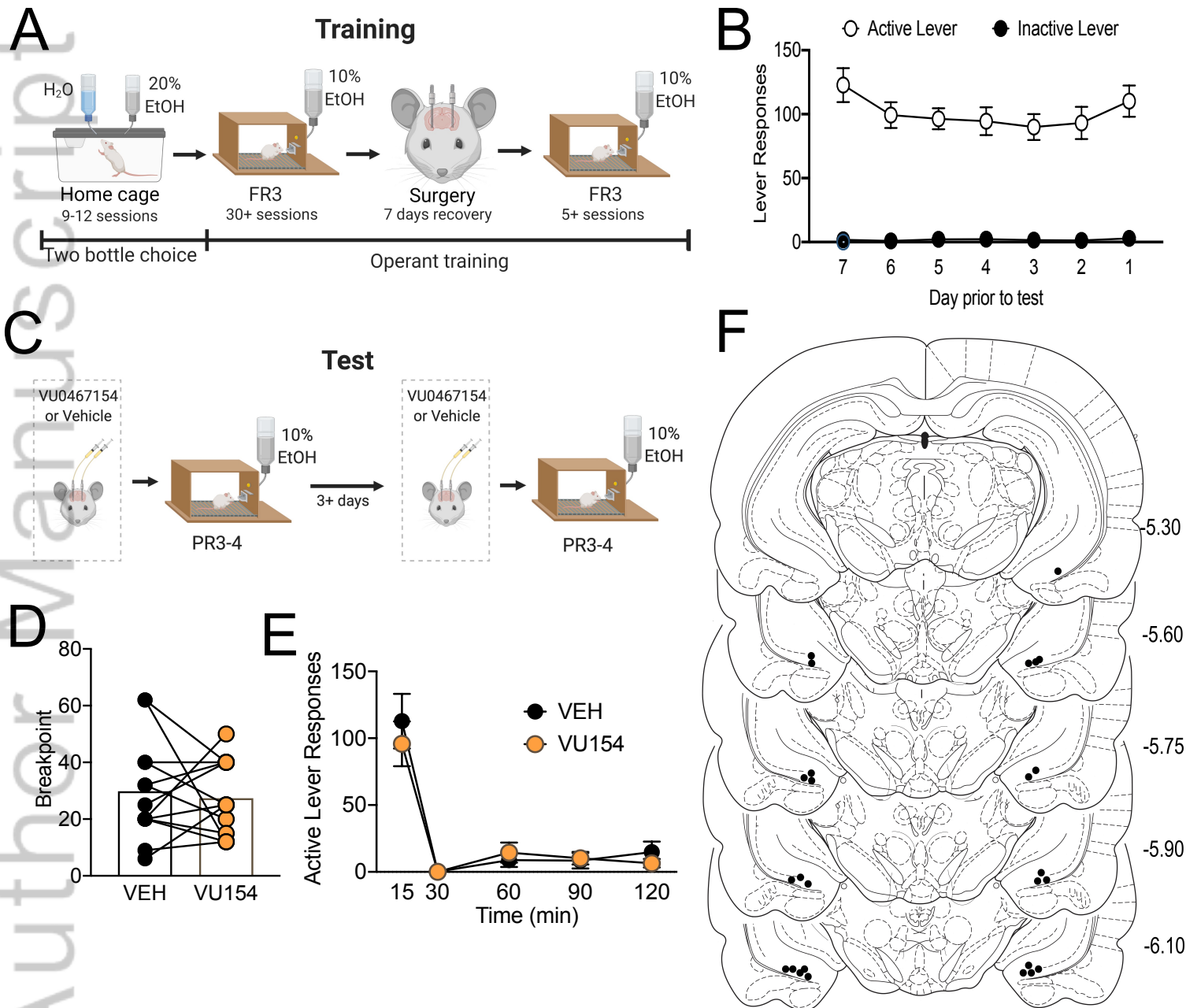
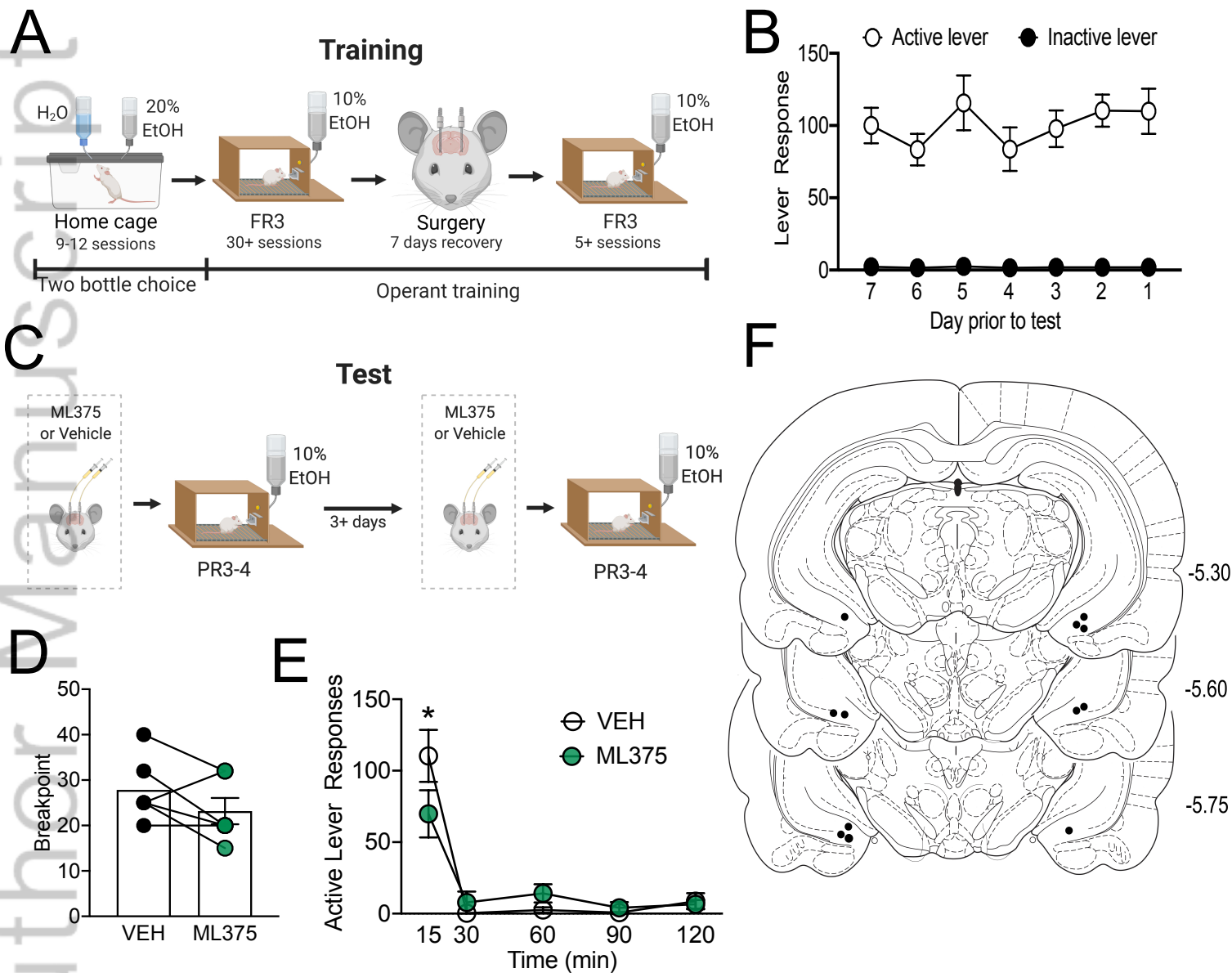
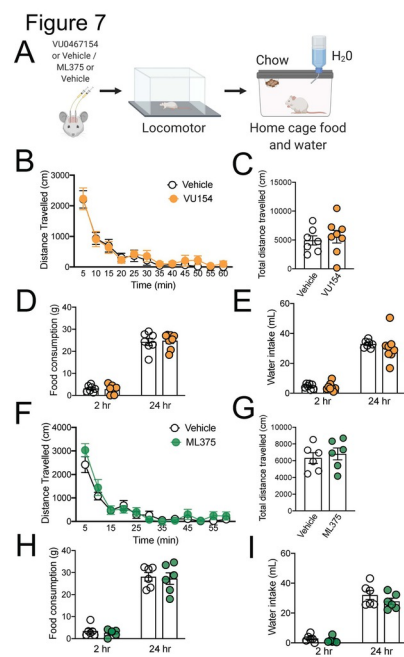


Figure 6





BPH_15513_Figure 7.jpg