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RESEARCH PAPER

Muscarinic M_4 and M_5 receptors in the ventral subiculum differentially modulate alcohol seeking versus consumption in male alcohol-preferring rats

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Background and Purpose: Muscarinic acetylcholine receptors mediate alcohol consumption and seeking in rats. While M_4 and M_5 receptors have recently been implicated to mediate these behaviours in the striatum, their role in other brain regions remain unknown. The ventral tegmental area (VTA) and ventral subiculum (vSub) both densely express M_4 and M_5 receptors 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. 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 receptors within the vSub in alcohol consumption, context-induced alcohol-seeking, locomotor activity, and food/water consumption.

Key Results: Long-term alcohol and abstinence dysregulated the expression of genes for muscarinic receptors in the vSub, not in the VTA. *Chrm4* was down-regulated following long-term alcohol and abstinence, while *Chrm5* was up-regulated following long-term alcohol consumption. Consistent with these data, a positive allosteric modulator (VU0467154) of intra-vSub M_4 receptors reduced context-induced alcohol-seeking, but not motivation for alcohol self-administration, while M_5 receptor 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 receptor allosteric modulators to reduce alcohol consumption or seeking.

Abbreviations: AcbSh, nucleus accumbens shell; AUD, alcohol use disorders; *Chrm4*, gene for muscarinic M_4 receptor; *Chrm5*, gene for muscarinic M_5 receptor; CTβ, cholera toxin-β; FISH, fluorescent in situ hybridisation; FR3, fixed ratio 3; *Hsp90*, gene for heatshock protein 90; iP, Indiana alcohol preferring rat; LH, lateral hypothalamus; 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.

Leigh C. Walker and Kate L. Huckstep contributed equally.

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

1 | INTRODUCTION

Despite the large social and economic burden of alcohol use disorders (AUD) on society (Bonomo et al., 2019; Rehm et al., 2017), current pharmacotherapeutic treatment options remain ineffective and insufficient at a population level (Walker & Lawrence, 2018; Witkiewitz, Litten, & Leggio, 2019). Using selective allosteric modulation, we have recently identified a role for **M_4** and **M_5 muscarinic acetylcholine receptors**, two **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 muscarinic receptor 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, Mufson, Wainer, & Levey, 1983), express muscarinic and **nicotinic** AChRs, including the M_4 and M_5 receptors (Lebois, Thorn, Edgerton, Popiolek, & Xi, 2018; Vilaró, Palacios, & Mengod, 1990), and have critical roles in relapse to drug seeking, specifically following exposure to a context previously associated with drug availability (Bossert et al., 2016; Bossert, Liu, Lu, & Shaham, 2004; Bossert & Stern, 2014; Crombag, Bossert, Koya, & Shaham, 2008; 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., 2016; Bossert, Gray, Lu, & Shaham, 2006; Crombag, Bossert, Koya, & Shaham, 2008; Cruz et al., 2014; Marchant, Kaganovsky, Shaham, & Bossert, 2015). 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 muscarinic receptors modulates the downstream accumbal **dopamine** release (Moss, Sharott, Goodhead, & Mitchell, 2003; Solecki et al., 2013), suggesting potential pathways in which cholinergic dysregulation may affect the mesocorticolimbic reward system. Given these data, the aim of the present study was to examine the roles of M_4 and M_5 receptors within the VTA and vSub in alcohol consumption and seeking behaviours, in rats.

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* (M_4 receptor) and *Chrm5* (M_5 receptor) expression in male Indiana alcohol-preferring (iP) rats. We found specific dysregulation of the expression of mRNA for muscarinic receptors 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-h 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 M_4 and M_5 receptors are expressed to a varying degree on vSub → AcbSh projections. Finally, we sought to modulate cholinergic signalling within the vSub via the M_4 receptor positive allosteric modulator (PAM), VU0467154 (Bubser et al., 2014) or M_5 receptor 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 with ligands that act at an orthosteric binding site (Langmead & Christopoulos, 2006, 2014). These low MW compounds may provide advantages including increased GPCR subtype selectivity and more nuanced modulation of downstream signalling (e.g., biased signalling and/or functional selectivity) (Conn, Christopoulos, & Lindsley, 2009; Langmead & Christopoulos, 2014). We found enhancement of M_4 receptors in the vSub with VU0467154 (to counteract *Chrm4* down-regulation in abstinence) specifically reduced context-induced reinstatement of alcohol seeking, while M_5 disruption of M_5 receptors with ML375

What is already known

- Allosteric modulation of M_4 and M_5 muscarinic receptors reduce alcohol consumption/seeking.
- The vSub and VTA provide loci where muscarinic receptors may mediate alcohol consumption/seeking.

What does this study adds

- Long-term alcohol consumption or abstinence alters muscarinic receptor expression in the vSub, but not VTA.
- vSub M_4 and M_5 allosteric modulation have divergent actions on alcohol consumption versus seeking.

What is the clinical significance

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

(to counteract *Chrm5* up-regulation 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 the allosteric modulation of muscarinic receptors to regulate vSub output pathways.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures 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 (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

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-h 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., 2020; Walker, Kastman, & Lawrence, 2019).

2.2 | Stereotaxic surgery

2.2.1 | AcbSh retrograde tracer injections

To determine vSub projections to the AcbSh, for experiment 2, rats were given an intracranial retrograde tracer (Cholera Toxin-β-488; CTβ-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-μl syringe (Hamilton, USA) was lowered into the AcbSh (anteroposterior, +1.6 mm; mediolateral, ± 2.3 mm; dorsoventral, − 7.5 mm), and 150 nl of CTβ-488 (1%, Thermofisher Scientific, CAT# C34775) was unilaterally infused at a rate of 75 nl/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 given meloxicam and baytril (3 mg kg^{-1} i.p.) every 24 h for 3 days.

2.2.2 | vSub cannulae implantation

For experiments 3–7, rats had a stereotaxic cannula implanted within the vSub, to allow direct drug administration. Rats were anaesthetized and prepared as described above. Next, four holes were drilled into the skull, and screws (1.4-mm 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, ± 5.3 mm; dorsoventral, − 7.8 mm on 4° angle) as previously described (Campbell et al., 2019; Marchant et al., 2016; Walker et al., 2017; Walker, Kastman, Krstew, Gundlach, & Lawrence, 2017). The cannula was 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 (3 mg kg^{-1} i.p.) every 24 h for 3 days.

2.3 | Drug microinfusion

Bilateral intra-vSub infusions of VU0467154 or ML375 were made using 40-cm polyethylene connectors (PlasticsOne) attached to 1-μl syringes (SGE Analytical Science, Ringwood). Rats were gently held while dummies were removed, and injectors inserted into cannulae. VU0467154 (0.5 μl), ML375 (0.5 μl), or their respective vehicle was infused bilaterally (0.25 μl min^{-1}) 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 randomised counterbalanced manner. Following behavioural testing to test cannula positioning, methylene blue (0.5 μl) 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 isopentane super cooled (approx. -20°C) on dry ice, sectioned (40 μm) 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%, and 100%) and X-3B (Olichem Pty Ltd.) before cover-slipping. An investigator blinded to treatment groups and behavioural outcomes performed injection site validations (Kastman et al., 2016; Walker, Kastman, Krstew, Gundlach, & Lawrence, 2017).

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.

2.4 | RT-qPCR

For experiment 1, rats were killed with Lethabarb (pentobarbital sodium) overdose (2 ml kg^{-1}; i.p.) 24 h 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 and 2.1 and RNA samples were analysed 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 three 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 three 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).

2.5 | 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 s on dry ice-cooled isopentane (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, and CTβ-488 was detected endogenously (Ch'ng et al., 2019; Gibson et al., 2018; Wang et al., 2012). Slides were fixed in 4% w/v paraformaldehyde for 15 min 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 min each at room temperature. Slides were then air-dried for 10 min at room temperature, and a hydrophobic barrier was drawn around the brain sections. Sections were then protease treated (pre-treatment 4) at room temperature 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 h. Following this, slides were incubated with preamplifier and amplifier probes (AMP1, 40°C for 30 min; AMP2, 40°C for 15 min; 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 CTβ-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 CTβ-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× objective was used to take overview images of the endogenous CTβ-488 fluorescence in the injection site, and placements were confirmed by two independent researchers. For FISH imaging, a 40× objective was used to take contralateral overview images of the vSub (pixel size 0.10um), which were quantified from three sections per rat (∼Bregma −6.0 mm; Paxinos & Watson, 2007) using image J (National Institutes of Health; RRID:SCR_003070).

TABLE 1 RT-qPCR primer sequences

Gene	Accession #	Forward	Reverse
Hsp90	NM_001004082	TCCTCCGGGAGTTGATCTCTA	TCAATCTTCAGCTCTTTCCCGC
Tbp	NM_001004198.1	AGAACAATCCAGACTAGCAGCA	GGGAACTTCACATCACAGCTC
Hprt1	NM_012583.2	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT
Chrm4	NM_031547.1	GAACCTCTGGCTTGTTCCGC	CCATGCTGAACCCAACTGGA
Chrm5	NM_017362.4	TTGCAGTTGTGACTGCGGTG	GAGAACCCAGCGTCCCATGA

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).

2.6 | 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 (Campbell et al., 2019; Walker et al., 2020, 2021). Alcohol solutions were prepared in tap water from 100% (v/v) ethanol. Daily sessions began at 10:00 AM. After 24 h, the alcohol bottle was replaced with a second water bottle for the subsequent 24- to 48-h 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).

2.7 | 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 (Kastman et al., 2016; Ryan et al., 2013; 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 (0.1 ml per delivery) into the receptacle via an 18-gauge blunt needle connected to a 60-ml 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.

2.8 | Locomotor activity apparatus

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

2.9 | Experimental design

2.9.1 | Experiment 1: Expression of mRNA for M_4 and M_5 receptors 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-abstinent ($n = 7$). Alcohol-experienced rats were then killed 24 h after the last ethanol access, while alcohol-abstinent 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).

2.9.2 | Experiment 2: Phenotyping expression of M_4 and M_5 receptors 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 M_4 and M_5 receptors 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 killed 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.

2.9.3 | Experiment 3 and 4: Examining M_4 and M_5 receptors 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 M_4 receptor activity (via positive allosteric modulation) or reducing M_5 receptor activity (via negative allosteric modulation) could prevent context-induced reinstatement of alcohol seeking. Following nine to 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 re-acquire 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 three consecutive days). Rats were then tested for alcohol seeking in context A (ABA) and context B (ABB), in a randomised and counterbalanced order 24 h apart (Hamlin, Newby, & McNally, 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 M_4 receptor PAM, VU0467154 ($n = 8$) or vehicle ($n = 6$), or for experiment 4, M_5 receptor NAM, ML375 ($n = 7$) or vehicle ($n = 5$), were microinfused into the vSub immediately prior to test.

2.9.4 | Experiment 5 and 6: Examining M_4 or M_5 receptors in the motivation to consume alcohol

To determine whether vSub M_4 and M_5 receptors regulate motivation to obtain alcohol, we next tested under a progressive ratio schedule. As per Experiments 3 and 4, rats underwent nine to 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 re-acquire 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, 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.

2.9.5 | Experiment 7: Examining M_4 and M_5 receptors 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 h), and subsequently, food (standard chow) and water consumption was measured in the home cage 2 and 24 h 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.

2.10 | Data and statistical analysis

GraphPad Prism (RRID:SCR_002798) statistical software was employed for all analysis. For context-induced reinstatement (context × treatment), progressive ratio time course (treatment × time), food and water intake (intake × 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 Student's *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 $2^{-\Delta\Delta Cq}$ approach, was used for qPCR data evaluation (Biogazelle, Zwijnaarde, Belgium; www.qbaseplus.com). In qbase+, Cq values were converted into normalized relative quantities (NRQs) based on the expression of the reference genes. One-way ANOVA was performed on NRQs 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 (Campbell et al., 2019; Kastman et al., 2016; Walker et al., 2017, 2021; Walker, Kastman, & Lawrence, 2019). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.11 | Materials

The selective M_4 receptor PAM, VU0467154 (5-amino-3,4-dimethyl-N-(4-((trifluoromethyl)sulfonyl)benzyl)thieno[2,3-c]-pyridazine-6-carboxamide) and selective M_5 receptor 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 was dissolved in 80% dimethyl sulfoxide (DMSO, v/v) in sterile saline and ML375 was formulated in 5% DMSO and 95% sterile saline (v/v). The doses used here (VU0467154, 3 μmol per hemisphere; ML375, 105 pmol per hemisphere) 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).

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Mathie et al., 2019).

3 | RESULTS

3.1 | Long-term alcohol consumption alters expression of *Chrm4* and *Chrm5* 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 that underwent long-term intermittent two-bottle choice and were culled 24 hours after their last alcohol session

(alcohol), and (3) rats that 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^{-1} per 24 h session (Figure 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. Bonferroni *post hoc* comparison revealed a significant decrease in *Chrm4* expression after abstinence compared with alcohol and naïve groups (Figure 1b). Further, one-way ANOVA of *Chrm5* expression also revealed a significant difference between treatment groups with *post hoc* analysis revealing a significant increase in *Chrm5* expression in alcohol compared with naïve groups (Figure 1c). In contrast, in the VTA, one-way ANOVA showed no significant difference in either *Chrm4* or *Chrm5* expression (Figure 1d).

3.2 | *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 were examined using RNAscope in combination with an intra-AcbSh injection of the retrograde tracer CTβ-488 (Figure 2a). Density calculations revealed greater expression of *Chrm4* than *Chrm5* in the vSub (Figure 2b). Within the vSub, most of the *Chrm5*-positive cells also expressed *Chrm4* while many fewer *Chrm4* expressing cells also expressed *Chrm5* (Figure 2c,d). Quantification of the percentage of CTβ-positive cells expressing muscarinic receptors showed that most CTβ-positive cells also expressed *Chrm4*, *with fewer* expressing *Chrm5*, or expressing both *Chrm4* and *Chrm5* (Figure 2f). A quarter of CTβ-positive cells expressed neither *Chrm4* nor *Chrm5*. Further, *Chrm4* and *Chrm5* mRNA were 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 approximatley one third of all vSub *Chrm4*-positive and one quarter ofall vSub *Chrm5*-positive cells expressed CTβ-488, suggesting a subpopulation of these cells project to the AcbSh (Figure 2g,h).

3.3 | M_4 receptors 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 M_4 receptors have a functional role in context-induced alcohol seeking. As *Chrm4* expression was decreased following abstinence, we microinjected the selective M_4 receptor PAM, VU0467154, directly into the vSub to enhance signalling. During training rats reached a stable level of responding in context A, with a high level of active lever responses (corresponding to an alcohol intake of 0.60 ± 0.06 g kg^{-1} per session), over the 7 days preceding extinction training (Figure 3a). Rats extinguished responding in context B (Figure 3b). After random assignment to treatment group, no significant differences were observed in responding for alcohol in context A prior to extinction (data not shown). On test day, repeated measures (RM) two-way ANOVA analysis revealed a main effect of

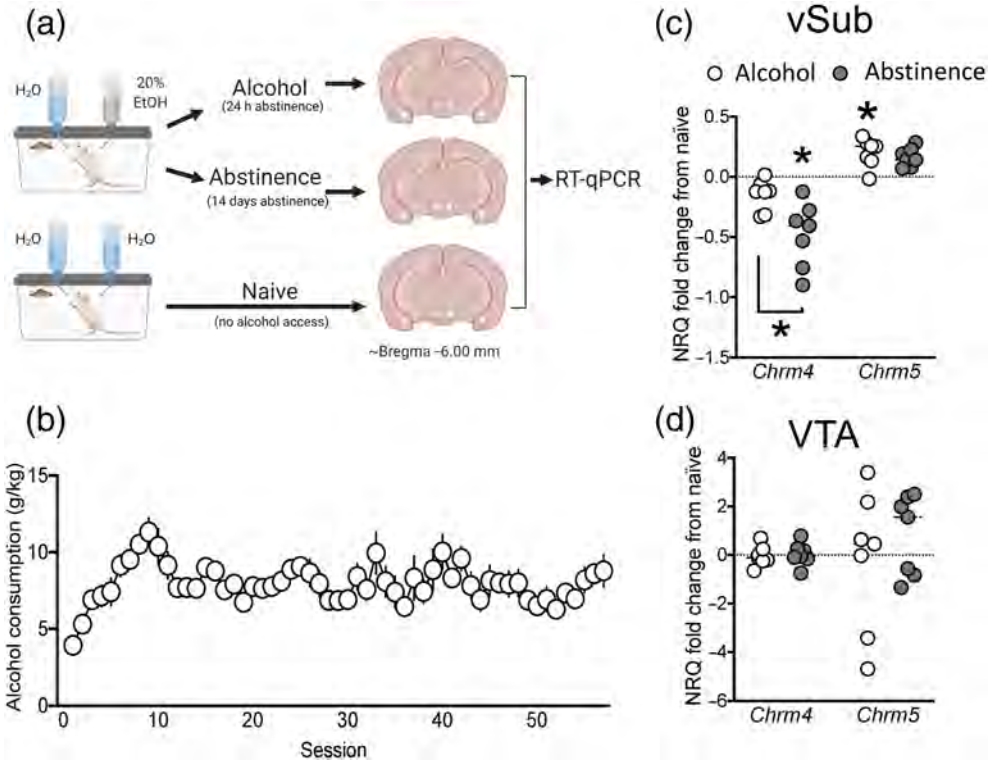

FIGURE 1 Chronic alcohol consumption alters the expression of *Chrm4* and *Chrm5* mRNA in the vSub, but not in the VTA. (a) Schematic of experimental procedures. (b) Rats consumed high level of alcohol for >57 24-h sessions of intermittent two-bottle choice. (c) Chronic intermittent alcohol consumption 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 shown as mean ± SEM or individual values; $n = 7$ per group. *$P < 0.05$, significantly different from naïve or as indicated

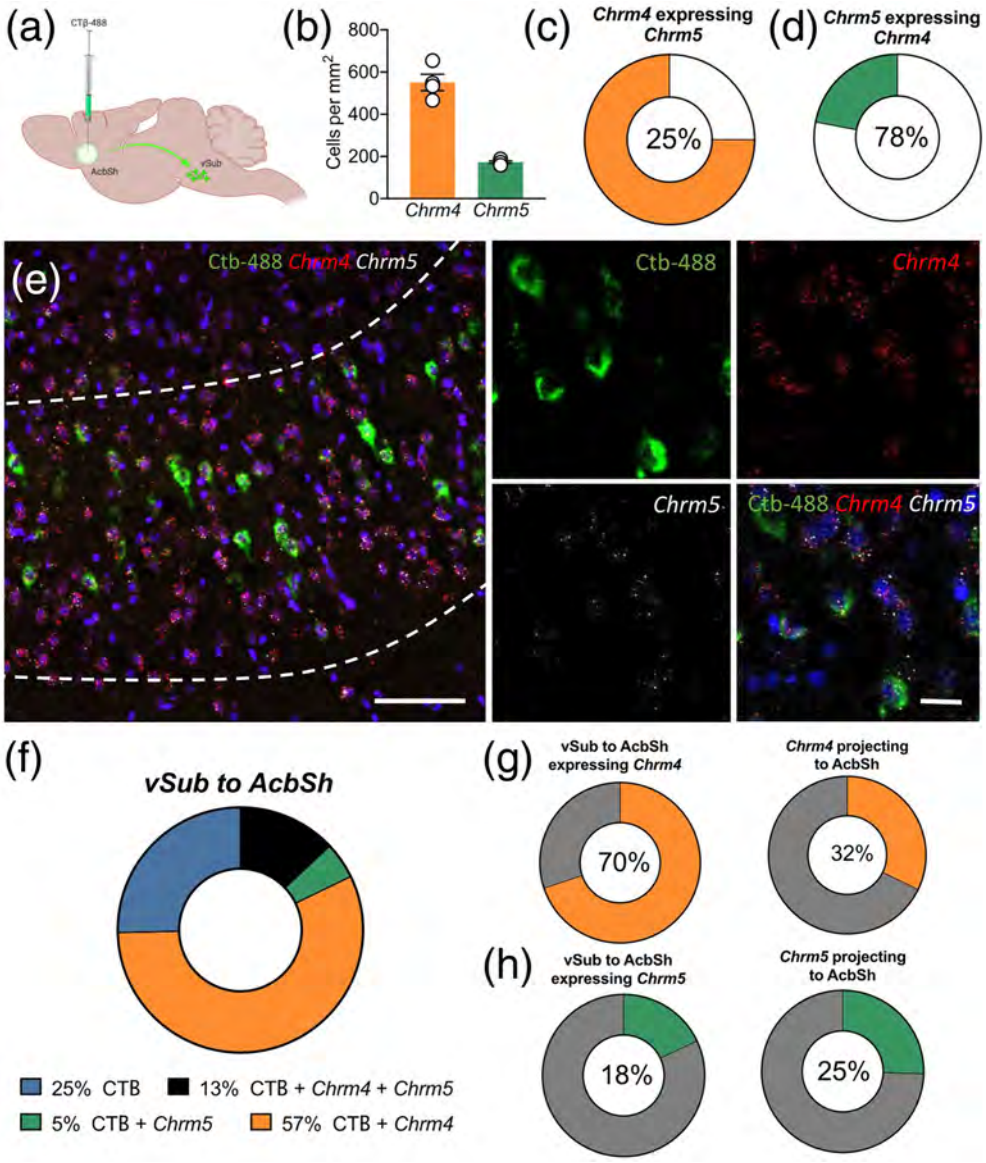

FIGURE 2 *Chrm4* and *Chrm5* expressing cells in the vSub projection to the AcbSh. (a) Schematic of CTβ-488 injection into the AcbSh and representative injection site. (b) Cell density of *Chrm4* and *Chrm5* expression within vSub. (c) Proportion of *Chrm4*-positive cells that express *Chrm5* within the vSub. (d) Proportion of *Chrm5*-positive cells that express *Chrm4* within the vSub. (e) Overview and high magnification representative microphotograph of CTβ-488, *Chrm4*, *Chrm5*, and overlay within the vSub. (f) Donut graph illustrating proportions of CTβ-488 positive, dual CTβ-488/*Chrm4*-positive, CTβ-488/*Chrm5*-positive and triple labelled CTβ-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 ± SEM. Scale bars = 200 μm (overview) and 50 μm (high magnification), $n = 4$

context, treatment, and context × treatment interaction. Bonferroni's *post hoc* analysis revealed that VU0467154 reduced the number of active lever presses in context A (ABA, Figure 3c) but not context B (ABB, Figure 3d). Analysis of latency to first active lever press in context A revealed a main effect of lever, but no significant effect of treatment or lever × treatment interaction (data not shown).

3.4 | M_5 receptors in the vSub do not mediate context-induced reinstatement of alcohol seeking

We next examined the functional role of the M_5 receptors in context-induced alcohol seeking. Based on the observation that *Chrm5* was up-regulated following alcohol, we microinjected the selective M_5 receptor NAM, ML375, within the vSub to reduce signalling. During training rats successfully acquired alcohol self-administration in context A and returned to pre-surgical levels of responding prior to extinction training, with a high level of active lever responses (corresponding to an alcohol intake of 0.76 ± 0.07 g kg^{-1} per session), over the final seven self-administration sessions in context A (Figure 4a). Rats extinguished responding in context B (Figure 4b). There was no difference in average active lever responding between treatment groups in context A. Bilateral intra-vSub microinjection of ML375 had no significant effect on context-induced alcohol seeking in context A (ABA, Figure 4c) or active lever pressing in context B (ABB, Figure 4d). RM two-way ANOVA revealed a main effect of context, but no significant effect of treatment, or context × treatment interaction. Analysis of latency to first active lever press in context A revealed a main effect of lever, lever × treatment interaction, but no significant effect of treatment. Bonferroni's *post hoc* analysis showed a significant increase in latency to inactive lever response, but not active lever latency (data not shown).

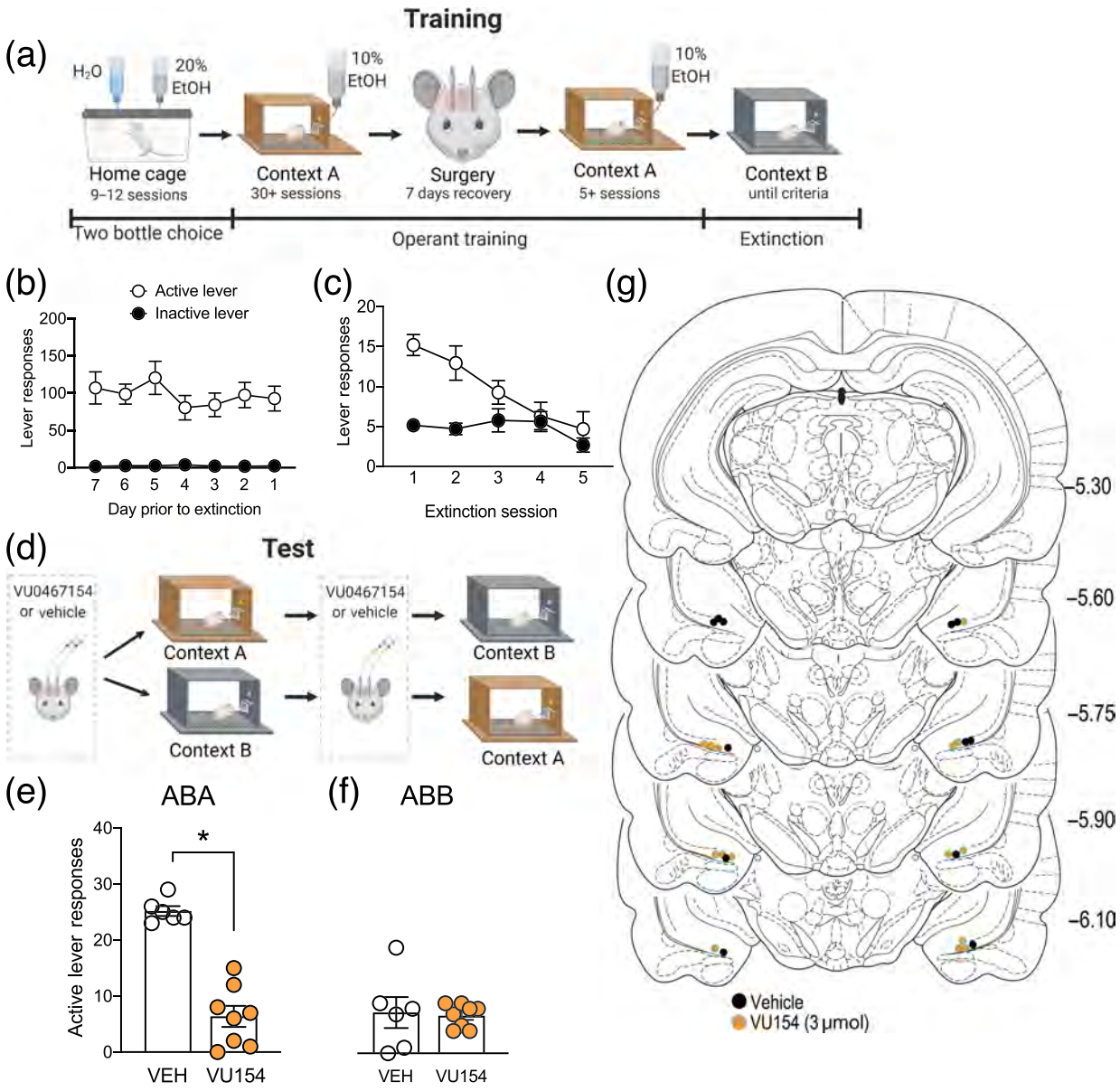

FIGURE 3 Intra-vSub administration of the M_4 receptor PAM VU0467154 reduces context-induced alcohol seeking. (a) Schematic of training procedures. (b) Lever responding during the seven operant self-administration training days prior to test in context A and (c) extinction in context B. (d) Schematic 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 of vehicle or of VU154; images from Paxinos and Watson (2007), position expressed as mm from bregma. Data are expressed as mean ± SEM, $n = 6$ vehicle-treated and $n = 8$ VU154-treated rats. $^*P < 0.05$, significantly different as indicated

3.5 | M_4 receptors in the vSub do not mediate motivation for alcohol self-administration

To determine whether the actions of the M_4 receptors in the vSub 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, with a high level of active lever responses (corresponding to an alcohol intake of 0.64 ± 0.04 g kg^{-1} per session) across the seven operant training sessions prior to testing (Figure 5a). There was no significant difference between groups in the breakpoint for alcohol (Figure 5b), or latency to first active lever press (data not shown). RM two-way ANOVA of time-course data revealed a main effect of time, but no significant effect of treatment or time × treatment interaction (Figure 5c).

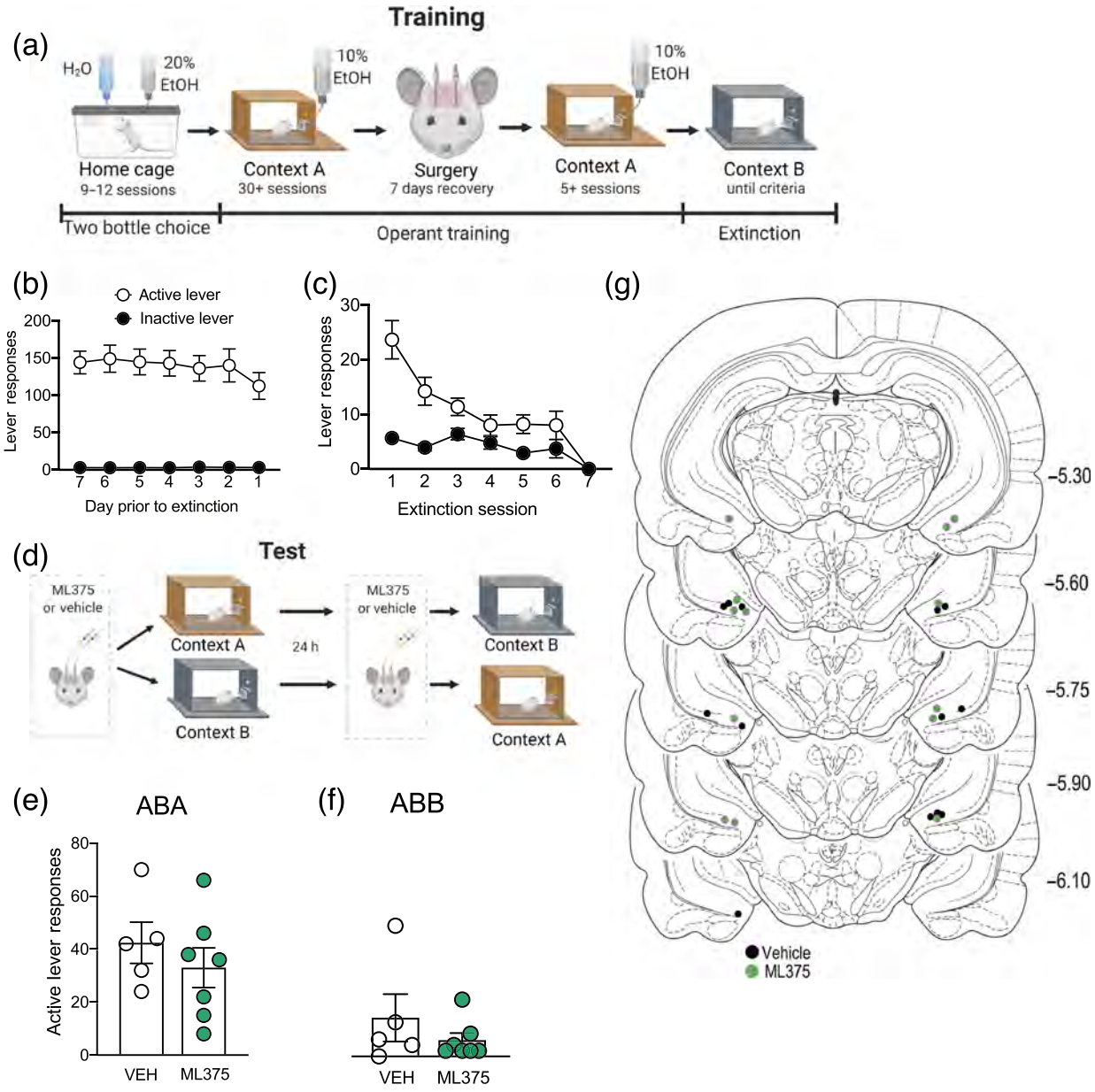

FIGURE 4 Intra-vSub administration of the M_5 receptor NAM ML375 does not alter context-induced alcohol seeking. (a) Schematic of training procedures. (b) Lever responding during the seven operant self-administration training days prior to extinction in context A and (c) extinction in context B. (d) 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, of vehicle , or of ML375; images from Paxinos and Watson (2007), position expressed as mm from bregma. Data are expressed as mean ± SEM, $n = 5$ vehicle-treated and $n = 7$ ML375-treated rats

3.6 | M_5 receptors in the vSub mediate motivation for alcohol self-administration

Based on our observation that *Chrm5* mRNA was up-regulated in the vSub, following alcohol but not after abstinence, we next sought to examine whether M_5 receptors mediated the motivation to obtain and consume alcohol. During training, rats reached a steady and high level of responding for alcohol, corresponding to an alcohol intake of 0.59 ± 0.05 g kg^{-1} per session, across the seven operant training sessions prior to testing (Figure 6a). There was no significant difference between groups in breakpoint for alcohol seeking (Figure 6b), nor latency to first active lever press (data not shown). However, RM two-way ANOVA of time-course data revealed an effect of time and time × treatment interaction, but not treatment. Bonferroni's *post hoc* analysis revealed a significant difference between vehicle and ML375 treated rats in the first 15 min, 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 (Figure 6c).

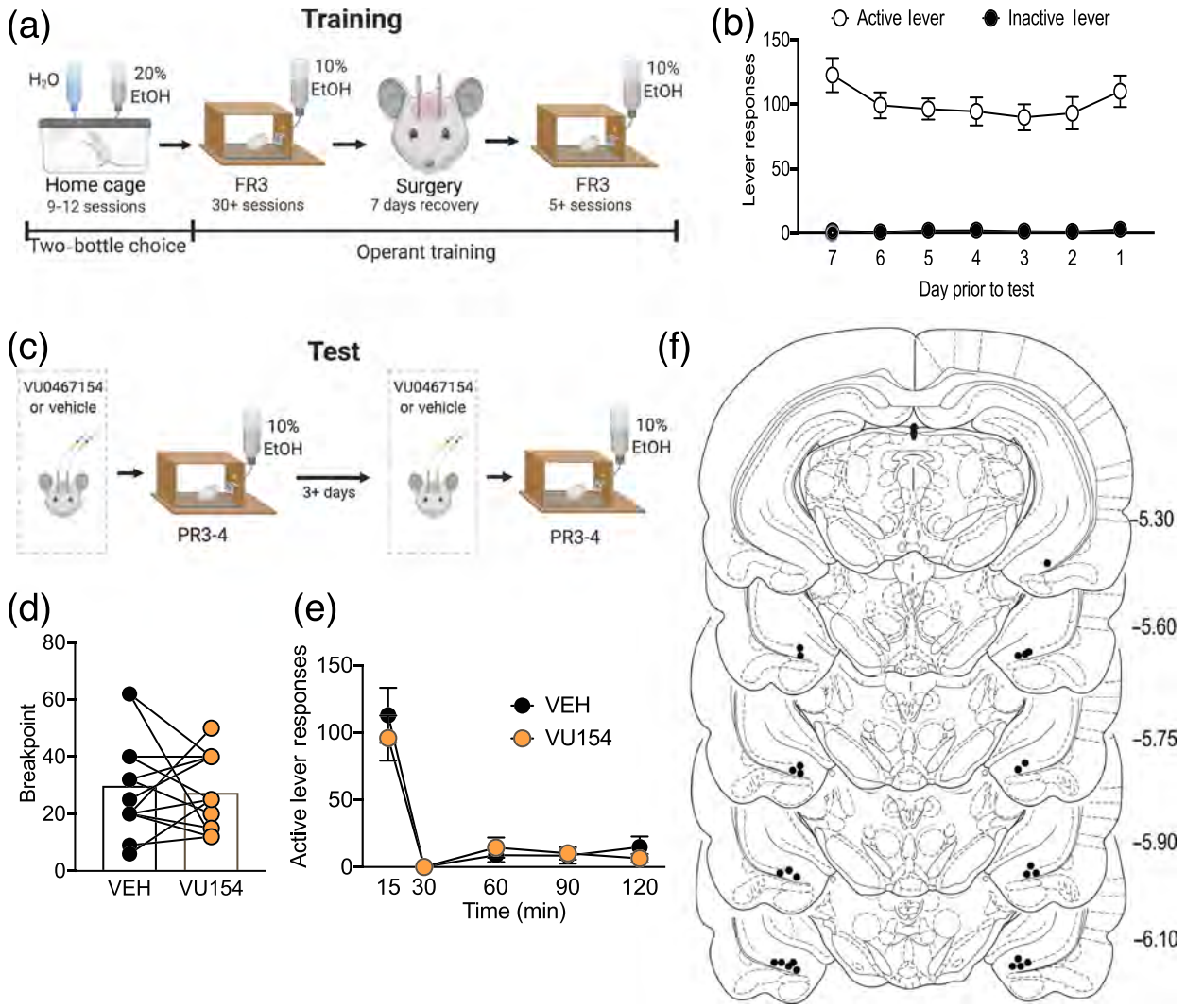

FIGURE 5 Intra-vSub administration of the M_4 receptor PAM VU0467154 does not alter motivation to self-administer alcohol. (a) Schematic of training procedures. (b) Lever responding during the seven operant self-administration training days prior to test. (c) Schematic of testing procedures. (d) VU0467154 (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 time course of the PR test. (f) Schematic of vSub infusions. Images from Paxinos and Watson (2007), position expressed as mm from bregma. Data are expressed as mean ± SEM, $n = 13$, tested in a randomised and counterbalanced manner

3.7 | M_4 and M_5 receptors 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 (Figure 7a) or across the 60-min period. RM two-way ANOVA showed a main effect of time, but no significant effect of treatment or interaction (Figure 7b). When we assessed home-cage chow intake, VU0467154 had no significant effect on consumption (RM two-way ANOVA; main effect of time, but no significant effect of treatment or time × treatment interaction; Figure 7c). Additionally, VU0467154 did not alter water intake (RM two-way ANOVA; main effect of time but no significant effect of treatment or time × treatment interaction; Figure 7d).

For ML375, there were no significant differences between treatment groups in total distance travelled (Figure 7e) or across the 60-min period (RM two-way ANOVA; main effect of time; no significant effect of treatment or interaction; Figure 7f). Further, ML375 had no significant effect on food intake (RM two-way ANOVA showed a main effect of time, but no significant effect of treatment or time × treatment interaction; Figure 7g) or water intake (RM two-way ANOVA; main effect of time but no significant effect of treatment or time × treatment interaction; Figure 7h).

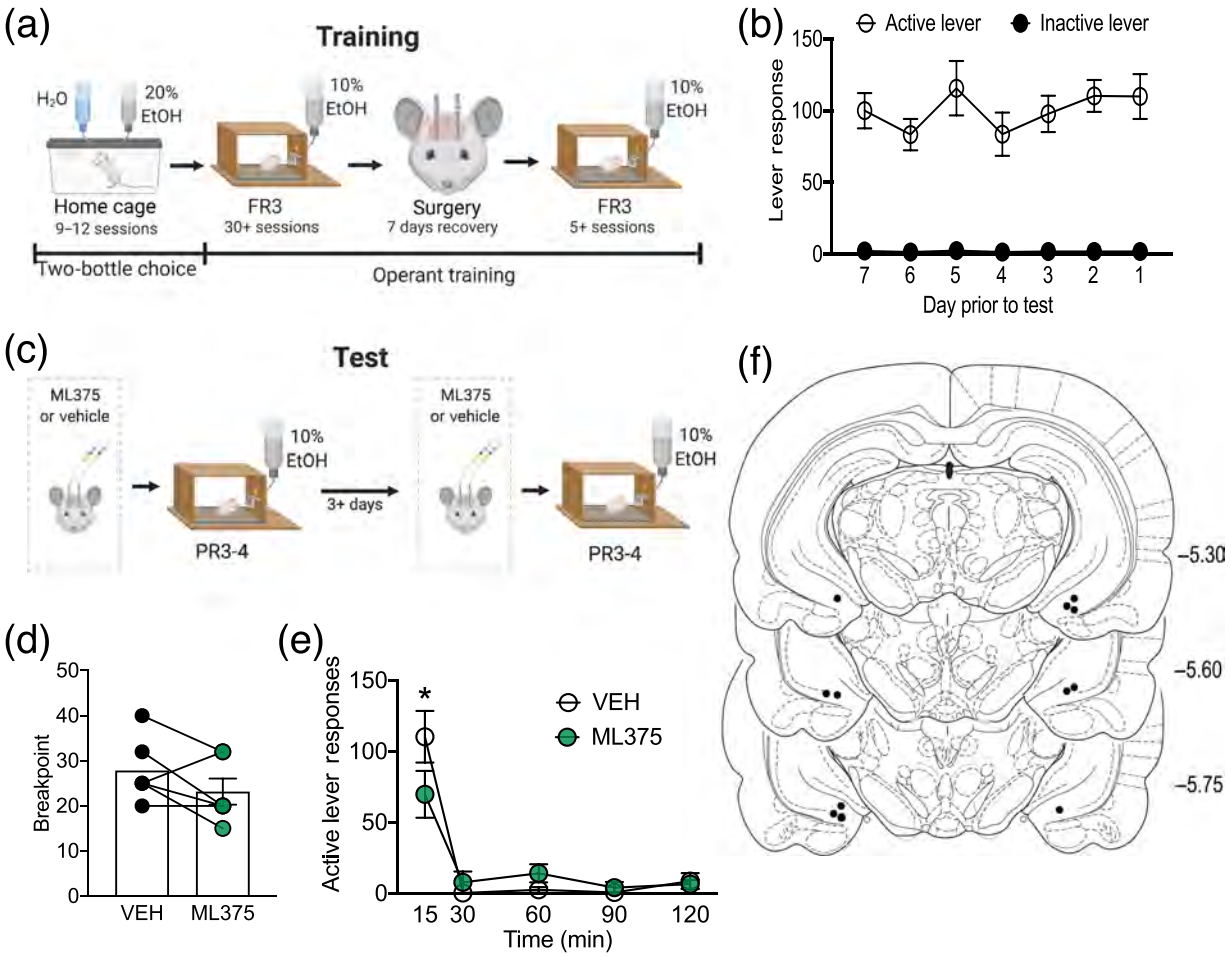

FIGURE 6 Intra-vSub administration of the M_5 receptor NAM ML375 transiently alters motivation to self-administer alcohol. (a) Schematic of training procedures. (b) Lever responding during the seven operant self-administration training days prior to test. (c) Schematic of testing procedures. (d) ML375 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. Images from Paxinos and Watson (2007), position expressed as mm from bregma. Data are expressed as mean ± SEM, $n = 6$ tested in a randomised and counterbalanced manner. $^*P < 0.05$, significantly different as indicated

4 | DISCUSSION

Our present study highlights new evidence for differences in the effects of the M_4 and M_5 receptors in the vSub in terms of alcohol seeking compared with alcohol consumption. Following long-term alcohol consumption, we observed striking dysregulation of the muscarinic receptors within the vSub, but not the VTA, demonstrating anatomical specificity. Specifically, after 14 days abstinence from alcohol, *Chrm4* expression was decreased, while after alcohol consumption, *Chrm5* expression was increased, within the vSub. The M_4 and M_5 receptors are GPCRs, with the M_4 receptors coupled to $G_{i/o}$ (inhibitory), while the M_5 receptors are coupled to $G_{q/11}$ (stimulatory) (Caulfield, 1993; Langmead, Watson, & Reavill, 2008). Therefore, while alcohol or abstinence dysregulated the expression of M_4 and M_5 receptors 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 (Taepavarapruk, Butts, & Phillips, 2015; Vorel, Liu, Hayes, Spector, & Gardner, 2001), while reversible inactivation decreases reinstatement of drug and alcohol seeking (Bossert & Stern, 2014; Sun & Rebec, 2003), suggesting increased vSub activity may enhance the propensity to relapse. This is consistent with our findings that long-term alcohol consumption or abstinence reduces inhibitory *Chrm4* expression and enhances excitatory *Chrm5* expression, presumably enhancing vSub activity.

Based on this dysregulation of muscarinic receptors in the vSub and the known role of the vSub → AcbSh projection in alcohol seeking (Marchant et al., 2016), we used RNAscope to characterise the expression of mRNA for M_4 and M_5 receptors within the vSub and specifically on vSub cells projecting to the AcbSh. Consistent with previous studies, we found differences in the expression of *Chrm4* and *Chrm5* within the vSub (Lebois, Thorn, Edgerton, Popiolek, & Xi, 2018; Vilaró, Palacios, & Mengod, 1990; Walker & Lawrence, 2020). We further quantified density and receptor overlap,

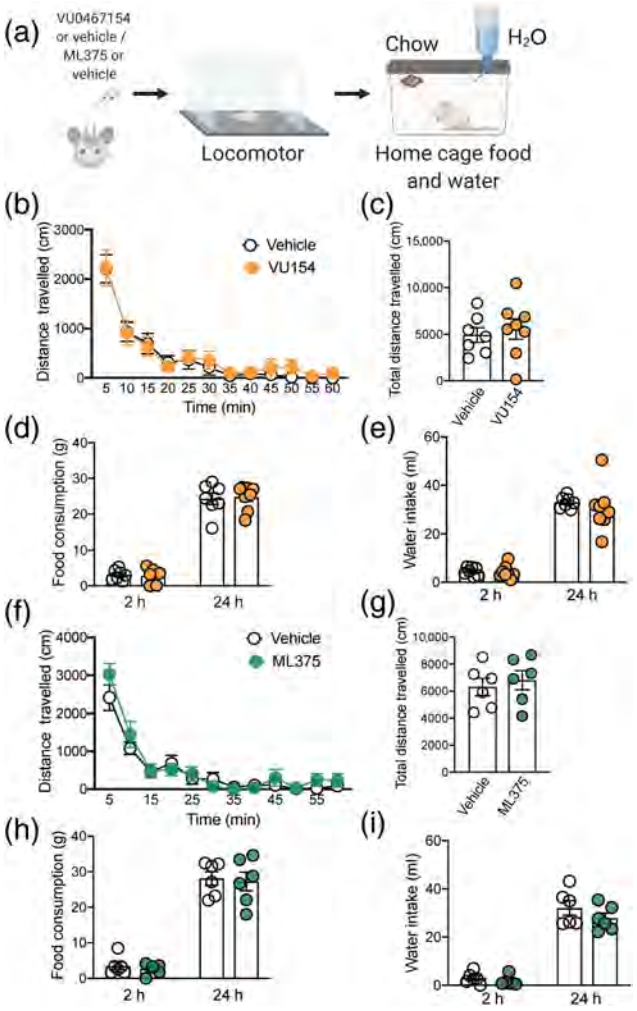

FIGURE 7 Intra-vSub administration of the M_4 receptor PAM VU0467154 or the M_5 receptor NAM ML375 does not alter locomotor activity, food or water consumption. (a) Schematic of testing procedures. (b) Following vSub VU0467154 (VU154) administration, no difference was observed in locomotor activity across time, nor (c) total locomotor activity. (d) No difference was observed in food consumption, nor (e) water intake. Data are expressed as mean ± 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 ± SEM, (b–e) $n = 8$ VU154-treated rats and $n = 7$ VU154 vehicle-treated controls. (f–i) $n = 6$ ML375 treated rats and $n = 6$ ML375 vehicle treated controls

observing a greater density of *Chrm4* than of *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 that discrete vSub → AcbSh inhibition (Marchant et al., 2016), suggesting other vSub outputs 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, further studies are required to determine whether the M_4 receptors on vSub → AcbSh projections are critical for alcohol seeking behaviour, or whether this may more broadly rely on M_4 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 involved in context-induced alcohol seeking, the vSub also regulates relapse to drug seeking and taking precipitated by drug primes, cues (Hiranita, Nawata, Sakimura, Anggadiredja, & Yamamoto, 2006; Sun & Rebec, 2003), 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, Blom, & Anthenelli, 2011), a major limitation of this study is the lack of female subjects. Little is known about sex differences in muscarinic receptor activity (Walker & 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). Further studies are required to determine the contribution of muscarinic signalling within the vSub in alcohol seeking in females.

Recently, several subtype-selective, allosteric modulators of muscarinic receptors have been developed, enhancing the ability to examine specific behavioural roles of the different muscarinic receptors in affective disorders (Bender, Garrison, & Lindsley, 2018; Berizzi et al., 2016; Gentry et al., 2013; Gould et al., 2019; Langmead & Christopoulos, 2014; Walker & Lawrence, 2020). The selective M_4 receptor 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 intake or 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 M_4 receptor activity (via the selective M_4 receptor 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 cholinergic signalling within the vSub. Further, no alterations in *Chrm4* expression were observed after alcohol without a period of abstinence, suggesting that M_4 receptor signalling is predominantly affected during periods of abstinence when relapse risk is highest and may also explain why enhancing M_4 receptor signalling had no significant effect on ongoing alcohol self-administration in the PR test.

In contrast, reduction of M_5 receptor signalling (via the corresponding 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* being up-regulated following alcohol, but not after alcohol and abstinence. However, the functional results showed a modest effect size, possibly due to the relatively lower density of M_5 receptor expression, and/or the degree of *Chrm5* dysregulation observed after long-term alcohol. In addition, the M_5 receptor NAM ML375 has less than optimal physicochemical and pharmacokinetic properties, limiting its 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 NAMs of M_5 receptors 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 modulating muscarinic receptors has surrounded their ability to act as cognitive enhancers and alleviate cognitive symptoms of schizophrenia (Carruthers, Gurvich, & Rossell, 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 (Charlton et al., 2019; Perry, 2016). Indeed, in vivo microdialysis revealed that chronic alcohol consumption significantly reduces **acetylcholine** release in the hippocampus (Melis, Stancampiano, Imperato, Carta, & Fadda, 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 to reverse 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 & Lawrence, 2020). For example, the M_4 receptor PAM (CVL-231, Cerevel) is currently undergoing Phase I clinical trials to assess safety, tolerability, and pharmacokinetics (clinicaltrials.gov ID: NCT04136873), while the M_4 receptor agonist, HTL0016878 (Sosei Heptares), has completed Phase I clinical trials to assess safety, however, results are yet to be reported (clinicaltrials.gov ID: NCT03244228). Additionally, KarXT (Karuna Therapeutics) a non-selective M_1/M_4 receptor agonist combined with a peripheral antagonist (to block peripheral adverse effects) has begun a randomized, double-blind, placebo-controlled, multicentre Phase III 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.

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AUTHOR CONTRIBUTIONS

The study was conceived and planned by A.J.L., L.C.W., and C.J.L. L.C.W. and A.J.L. jointly supervised students. L.C.W., K.L.H., and N.A.C. performed qPCR and fluorescent in situ hybridization; L.C.W., K.L.H., N.A.C., and L.J.H. performed behavioural studies. L.C.W. and K.L.H. analysed data. C.K.J. supplied VU0467154 and ML375. The manuscript was written and revised by L.C.W., K.L.H., and A.J.L., with input from C.J.L. and C.K.J. All other authors approved the manuscript prior to submission.

CONFLICT OF INTEREST

C.W.L. 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 RIGOUR

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.

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