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Enhanced self-administration of alcohol in muscarinic acetylcholine M_4 receptor knockout mice

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ABSTRACT

Modulation of cholinergic neurotransmission *via* nicotinic acetylcholine receptors is known to alter alcohol-drinking behavior. It is not known if muscarinic acetylcholine receptor subtypes have similar effects. The muscarinic M_4 receptor is highly expressed in the brain reinforcement system and involved in regulation of cholinergic and dopaminergic transmission. Here we investigate, for the first time, the role of the M_4 receptor in alcohol consumption using M_4 knockout ($M_4^{-/-}$) and wild-type ($M_4^{+/+}$) mice.

Experimentally naïve $M_4^{-/-}$ and $M_4^{+/+}$ mice were trained to orally self-administer 5%, 8% and 10% alcohol in 60 min sessions, 6 days/week, after having undergone a standard sucrose fading training procedure on a fixed ratio schedule. The mice were further subjected to an extinction period followed by a 1 day reinstatement trial.

$M_4^{-/-}$ mice consumed more alcohol at 5% and 8% compared to their $M_4^{+/+}$ littermates. The highest alcohol concentration used (10%) did not immediately result in divergent drinking patterns, but after 4 weeks of 10% alcohol self-administration, baseline levels as well as a pattern of $M_4^{-/-}$ mice consuming more alcohol than their $M_4^{+/+}$ controls were re-established. Moreover, the $M_4^{-/-}$ mice displayed a reduced capacity to extinguish their alcohol-seeking behavior.

Taken together, alcohol consumption is elevated in $M_4^{-/-}$ mice, indicating that the M_4 receptor is involved in mediating the reinforcing effects of alcohol. The M_4 receptor should be further explored as a potential target for pharmacological (positive allosteric modulators or future agonists) treatment of alcohol use disorders.

1. Introduction

Excessive alcohol consumption is strongly driven by the activity of an individual's brain reinforcement system. The mesolimbic dopamine system, including the ventral tegmental area, the nucleus accumbens and the prefrontal cortex, constitutes the main site of the brain reinforcement system, with the effects of alcohol being particularly linked to dopamine activation in the nucleus accumbens (Koob et al., 1994; Koob and Bloom, 1988; Wise and Bozarth, 1987). Alcohol-induced increase of extracellular dopamine in the nucleus accumbens is, in turn, dependent on the cholinergic activity within the ventral tegmental area, the posterior pedunculopontine nucleus and the laterodorsal tegmental nucleus (Ericson et al., 2003). Indeed, local administrations of nicotinic acetylcholine receptor antagonists into the ventral tegmental area reduce alcohol consumption and alcohol-induced dopamine overflow (Ericson et al., 2003, 1998; Jerlhag et al., 2006; Larsson and Engel, 2004) and modulation of cholinergic transmission within the posterior pedunculopontine nucleus and ventral tegmental area has been shown to affect alcohol-drinking behavior in rats (Katner et al., 1997).

In addition to its pharmacological action on ligand-gated nicotinic acetylcholine receptors, acetylcholine also acts on G protein-coupled muscarinic acetylcholine receptors. Among the five muscarinic acetylcholine receptor subtypes (M_1–M_5), with central as well as peripheral functions, the M_4 receptor subtype is highly expressed in the posterior pedunculopontine nucleus, the laterodorsal tegmental nucleus and in the nucleus accumbens (Sugaya et al., 1997; Levey et al., 1993; Wess et al., 2007). Expressed primarily on cholinergic interneurons and GABAergic medium spiny projection neurons, the M_4 receptor is believed to be involved in direct regulation of cholinergic neurotransmission and consequently also in indirect regulation of dopaminergic transmission.

Moreover, global M_4 knockout ($M_4^{-/-}$) mice display a dopamine-hypersensitive behavioral phenotype and show increased self-administration of cocaine as well as highly palatable liquid food

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compared to M_4 wild-type mice ($M_4^{+/+}$) (Felder et al., 2001; Gomeza et al., 1999; Schmidt et al., 2011; Tzavara et al., 2004) and the M_4 positive allosteric modulator VU100152 inhibits cocaine self-administration (Dencker et al., 2012) supporting a role of the M_4 receptor in the reinforcing effects of alcohol.

To investigate the functional role of the M_4 receptor subtype in alcohol consumption, $M_4^{-/-}$ and $M_4^{+/+}$ mice were first introduced to increasing concentrations of alcohol on a fixed-ratio 1 (FR1) schedule of operant responding and then subjected to an extinction period followed by a 1 day cue-induced reinstatement trial.

2. Material and methods

2.1. Animals

Male $M_4^{-/-}$ mice were generated as previously described (Gomeza et al., 1999) and bred at the Panum Institute, University of Copenhagen. Founder mice of mixed genetic background (129SvEv/CF1) were backcrossed to the C57BL/6Ntac strain for 13 generations and genotyping was performed on mouse-tail DNA using the polymerase chain reaction. Male $M_4^{+/+}$ littermates were used as controls. All animals were housed in groups of four to eight in Makrolon cages ($20 \times 35 \times 15$ cm^3) enriched with cardboard housing and nesting material and allowed free access to standard chow and water. The animal room was maintained on a 12 h light/dark cycle (lights on at 7:00 am) and kept at a constant temperature (22–24 °C). All experiments were performed in the light cycle between 8:00 am and 4:00 pm. The mice were allowed to acclimatize to the animal facility for at least 1 week prior to initiation of the experiments. All procedures were conducted in accordance with guidelines from the Animal Experimentation Inspectorate, the Ministry of Justice, Denmark.

2.2. Apparatus

Mouse operant chambers (Med Associates, USA) contained two nose-poke openings 10 mm above a grid floor, both equipped with photocells and a discriminative cue light, positioned on either side of a small dish-shaped plate into which liquid could be delivered. Responding in the right (active) opening resulted in delivery of a droplet of alcohol (25 μl) as well as illumination of the cue light for 15.25 s, during which additional responses were counted but had no scheduled consequences (time out period). Responding in the left (inactive) opening was counted but produced no light or alcohol (inactive responding). A house light positioned near the top of the chamber opposite the nose-poke openings was lit throughout the session.

2.3. Drugs

Sucrose (D-) and/or alcohol (99%, SigmaAldrich, Denmark) were diluted with tap water to produce solutions of sucrose 5% *per se*, (sucrose 5%+alcohol 5%), (sucrose 5%+alcohol 8%) and alcohol 5, 8 and 10% *per se*.

2.4. Operant alcohol self-administration

Experimentally naïve mice were trained to orally self-administer 5%, 8% and 10% alcohol in 60 min sessions, 6 days/week, after having undergone a standard sucrose fading training procedure on a FR1 schedule. This scheme involved 10 days of sucrose 5% solution *per se*, 5 days of (sucrose 5%+alcohol 5%) and subsequent alcohol 5% *per se*, (sucrose 5%+alcohol 8%), alcohol 8% *per se* and finally alcohol 10% *per se*. Only mice which performed a mean of ≥ 15 reinforced responses over the last 3 days of sucrose training were included in the study and continued to alcohol exposure.

2.5. Extinction and cue-induced reinstatement of alcohol-seeking behavior

Animals self-administered 10% alcohol for 4 weeks, where after the reinforced nose-poke opening was inactivated along with the concomitant light cue. When the control group reached a significantly lowered and stable response rate over the last week of extinction, the reinforced opening was again activated and coupled with the light cue for one session to examine cue-induced reinstatement.

2.6. Statistical analyses

All data are presented as mean ± the standard error of the mean (S.E.M.) and a significance level of $P < 0.05$ was used throughout this study. Mean comparisons were performed by the one-way ANOVA analysis or a two-way ANOVA for repeated measures, when necessary. Active *vs.* inactive responding was compared with Student's *t*-tests for dependent samples, for each genotype.

3. Results

3.1. Operant alcohol self-administration

To study the reinforcing properties of alcohol in $M_4^{-/-}$ mice and their $M_4^{+/+}$ littermates, all mice were tested on a FR1 schedule of operant self-administration. A total of 70% (7 $M_4^{-/-}$ and 6 $M_4^{+/+}$) of the mice screened in sucrose training met the pre-set criterion for inclusion in the study. During the last week of sucrose training these mice displayed a 77.2 ± 2.6% preference for the active opening.

The $M_4^{-/-}$ and $M_4^{+/+}$ mice showed no significant difference in body weight (25.8 ± 1.13 g and 26.2 ± 1.22 g, respectively) when measured in the middle of the alcohol-responding period (approximately 18 weeks old). The $M_4^{-/-}$ group performed an average of 23.4 ± 2.6 reinforced nose-pokes per 60 min alcohol session, whereas the corresponding result for the $M_4^{+/+}$ group was 16.6 ± 1.01, calculated as the average responding for 5%, 8% and 10% alcohol. This equals an alcohol consumption of 1.3 g/kg bodyweight for the $M_4^{-/-}$ mice and 1.0 for the $M_4^{+/+}$ mice.

A considerably lower number of responses were observed in the inactive opening, 5.6 ± 0.6 and 5.0 ± 0.2 for the $M_4^{-/-}$ and the $M_4^{+/+}$ mice, respectively. Two separate Student's *t*-tests for dependent samples comparing the average of active and inactive nose-pokes for each genotype revealed that both groups displayed a significantly higher number of operant responses in the active opening $t=(8.209)$, $P < 0.001$ and $t=(14.064)$, $P < 0.001$ for $M_4^{-/-}$ and $M_4^{+/+}$ mice, respectively. However, there was no difference in inactive responding between the two genotypes (F(genotype): $F(1,11)=0.0268$, $P=0.872$), nor did responding in the inactive opening change with alcohol concentration (F(alcohol-concentration): $F(1,2)=0.179$, $P=0.837$) as measured over 60 min.

During exposure to 5% alcohol, the $M_4^{-/-}$ mice consumed significantly more alcohol than their $M_4^{+/+}$ littermates (F(genotype): $F(1,11)=6.316$, $P=0.029$) (Fig. 1), a pattern that was sustained when the mice were offered 8% alcohol (F(genotype): $F(1,11)=5.147$, $P=0.044$) (Fig. 2). This genotype effect was further pronounced during the first 30 min of alcohol responding, 5% alcohol (F(genotype): $F(1,11)=11.107$, $P=0.007$) and 8% alcohol (F(genotype): $F(1,11)=8.582$, $P=0.014$) (*data not shown*). However, during the first days of exposure to 10% alcohol, $M_4^{-/-}$ and $M_4^{+/+}$

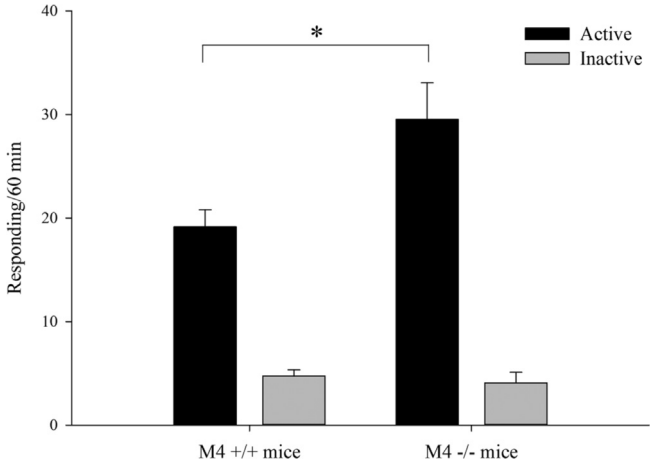

Fig. 1. Operant alcohol self-administration (FR1) of 5% alcohol in $M_4^{-/-}$ and $M_4^{+/+}$ mice. Data depict mean numbers (±S.E.M.) of active (dark) and inactive (light) responses by $M_4^{-/-}$ and $M_4^{+/+}$ mice accumulated over 5 days. * indicates $P<0.05$ between genotypes. $n=6$–7.

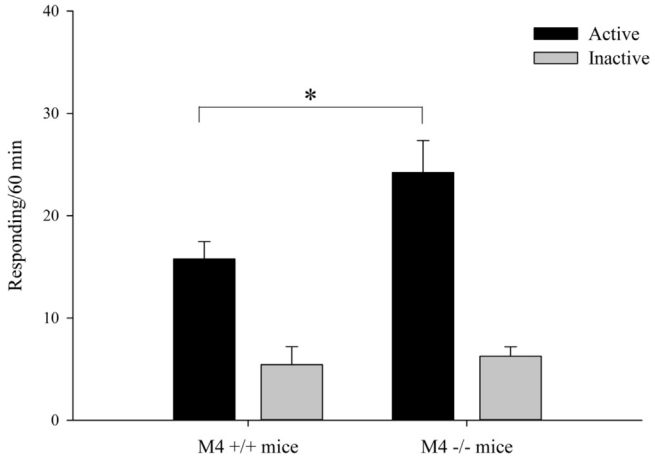

Fig. 2. Operant alcohol self-administration (FR1) of 8% alcohol in $M_4^{-/-}$ and $M_4^{+/+}$ mice. Data depict mean numbers (±S.E.M.) of active (dark) and inactive (light) responses by $M_4^{-/-}$ and $M_4^{+/+}$ mice accumulated over 5 days. * indicates $P<0.05$ between genotypes. $n=6$–7.

mice responded similarly (F(genotype): $F(1,11)=0.552$, $P=0.473$) and both groups showed a transient reduction in alcohol consumption compared to previous levels (5% and 8% alcohol) (*data not shown*). The responding for 10% alcohol returned to baseline levels after approximately 4 weeks and a rise was noticed in the $M_4^{-/-}$ group compared to their $M_4^{+/+}$ controls (F(genotype): $F(1,11)=2.930$, $P=0.115$) as measured over 60 min (Fig. 3) and (F(genotype): $F(1,11)=5.841$, $P=0.035$) as measured over 30 min (*data not shown*).

3.2. Extinction and cue-induced reinstatement of alcohol-seeking behavior

After 4 weeks of 10% alcohol self-administration, 6 $M_4^{-/-}$ and 6 $M_4^{+/+}$ mice were exposed to an 18-day extinction and 1-day cue-induced reinstatement trial (Fig. 4). A two-way repeated measures ANOVA (genotype × days) was performed yielding significance for the day effect ($F(1,17)=3.330$, $P<0.001$), a genotype effect of ($F(1,10)=4.606$, $P=0.057$) and a non-significant (genotype × days) interaction as measured over 60 min. Subsequent paired t-tests revealed a significant reduction in extinction responding for days 9, 11, 12, 13, 14, 17

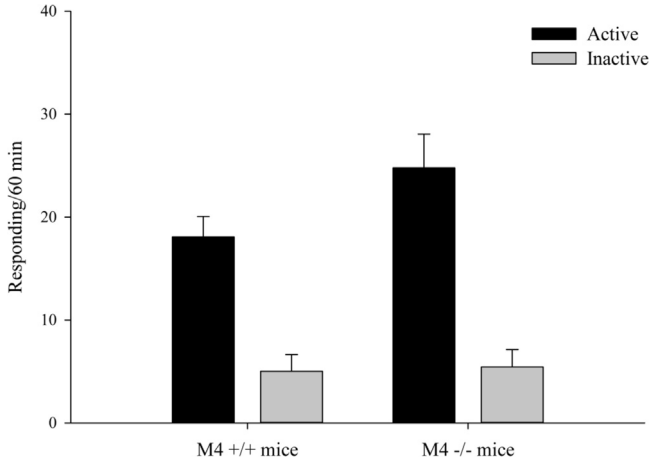

Fig. 3. Operant alcohol self-administration (FR1) in the last week of 10% alcohol in $M_4^{-/-}$ and $M_4^{+/+}$ mice. Data depict mean numbers (±S.E.M.) of active (dark) and inactive (light) responses by $M_4^{-/-}$ and $M_4^{+/+}$ mice accumulated over 6 days. $n=6$–7.

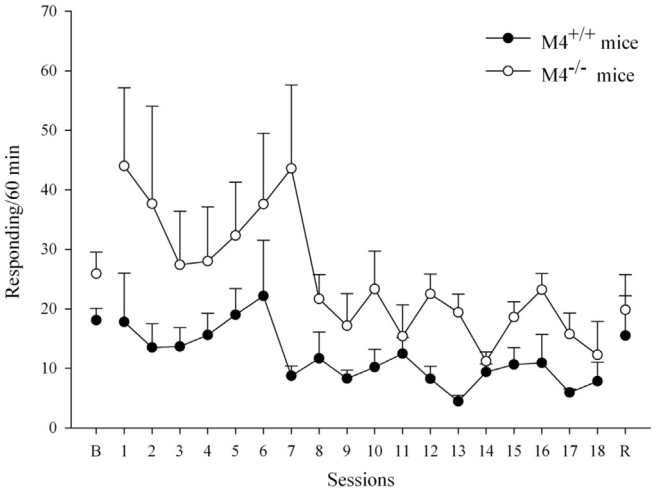

Fig. 4. Extinction and reinstatement of operant alcohol self-administration in $M_4^{-/-}$ and $M_4^{+/+}$ mice. Data depict mean numbers (±S.E.M.) of responses by $M_4^{-/-}$ (open) and $M_4^{+/+}$ (closed) mice at baseline (B, last 6 days of 10% alcohol), 18 extinction sessions and 1 day of cue-induced reinstatement (R). $n=6$, 1 mouse was excluded after the self-administration sessions due to technical limitations.

and 18 as compared to baseline (10% alcohol) in the $M_4^{+/+}$ group while the $M_4^{-/-}$ group failed to display such significance, suggesting that the extinction procedure was more effective in the $M_4^{+/+}$ group than in the $M_4^{-/-}$ group. Both $M_4^{-/-}$ and $M_4^{+/+}$ mice showed a trend-level increase in their responding when the operant contingency was re-established. A two-way repeated measures ANOVA (genotype × days) revealed ($F(1,1)=4.561$, $P=0.061$) when comparing the number of nose-pokes on the last day of extinction to that of the reinstatement day. There was a complete lack of effect on genotype ($F(1,10)=0.235$, $P=0.638$) as well as (genotype × days) interaction ($F(1,9)=0.0572$, $P=0.861$) as measured over 60 min in the responses after reinstatement.

4. Discussion

The present study investigates, for the first time, alcohol self-administration and relapse behavior in $M_4^{-/-}$ mice using an operant FR1 paradigm. Our results demonstrate elevated alcohol

consumption and reduced capacity to extinguish alcohol-seeking behavior in $M_4^{-/-}$ compared to $M_4^{+/+}$ mice.

The delivery of increasing concentrations of alcohol, 5% and 8%, revealed significantly elevated alcohol consumption in $M_4^{-/-}$ mice compared to their $M_4^{+/+}$ home-cage littermates. The highest concentration, 10% alcohol, did not immediately result in divergent drinking patterns and both groups lowered their intake level compared to 5% and 8% alcohol. This could reflect an aversive response to the taste of high alcohol concentrations frequently encountered in mice (Faccidomo et al., 2009; Heidbreder et al., 2007). Nevertheless, the effect faded and after 4 weeks of training, normal levels of responding for alcohol were re-established as well as the pattern of increased alcohol consumption in the $M_4^{-/-}$ compared to the $M_4^{+/+}$ group. Interestingly, the $M_4^{-/-}$ mice displayed intense self-administration behavior, shortly after alcohol was made available at the beginning of each session and the genotype effect was pronounced during the first 30 min of responding for 5%, 8% and 10% alcohol (last week) (*data not shown*). A similar drinking pattern is commonly observed during reinforced responding for alcohol and in alcohol-dependent mice (Lê et al., 2014; Samson et al., 2000).

In the present study, no difference in inactive responding or in body weight was observed between the two genotypes. No compensatory changes such as increased or decreased expression levels of dopaminergic or muscarinic receptors have been reported in $M_4^{-/-}$ mice (Gomeza et al., 1999; Schmidt et al., 2011; Wess, 2004; Zhang et al., 2002). All mice in our study were presented with a large volume of alcohol (25 μl) per reinforced nose-poke. A relatively low number of nose-pokes therefore generated a large total volume per session. Previous experiments as well as published data on operant alcohol intake show that approximately 1.0 g/kg, correlating to the level in the $M_4^{+/+}$ group, yield blood alcohol levels of 25–40 mg/dl, a range that produces pharmacologically relevant effects (Roberts et al., 2001; Zghoul et al., 2007). It is therefore unlikely that the present results are derived from other phenomena than reinforced responding for alcohol.

Previous studies suggest that an activation of the cholinergic system increases self-administration of alcohol. Sharma et al. (2014) showed increased consumption after local administration of nicotine into the forebrain and Hauser et al. (2014) showed the same outcome after microinjections of nicotine into the ventral tegmental area. Pharmacological inhibition of the cholinergic activity in the ventral tegmental area through nicotine or ghrelin antagonists has the opposite effect and reduces both voluntary and operant responding for alcohol (Hendrickson et al., 2013; Landgren et al., 2012). One can therefore assume that muscarinic receptors expressed in the brain reinforcement system, involved in regulation of cholinergic and dopaminergic activity, are involved in the regulation of alcohol consumption as well. A possible explanation for the increased alcohol consumption in the $M_4^{-/-}$ mice is that these mice display a "dopamine-hypersensitive" phenotype. Inactivation of presynaptic M_4 receptors in the posterior pedunculopontine nucleus may result in loss of the brake function controlling cholinergic activity in the ventral tegmental area (Tzavara et al., 2004). When exposed to alcohol or other psychostimulants the $M_4^{-/-}$ mice respond more easily than the $M_4^{+/+}$ mice, resulting in elevated cholinergic and consequently dopaminergic output levels in the ventral tegmental area and the nucleus accumbens respectively. Such an explanation is in line with recent findings showing that basal dopamine levels in $M_4^{-/-}$ and $M_4^{+/+}$ mice are equal (Schmidt et al., 2011; Turner et al., 2010). However, when challenged with cocaine, amphetamine or phencyclidine, accumbal dopamine levels increase significantly more in $M_4^{-/-}$ as compared to control mice (Schmidt et al., 2011; Tzavara et al., 2004). One should not exclude the possibility that lack of functional M_4 receptors in the nucleus accumbens, directly or indirectly, may affect neurotransmission in the nucleus accumbens and the ventral tegmental area as well. Clearly, additional studies are warranted for a detailed understanding of the mechanisms responsible for the present results.

Having observed elevated alcohol consumption in the $M_4^{-/-}$ mice, we continued to investigate alcohol seeking (craving) and relapse in the reinstatement model that holds translational value to human alcohol dependence (Stewart and de Wit, 1987). After having obtained a stable baseline consumption of 10% alcohol, both groups were introduced to an 18-day extinction period. Extinction was successful in the $M_4^{+/+}$ mice, which lowered their responding significantly during the extinction period and in comparison to baseline drinking. In contrast, the $M_4^{-/-}$ mice failed to reach the same level of extinction responding over 18 days. A delay in extinction was also observed in $M_4^{-/-}$ mice trained to self-administer cocaine (Schmidt et al., 2011) and may further reflect increased reinforcing effects of alcohol in $M_4^{-/-}$ mice. Lack of a functional M_4 receptor may affect extinction learning mechanisms mediated through the prefrontal cortex. The medial prefrontal cortex participates in behavioral adaptations seen in both appetitive and aversive-cue-mediated responding (Sparta et al., 2014) and the M_4 receptor is known to be expressed in moderate levels in cortex (Levey, 1993). The effect on extinction learning could also be derived from a lack of striatal or hippocampal M_4 receptors in an indirect fashion (Hasselmo, 2006; Power et al., 2003).

Following the extinction period, all mice were re-exposed to 1 day of cue-induced responding for 10% alcohol. The $M_4^{-/-}$ and $M_4^{+/+}$ mice showed a trend-level significance ($P=0.061$) of reinstating their responding in the reinstatement trial. It is worth noting that 5 out of 6 animals in the $M_4^{+/+}$ group more than doubled their responding on the reinstatement day compared to the last day of extinction and therefore likely reinstated their alcohol-seeking behavior. Likewise, 3 out of 6 animals in the $M_4^{-/-}$ group more than doubled their responding on the reinstatement day compared to the last day of extinction. However and in contrast to the $M_4^{+/+}$ group these mice failed to extinguish their responding for a longer time-period than 1 day only. It is likely that the resistance to extinguishing self-administration behavior in the $M_4^{-/-}$ mice affected the capacity to reinstate alcohol-seeking and it explains the lack of genotype effect on the reinstatement day. It should be noted that the magnitude of reinstatement including oral alcohol delivery may be modest and the choice of a different protocol on the reinstatement day or a prolonged extinction period maybe could have potentiated the overall outcome (Ford et al., 2011). For example, studies associating alcohol delivery, plus concomitant cue light, with a contextual cue followed by an extinction period in a different context reinstate successfully when mice are exposed to the alcohol-paired context (Burattini et al., 2006). Moreover, other studies show successful reinstatement when mice are exposed to the cue light only on the reinstatement day (Zghoul et al., 2007). Clearly, additional experiments on $M_4^{-/-}$ mice and their alcohol-relapse behavior should be performed, either *via* addition of a contextual cue or *via* exposure to alcohol deprivation in a home-cage drinking model.

Taken together, the present data show elevated alcohol consumption and reduced capacity to extinguish alcohol-seeking behavior in $M_4^{-/-}$ compared to $M_4^{+/+}$ mice. It is likely that the M_4 receptor is involved in mediating the reinforcing effects of alcohol and the M_4 receptor should be further explored as a potential target for pharmacological (positive allosteric modulators or future agonists) treatment of alcohol use disorders.

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