Inactivation of the cholinergic M₄ receptor results in a disinhibited endophenotype predicting alcohol use

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ABSTRACT

The muscarinic cholinergic M₄ receptor subtype (M₄ mAChR) is densely expressed in brain areas known to be involved in the reinforcing effects of drugs of abuse and we were the first to show that mice lacking M₄ mAChRs exhibit elevated operant responding for alcohol and reduced capacity to extinguish this alcohol-seeking behaviour. Here we explore possible underlying determinants of this phenotype. We subjected M₄ mAChR knockout mice and their littermate wildtype controls to tests of spontaneous activity, learning and memory, novelty seeking, as well as anxiety and examined the relationship of a newly discovered “disinhibited” endophenotype of these mice with voluntary alcohol consumption and relapse. We found a positive correlation between “disinhibited” behaviour on the plus maze and alcohol preference as well as relapse to alcohol drinking after a period of abstinence.

Taken together, these data point to M₄ mAChRs as a potential target for improved treatment strategies for alcohol use disorder. This receptor should be further investigated for its involvement in modulating behavioural inhibition in relation to loss of control over consumption of alcohol.

1. Introduction

Despite remarkable progress in the understanding of susceptibility factors contributing to the development of alcohol use disorder, the exact mechanisms behind this phenomenon remain to be elucidated. Today we know that consumption of alcohol is linked to the activity of an individual’s brain reinforcement system [1]. The ventral tegmental area (VTA), the nucleus accumbens (NAc) and the prefrontal cortex (PFC) constitute the main sites of the brain reinforcement system, with the effects of alcohol being particularly linked to dopamine activity in the NAc [1–5]. Alcohol-induced increase of extracellular dopamine in the NAc is, in turn, dependent on cholinergic activity within the VTA, the posterior pedunculopontine nucleus (PPN) and the laterodorsal tegmental nucleus (LDT) [6]. Nicotinic acetylcholine receptors in the VTA were previously shown to be involved in the control of alcohol drinking behaviour and alcohol-induced dopamine overflow in rodent models [6–9]. However, until recently, the G protein-coupled muscarinic acetylcholine receptor subtypes (M₁–M₅) were not investigated in this regard. Since the M₄ receptor subtype is largely expressed in the PPN, the LDT and the NAc, it may constitute an important target for regulation of alcohol-induced dopamine activity and alcohol drinking behaviour [10–13]. Indeed, we have previously reported that M₄⁻⁄⁻ mice, in which the M₄ receptor is inactivated, display an increased operant response for alcohol and a reduced capacity to extinguish their alcohol-seeking behaviour [14]. Also, other laboratories have shown that the M₄ receptor is downregulated in some brain regions in individuals with alcohol use disorder and in rats after long-term alcohol consumption and that positive allosteric modulators of M₄ receptor signaling reduced both home cage and operant alcohol self-administration in rats [15–17]. However, the mechanisms by which this is established remain to be fully understood. Possibly the M₄ receptor is involved in modulating the reinforcing value of drugs of abuse or it may be involved in the regulation of behaviours such as anxiety and novelty seeking often referred to as vulnerability markers for the
development of substance use disorders. Such behavioural traits have previously been associated both with midbrain dopamine function and drug-taking behaviour \[18,19\] and M4\(^{-/-}\) mice have previously been referred to as displaying a dopamine-hyper reactive behavioural phenotype \[20–23\]. Differences in novelty-seeking behaviour have also been associated with alcohol and substance use disorders in humans \[24–27\]. The role of anxiety in the development of these disorders is less clear; however, anxiety-like behaviours have been linked to both changes in dopamine function and drug self-administration behaviours \[28,29\] and M4\(^{-/-}\) mice were previously found to exhibit decreased burying behaviour in the shock-probe burying model of anxiety-like behaviour, while they did not differ from wild type mice in the light-dark transition test \[30,31\].

Addiction may also be viewed as a disorder of learning and memory, as converging evidence points to addiction representing a pathological usurpation of normal mechanisms of learning and memory \[32\] and the cholinergic system is clearly implicated in these functions \[33\]. Consequently, it is plausible that the M4 receptor influences drug-taking behaviour via this avenue. Indeed, it has been reported that a positive allosteric modulator of the M4 receptor enhances some memory functions \[34–36\], while M4\(^{-/-}\) mice were found to have normal memory in various models \[30,31,37\]. However, M4\(^{-/-}\) mice have not previously been assessed on the Barnes maze.

To further our understanding of the role of M4 receptors in the regulation of alcohol consumption we assessed M4\(^{-/-}\) mice and their littermate WT controls in tests of learning and memory, anxiety-like behaviour, spontaneous activity and novelty seeking, and examined the relationship of a newly discovered “d uninhibited” endophenotype of these mice with voluntary alcohol consumption and relapse.

2. Materials and methods

2.1. Animals and housing conditions

Male M4\(^{-/-}\) mice were generated as previously described \[22\] and bred at the Panum Institute, University of Copenhagen in a fully AAALAC accredited facility. Founder mice of a mixed genetic background (129SvEv/CF1) were backcrossed to the C57BL/6Ntac strain for 13 generations, and genotyping was performed on mouse-ear DNA using the polymerase chain reaction. Male M4\(^{-/-}\) littersmates were used as controls. After weaning, mice were housed with littersmates in groups of 4–8 in Makrolon cages (20 × 35 × 15 cm\(^3\), Tecniplast, Varese, Italy). They were provided pelleted feed (Altromin 1319; Brogaarden, Gentofte, Denmark) and acidified tapwater ad libitum. Cages were provided with paper enrichment (Lilico), bite blocks (Tapvei Oy) and cardboard tubes (Lilico). The animal room was kept at a constant temperature (22–24 °C) and the light regimen was a 12:12 h dark/artificial light cycle with 30 min of "twilight" and the lighting period starting at 7:00 AM. After being transferred from the breeding facility, the animals were allowed to acclimatize to the experimental facility for at least 1 week prior to initiation of any experiments. All behavioral experiments were performed during the light cycle between 8:00 AM and 4:00 PM. Separate cohorts of mice were used for the different behavioural tests and mice continued to be group housed with littersmates unless otherwise indicated.

2.2. Ethics

The animal experiments were approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries (license number 2012-15-2934-00038). All procedures were performed in accordance with the EU directive 2010/63/EU in a fully AAALAC accredited facility under the supervision of a local animal welfare committee. All efforts were made to minimize pain or discomfort as well as the number of animals used during the experiments.

2.3. Drugs

Alcohol (96%, Sigma Aldrich, Denmark) was diluted with tap water to produce solutions of 2–16% of alcohol.

2.4. Spontaneous locomotor activity

Assessment of spontaneous locomotor activity was conducted in locomotor activity cages (approximately 100 lx inside the cages) (Ellegaard Systems, Denmark) equipped with 5 × 8 infrared light sources plus photocells. The light beams crossed the cage 1.8 cm above the bottom of the cage. The recording of a motility count required the interruption of two adjacent light beams, thus avoiding counts generated by stationary movements of the mice. All experiments were conducted in a clean cage with a scant lining of bedding material. Mice were transported to the test room and allowed to acclimatize for 30 min before being placed individually into the apparatus and activity was recorded for 60 min (N = 12 for both genotypes).

2.5. Barnes maze

The Barnes maze consisted of a brightly illuminated (280 lx) circular white-coated platform 100 cm in diameter and elevated 72 cm above the ground. Sixteen 3.9 cm-diameter holes were evenly distributed around the perimeter, 2 cm from the edge. One of those holes allowed the mouse to enter the escape box, a dark plastic storage box (12 × 8 × 6 cm) located under the escape hole and containing bedding material from the home cage of the mouse being tested. All trials were recorded by a camera mounted above the maze and analysed by the video tracking program EthoVision (Noldus Information Technology, Wageningen, The Netherlands, version 11). The mice underwent three days of shaping to enter the escape box before training started. On day 1 and 2 of shaping, the mice were released in close proximity of the escape hole and confined there for 3 min by a circular semitransparent beaker (diameter 20 cm) surrounding mouse and escape hole. Mice that had not entered the escape hole after the 3 min had elapsed were gently guided there by the experimenter. After 30 s the escape box was removed, and the mouse was transported in the escape box to its home cage. This was repeated 2 times on day 1 and 3 times on day 2. On day 3 of shaping two 40 cm high wooden walls delineated a wedge-shaped corridor from the middle of the maze to the escape hole and its two adjacent holes. The mice were now released in the center of the maze and allowed 3 min to find their way into the escape box. Mice that had not entered the escape box when the 3 min had elapsed were gently guided there by the experimenter. After 30 s the escape box was removed, and the mouse was transported in the escape box to its home cage. This was repeated 2 times. Training consisted of two trials a day for 6 consecutive days. For each trial, the mouse was released in the middle of the maze oriented in a random direction and allowed to explore the maze freely until it entered the hidden escape box or until 3 min had passed. Mice that did not find the escape box within the 3-minute trial were guided there gently by the experimenter. Twenty four hours after the last training session a probe trial was conducted, where the escape box was removed, the mouse was released in the middle of the maze, and allowed to explore freely for 3 min. The video tracking system recorded the number of errors (number of visits to other holes before visiting the escape hole for the first time) and distance travelled (distance before the first visit to the escape hole) \(N = 8\) M4\(^{-/-}\) and \(N = 15\) M4\(^{+/-}\) mice were used for this experiment.

2.6. Novel object exploration

An open black plastic arena measuring 77 × 56 × 41 cm was used and illuminated indirectly (80 lx). Behaviour was recorded by a video camera mounted vertically above the test arena and analysed using EthoVision. First a mouse was allowed to explore the empty arena for 30 min. At that point it was briefly removed, while a novel object was
placed in the middle of the arena. Then the mouse was re-introduced and allowed to explore for another 5 min [38]. Latency to approach the novel object and time spent exploring the novel object was recorded. N = 8 M\textsuperscript{+/-} and N = 10 M\textsuperscript{-/-} mice were used for this experiment.

2.7. Novel environment exploration

The apparatus consisted of two equally sized (19 × 19 × 43 cm) compartments with distinct visual cues, separated by a wall with a centrally placed door (3.5 × 3.5 cm) that was closed during training and open during testing and illuminated indirectly (80 lx). The compartment used for training was counter-balanced between genotypes and each mouse was allowed to explore its training compartment for 15 min. At that point it was briefly removed, while the door was opened. Then the mouse was re-introduced to its training compartment and allowed to explore for another 5 min. The time spent (s) in each compartment, the total distance moved and the latency to enter (s) the new compartment was recorded. [39] N = 15 M\textsuperscript{+/-} and N = 15 M\textsuperscript{-/-} mice were used for this experiment.

2.8. The elevated plus maze

The elevated plus maze consisted of two opposing open arms (21 × 8 cm) connected by a central square (8 × 8 cm) to two opposing enclosed arms of the same size with 32 cm high walls. The apparatus was elevated 50 cm above the floor in a large room with an ambient light intensity of 80–100 lx. Behaviour was recorded by a video camera mounted vertically above the maze and analysed using EthoVision. For testing, the animal was placed in the centre of the maze and behaviour was recorded for 5 min. The following parameters were calculated: total time (s) spent in open and closed arms, total time (s) spent on the outer third of the open arms, as well as the frequency of headdips (extending the head over the edge of the open arm). N = 12 M\textsuperscript{+/-} and N = 10 M\textsuperscript{-/-} mice were used for this experiment.

2.9. Voluntary alcohol consumption in the two-bottle free-choice model

A new cohort of mice was tested in the elevated plus maze as described above and went on to have continuous free access to increasing concentrations of an aqueous alcohol solution and water in the home cage. For this purpose, the mice were housed singly.

For the first seven days, access was given to two bottles containing tap water, and intake was examined for possible side preference (which was not detected). Alcohol was then provided in one of the bottles, placed randomly, and faded in (2% and 4%, vol/vol, for 5 days each), after which consumption was measured at 8%, 10%, 12% and 16%. Intake was assessed by weighing the bottles each day between 8 and 10 AM, with occasional omissions, obtaining 5–6 data points at each concentration over the course of 8–9 days. The amount of alcohol ingested was expressed as alcohol preference (% alcohol consumed of total fluid intake) and intake (g/24 h/kg body weight). N = 11 M\textsuperscript{+/-} and N = 11 M\textsuperscript{-/-} mice were used for this experiment. However, one M\textsuperscript{-/-} mouse got sick and had to be euthanized during the course of the experiment.

2.10. Deprivation-induced alcohol consumption

Approximately four weeks after the introduction of 16% of alcohol, a deprivation phase was introduced where the alcohol bottles were removed for a time period of two weeks. At the end of the deprivation phase, the 16% alcohol bottles were reintroduced, and the alcohol deprivation effect (ADE)-induced alcohol intake was measured daily over five days.

2.11. Determination of blood alcohol concentration

After systemic administration of alcohol (~30 min; 2 g/kg, i.p.) trunk blood alcohol levels were analyzed using an Analox GL6 instrument (Analox Instruments Ltd, 22 Acton Park Estate, The Vale London). The Analox instrument analyzes blood alcohol levels via alcohol oxidase (AOD) catalysed oxidation of alcohol (ethyl alcohol) to acetaldehyde and hydrogen peroxide (H2O2). Five microliters of EDTA plasma per mouse were injected into the Analox system, then the sample was mixed with air and lyophilized enzyme. The amount of H2O2 generated is directly proportional to the alcohol concentration measured over a 5.0% W/V alcohol standard.

2.12. Statistical analysis

The two experimental groups (M\textsuperscript{+/-} and M\textsuperscript{-/-} mice) were compared by independent samples t-tests, when only one dependent variable was considered and by mixed-model ANOVA in cases where two curves were compared (locomotor activity, learning in the Barnes maze, drinking of increasing concentration of alcohol, relapse-like drinking). When appropriate this was followed by post-hoc Student t-tests. Additionally, the relationship between performance on the elevated plus maze and alcohol preference was assessed by Spearman’s rank correlation coefficient (p). All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 25.0 (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as p < 0.05. All statistical tests were two sided and data are presented as mean ± SEM.

3. Results

3.1. Spontaneous locomotor activity

M\textsuperscript{+/-} and M\textsuperscript{-/-} mice did not differ with respect to spontaneous locomotor activity or habituation of this behaviour. Both groups showed an equal and significant reduction in activity over time (F(10,110) = 54.215, p < 0.001, Fig. 1A).

3.2. Barnes maze

Mixed-model ANOVA (with training day as the repeated measure) of the distance travelled on the Barnes maze before entering the escape box showed a significant interaction between training day and genotype (F (5105) = 3.004, p = 0.014; Fig. 1B), indicating that the M\textsuperscript{+/-} and M\textsuperscript{-/-} mice differed in performance on some, but not other, days. Post-hoc Student t-tests revealed that the M\textsuperscript{+/-} mice covered a longer distance than the M\textsuperscript{-/-} mice on day 2 of testing (t(21) = 3.224, p = 0.004), however, the overall effect of genotype was not significant. Both groups improved their performance significantly over time. Mixed-model ANOVA of the number of errors did not reveal a significant effect of genotype either, nor was the interaction term significant (Fig. 1C). Both groups improved their performance significantly over time. During the probe trial the performance of M\textsuperscript{+/-} and M\textsuperscript{-/-} mice did not differ, neither with respect to distance travelled nor the number of errors.

3.3. Novel object exploration

When the novel object was introduced to the testing chamber M\textsuperscript{+/-} mice displayed a significantly and markedly shorter latency to approach it, compared to their M\textsuperscript{-/-} littermates (t(16) = 3.034, p = 0.008; Fig. 2A). The total time spent exploring the novel object did not differ between groups (Fig. 2B) and neither did the distance travelled in the testing chamber (data not shown).

3.4. Novel environment exploration

When the door between the two compartments of the apparatus was opened, M\textsuperscript{+/-} mice displayed a slightly shorter latency to enter the new environment, compared to their M\textsuperscript{-/-} littermates, however, this was not
significant (t(28) = 1.843, p = 0.076; Fig. 2C). The total time spent exploring the novel environment did not differ between groups (Fig. 2D) and neither did the distance travelled in the testing chamber (data not shown).

3.5. The elevated plus maze

The M4/− mice spent less time in the closed arms (t(21) = 2.346, p = 0.029; Fig. 3C), more time in the open arms (this was, however, not significant, p = 0.056; Fig. 3A), more time on the outer third of the open
arms (t(21) = 2.294, p = 0.032; Fig. 3B), and made significantly more head-dips (t(21) = 2.300, p = 0.032; Fig. 3D), compared to their M4+/+ littermates. Total distance moved, velocity and total number of arm-entries did not differ between the groups (see supplementary materials).

3.6. Voluntary alcohol consumption in the two-bottle free-choice model

Initially, also this cohort of mice was tested in the elevated plus maze. The M4−/− mice spent less time in the closed arms of the elevated plus maze (t(20) = −3.843 p = 0.001; Fig. 4C), more time on the open arms (t(20) = 4.414, p = 0.000; Fig. 4A), more time on the outer third of the open arms (t(20) = 4.672, p = 0.000; Fig. 4B), and made significantly more head-dips (t(20) = 2.755, p = 0.013; Fig. 4D), compared to their M4+/+ littermates. Total distance moved, velocity and total number of arm-entries did not differ between the groups (see supplementary materials).

Thereupon the mice were offered a voluntary choice of drinking either increasing concentrations of alcohol or water in their home cage. In this two-bottle free-choice model, mixed-model ANOVA (with alcohol concentration as the repeated measure) revealed that the M4−/− mice displayed a significantly higher alcohol preference (main effect of genotype: F(1,19) = 9.480, p = 0.006, Fig. 5A) and intake (main effect of genotype: F(1,19) = 4.415, p = 0.039, Fig. 5B) compared to the litter-mate control M4+/+ mice. There was also a significant main effect of alcohol concentration on drinking behaviour (F(3,57) = 19.535, p = 0.000 for alcohol preference and F(3,57) = 3.603, p = 0.000 for alcohol intake), reflecting the fact that alcohol consumption declined at increasing concentrations. Additionally, there was a significant interaction between the effects of genotype and alcohol concentration (F(3,57) = 3.384, p = 0.024 for alcohol preference and F(3,57) = 1.287, p = 0.023 for alcohol intake), indicating that the difference in alcohol preference and alcohol intake between M4−/− mice and M4+/+ littermate controls was more prominent at lower concentrations of alcohol. This was confirmed by post-hoc Student t-tests, which showed that the difference in alcohol preference between the genotypes was significant at 8% (t(19) = 2.325, p = 0.031), 10% (t(19) = 2.810, p = 0.011), and 12% (t(19) = 2.599, p = 0.018), but not at 16% alcohol concentration (see Fig. 5A). With respect to alcohol intake, the difference between the genotypes was only significant at the 8% alcohol concentration (t(19) = 2.446, p = 0.024, see Fig. 5B). Water intake did not differ significantly between the two genotypes, although the water intake level changed over the period when the mice were offered increasing concentrations of alcohol.

Fig. 3. Constitutional deletion of the muscarinic M4 receptor decreases anxiety-like behavior as indicated by increased time spent in the outer parts of the open arms (B) and decrease time spent in the closed arms (C) of the elevated plus maze as well as an increased number of head dips (D). M4+/+ (N = 12) and M4−/− (N = 10), data are shown as mean ± SEM, * p < 0.05.
alcohol (main effect of alcohol concentration: $F(3,57)=21.83$, $p<0.001$). There was no significant difference in total fluid intake between the two genotypes and the total fluid intake level did not change over the period when the mice were offered increasing concentrations of alcohol (see supplementary materials).

3.7. Deprivation induced alcohol consumption

After a two week period of alcohol deprivation, the mice’ preference for and intake of the 16% alcohol concentration was reassessed. Mixed-model ANOVA (with day as the repeated measure) revealed that the $M_4^{-/-}$ mice displayed a significantly higher preference for 16% alcohol after the deprivation phase compared to the littermate control $M_4^{+/+}$ mice (main effect of genotype: $F(1,19)=8.853$, $p=0.008$, Fig. 6A). This was not significant with respect to intake (main effect of genotype: $F(1,19)=4.056$, $p=0.058$, Fig. 6B). There was also a significant main effect of day on drinking behaviour ($F(3,57)=10.031$, $p=0.005$ for alcohol preference and $F(3,57)=3.996$, $p=0.005$ for alcohol intake), reflecting the fact that alcohol consumption declined over days. There was no significant interaction between the effects of genotype and day with respect to deprivation induced drinking. Post-hoc Student t-tests showed that $M_4^{-/-}$ mice displayed a greater preference for 16% alcohol on day 3 after the end of the deprivation period ($t(19)=3.928$, $p=0.001$), while the difference between genotypes did not reach statistical significance on the other days. (see Fig. 6A).

3.8. Correlation between plus maze behaviour and alcohol consumption

We examined the relationship between time spent on the elevated plus maze open arms and alcohol preference as well as intake (mean over the four alcohol concentrations offered in the two-bottle free-choice model) by calculating Spearman’s rank correlation coefficient. We found that mice that consumed larger amounts of alcohol and showed greater alcohol preference had spent more time on the elevated plus maze open arms ($\rho(19)=0.586$, $P=0.005$ for preference and $\rho(19)=0.490$, $P=0.024$ for intake, see Fig. 7 A and B).

We also examined the relationship between time spent on the elevated plus maze open arms and alcohol preference as well as intake (mean over the five days) during deprivation induced alcohol consumption by calculating Spearman’s rank correlation coefficient. We found that, also during this phase of the experiment, mice that consumed larger amounts of alcohol and showed greater alcohol preference had spent more time on the elevated plus maze open arms ($\rho(19)=0.555$, $P=0.009$ for preference and $\rho(19)=0.487$, $P=0.025$ for intake, see Fig. 7 C and D).
3.9. Blood alcohol concentration

Blood alcohol levels were similar between the M₄⁻/⁻ mice and the littermate control M₄⁺/+ mice measured thirty minutes after systemic administration of 2 g/kg alcohol (see Fig. 8).

4. Discussion

The findings of the present study indicate that muscarinic acetylcholine M₄ receptors impact alcohol preference and alcohol intake, as well as alcohol deprivation induced relapse-like drinking behaviour by a mechanism involving disinhibited approach behaviour.

This is supported by three lines of evidence: First, the M₄⁻/⁻ mice displayed disinhibited approach behaviour in a test of novelty preference and a tendency to do so in another, second, the M₄⁻/⁻ mice had disinhibited exploratory behaviour on the elevated plus maze, and third this "disinhibited" endophenotype correlated with alcohol preference and alcohol intake, as well as alcohol deprivation induced relapse-like drinking behaviour measured subsequently in the same mice. Thus, the increased propensity to consume drugs of abuse observed previously in M₄⁻/⁻ mice may be driven by an altered regulation of approach behaviour [14,40].

The M₄⁻/⁻ and M₄⁺/+ genotypes showed no difference in basal locomotor activity in a novel environment and habituated gradually with an equal pattern over time. This result is in line with previous findings investigating locomotor activity in M₄⁻/⁻ mice fully backcrossed to the C57BL/6NTac or C57Bl/6 J strain [40–43] but contrasts with Gomenza et al. and Koshimizu et al., who found increased novelty stimulated activity in the M₄⁻/⁻ genotype on a on mixed 129SvEv/CF-1 or pure129SvEv genetic background, respectively [22,31,44]. We conclude that neither spontaneous, novelty stimulated locomotor activity nor habituation to a novel environment, a non-associative form of learning, is affected in fully backcrossed M₄⁻/⁻ mice. Also, M₄⁻/⁻ mice did not differ from their littermate M₄⁺/+ controls with respect to spatial learning and memory on the Barnes maze, which confirms previous findings with the Morris water-maze in M₄⁻/⁻ mice maintained on a pure 129SvEv background [31]. This indicates that the disinhibited phenotype of M₄⁻/⁻ mice revealed in the present study is not driven by a deficit in habituation, nor is the higher intake and preference for alcohol related to deficits in learning and memory.

When exposed to two different tests of novelty preference, novelty-object and novel-environment exploration, the M₄⁻/⁻ mice showed "disinhibited" approach behaviour, in as much as their latency to approach the novel stimulus was substantially shorter than that of their M₄⁺/+ littermates, while the total time spent exploring the novel stimulus did not differ between genotypes. We suggest that this reflects increased novelty seeking/sensation seeking which has been associated with alcohol use [45]. We therefore subjected the M₄⁻/⁻ mice to the elevated plus maze test, as it has been suggested that time spent in an
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environment that mice perceive as more hazardous (here, the open arms of the elevated plus maze) represents a balance between the innate drive to explore novel environments as opposed to anxiety about the dangers of that novel environment [46, 47]. Thus, behavior on the open arms of the elevated plus maze can be used as an index of disinhibition and impulsivity in addition to anxiety. We found that M4−/− mice spent less time in the closed arms, more time on the outer third of the open arms and made more head dips (extending the head over the edge of the open arms) than their littermate M4+/+ controls. We believe that this supports our hypothesis of the M4−/− mice displaying a "disinhibited" endophenotype rather than simply being less anxious. This is further supported by the finding that M4−/− mice did not differ from wild type mice in another test of anxiety, the light-dark transition test [31]. In order to examine how this relates to the M4−/− mice’s propensity to consume alcohol we subjected a new cohort of M4−/− mice and their littermate M4+/+ controls to the elevated plus maze test and subsequently offered these mice the choice to consume increasing concentrations of alcohol or water in their home cage. We found that the M4−/− mice consumed more alcohol and showed a greater preference for alcohol as compared to their M4+/+ littermates and that this correlated moderately with the time the mice had spent on the open arms of the elevated plus maze. We propose that this resembles previous findings of a correlation between "disinhibited", sensation seeking, behaviour and the propensity to consume alcohol and other drugs of abuse [48, 49] and suggests that the M4 receptors role in drug seeking behaviour [14, 40] might in part be mediated via an effect on the response to novelty. It is well known that exposure to novelty increases midbrain dopamine levels [50] and we have previously shown that M4−/− mice exhibit a dopamine "hyperreactive" phenotype [40, 43].

The difference between the genotypes with respect to alcohol intake and preference was more pronounced at lower alcohol concentrations and was abolished at the highest (16%) concentration of alcohol. This confirms previous findings with M4−/− and M4+/+ mice allowed to respond for alcohol on an FR1 schedule of oral operant self-administration [14] and could reflect an aversive response to the taste of high alcohol concentrations frequently encountered in mice [51, 52]. Since alcohol and drug dependences are chronic relapsing disorders, the study of vulnerability to relapse is of particular importance. We therefore subjected the mice to a 2-week period of abstinence whereupon we reintroduced the choice to consume alcohol at the 16% concentration. This is known to induce a relapse-like pattern of increased alcohol consumption and has been termed "the alcohol deprivation

Fig. 7. The time spent on the elevated plus maze open arms many weeks prior correlated with alcohol preference (A and C) and alcohol intake (B and D), both during initial voluntary consumption of 8–16% alcohol in the two-bottle free-choice model (A and B) and during later deprivation induced relapse-like alcohol consumption (16%). Empty circles M4+/+ (N = 11) and black squares M4−/− (N = 10).
therefore suggest that the changed drug-taking behaviour during base environment rather than a change in general learning ability. We additionally show that this phenotype correlates with disinhibited approach behaviour, providing a potential mechanistic explanation. Taken together, these findings further support the potential usefulness of targeting M4 receptors in the treatment of substance use disorder.

**CRediT authorship contribution statement**

Anna Molander: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. Ditte Dencker Thorbek: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization. Christian Lysne: Investigation, Visualization. Pia Weikop: Investigation, Visualization. Anders Fink-Jensen: Conceptualization, Supervision, Funding acquisition. Gitta Wörtwein: Conceptualization, Methodology, Validation, Formal analysis, Visualization, Writing – review & editing.

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**Fig. 8.** Blood alcohol levels did not differ between M4−/− (N = 10) and M4+/+ (N = 10) mice, 30 min after systemic administration of 2 g/kg, i.p.
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