Cholinergic mu-opioid receptors participate in nicotine, but not ethanol, drinking behaviors

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Abstract

Heavy alcohol use and binge drinking are important contributors to alcohol use disorder (AUD). The endogenous opioid system has been implicated in alcohol consumption and preference in both humans and animals. The mu opioid receptor (MOR) is expressed on multiple cells in the striatum, however little is known about the contributions of specific MOR populations to alcohol drinking behaviors. The current study used mice with a genetic deletion of MOR in cholinergic cells (ChAT-Cre/Oprm1$^{fl/fl}$) to examine the role of MORs expressed in cholinergic interneurons (CINs) in home cage self-administration paradigms. Male and female ChAT-Cre/Oprm1$^{fl/fl}$ mice were generated and heterozygous Cre+ (knockout) and Cre- (control) mice were tested for alcohol and nicotine consumption. In Experiment 1, binge-like and quinine-resistant drinking was tested using 15% ethanol (EtOH) in a two-bottle, limited-access Drinking in the Dark paradigm. Experiment 2 involved a six-week intermittent access paradigm in which mice received 20% EtOH, nicotine, and then a combination of the two drugs. Deleting MORs in cholinergic cells did not alter consumption of EtOH in Experiment 1 or 2. In Experiment 1, the MOR deletion resulted in greater consumption of quinine-adulterated EtOH in male Cre+ mice (vs. Cre-). In Experiment 2, Cre+ mice demonstrated a significantly lower preference for nicotine but did not differ from Cre- mice in nicotine or nicotine + EtOH consumption. These data suggest that cholinergic MORs are involved in nicotine, but not EtOH, drinking behaviors and may contribute to aversion resistant EtOH drinking in a sex-dependent manner.

Keywords: alcohol, nicotine, cholinergic neurons, mu opioid receptor, binge drinking, aversion-resistance

Abbreviations: AUD: Alcohol Use Disorder; MOR: mu opioid receptor; EtOH: ethanol; VTA ventral tegmental area; DS: dorsal striatum; NAc: nucleus accumbens; MSN: medium spiny neuron; CIN: cholinergic interneuron; DA: dopamine; nAChR: nicotinic acetylcholine receptor; DID: drinking in the dark; ChAT: choline acetyltransferase; IA: intermittent access

1. Introduction

Alcohol use disorder (AUD) is a significant public health crisis with 28.6 million adults in the United States ages 18 and older diagnosed with the disease (NIAAA, 2023). Binge drinking and heavy alcohol use contribute to the risk for developing AUD and are key symptoms of the disorder (NIAAA, 2023). Notably, compulsive drinking, defined as heavy drinking despite negative consequences, is an important characteristic of AUD (Association and Others, 2013). The prevalence of AUD and the resulting alcohol-related problems make research surrounding the neural underpinnings of this disease vital to developing novel and effective treatments and therapies.

The endogenous opioid system is known to be a critical regulator of alcohol consumption and preference in humans and animals (Gianoulakis, 2009; Méndez and Morales-Mulia, 2008). Increases in mu opioid receptor (MOR) expression and affinity have been associated with enhanced alcohol intake and dependence in rodents, non-human primates, and humans (Barr et al., 2007; Bart et al., 2005; Learn et al., 2001; Oslin et al., 2003). Genetic deletion of the mu opioid receptor (MOR) in mice reduces consumption (Becker et al., 2002; Hall et al., 2001; Roberts et al., 2000) as well as the acute stimulant and anxiolytic actions of ethanol (EtOH) (Ghozland et al., 2005). Further, systemic MOR antagonists decrease alcohol consumption in rodents (Gilpin et al., 2008; Heyser et al., 1999; Hyytiä and Kiianmaa, 2001) and the MOR antagonist naltrexone is one of three medications approved in the United States to treat AUD.

Despite this clear role of the endogenous opioid system and the MOR in alcohol drinking behaviors, the contributions of various MOR populations have yet to be fully identified. MORs are broadly expressed throughout the nervous system (Erbs et al., 2015), including in addiction-relevant nuclei such as the ventral tegmental area (VTA), dorsal striatum (DS), nucleus accumbens (NAc), and amygdala. Within each of these regions, MORs are expressed in multiple cell types. For example, in the striatum, MOR expression has been observed in both D1 and D2 subtypes of medium spiny neurons (MSNs) (i.e., direct and indirect pathway neurons) and in the cholinergic interneurons (CINs) (Cui et al., 2014; Jabourian et al., 2005; Oude Ophuis et al., 2014; Ponterio et al., 2013). MOR expression on D1-expressing, but not D2-expressing, MSNs contributes to morphine-induced locomotor sensitization

(Severino et al., 2020). Furthermore, selective restoration of MOR expression in direct-pathway MSNs only partially restores opioid seeking behavior (Cui et al., 2014), suggesting that MOR-expressing neuronal subpopulations play distinct roles in behavior.

Although they comprise only 1-5% of striatal neurons, CINs are important regulators of dopamine (DA) release from terminals via nicotinic acetylcholine (ACh) receptor (nAChR) activation (Liu et al., 2022; Mohebi et al., 2023). MORs exhibit an inhibitory effect on striatal CIN excitability and striatal ACh release (Arttamangkul et al., 2021; Jabourian et al., 2005; Kiguchi et al., 2016; Ponterio et al., 2013), indirectly inhibiting DA overflow caused by a single-spike stimulus (Britt and McGehee, 2008; Yorgason et al., 2017). Thus, activation of CIN MORs could influence addictive behaviors by modulating DA signals associated with the response to rewarding stimuli such as drugs of abuse (Al-Hasani et al., 2021; Berlanga et al., 2003; Gonzales and Smith, 2015). Indeed, targeted deletion of MORs on cholinergic cells has been shown to selectively impair extinction of opioid-seeking (Severino et al., 2020). Additionally, alcohol exposure can activate CINs (Herring et al., 2004; Wadsworth et al., 2023) while optogenetic stimulation of NAc CINs has been shown to increase EtOH consumption (Kolpakova et al., 2022).

Considering the role that striatal MORs are thought to play in regulating alcohol-drinking behaviors via alcohol-induced release of endogenous opioids (Heinz et al., 2005; Hermann et al., 2017; Lam et al., 2010; Méndez et al., 2010; Mitchell et al., 2012; Nealey et al., 2011; Olive et al., 2001; Perry and McNally, 2013; Richard and Fields, 2016; Zhang and Kelley, 2002), it is important to understand the relative contribution of MSN vs. CIN MOR expression to alcohol consumption. As such, we targeted CIN MORs by generating a transgenic line of mice with a deletion of the MOR on cholinergic cells. In Experiment 1, we used a two-bottle, limited-access “drinking in the dark” (DID) paradigm (Sneddon et al., 2019) to model binge-and compulsive-like EtOH drinking. Three hours into the dark cycle, when mice are most active and likely to drink EtOH (Sprow and Thiele, 2012), they are given access to water and 15% EtOH in their home cages for a period of two hours. To model aversion-resistant drinking in this paradigm, we adulterated the EtOH with quinine, a bitter tastant (Hopf and Lesscher, 2014; Lesscher et

al., 2010; Radke et al., 2021; Sneddon et al., 2019). Experiment 2 tested consumption and preference of EtOH in a 24-h, two-bottle intermittent access (IA) paradigm. To determine whether any observed effects were specific to alcohol, and because acetylcholine release from CINs has been implicated in nicotine seeking (Leyrer-Jackson et al., 2021; Williams et al., 2017), the effect of cholinergic MOR deletion on nicotine consumption was also assessed. In this experiment, mice received EtOH, nicotine, and then a combination of the two drugs over a six week period. Our results suggest that cholinergic MORs are involved in nicotine, but not EtOH drinking behaviors.

2. Materials and Methods

2.1. Subjects

ChAT-Cre/Oprm1$^{fl/fl}$ knockout mice were generated from breeding pairs purchased from the Jackson Laboratory (Bar Harbor, ME). Heterozygous ChAT-Cre males (B6.129S-Chattm1(cre)Lowl/MwarJ, strain #031661) were bred with homozygous *Oprm1* floxed females (B6;129-Oprm1tm1.1Cgrf/KffJ, strain #030074) to produce a Cre-mediated deletion of exons 2-3 of the MOR gene (*Oprm1*) on choline acetyltransferase (ChAT)-expressing neurons (**Figure 1A**). Following three crosses, the line was established and Cre- females were bred with Cre+ males to produce offspring that were Cre- or Cre+. All offspring were homozygous for floxed *Oprm1*.

For Experiment 1, 55 male and female ChAT-Cre/Oprm1$^{fl/fl}$ mice were used. For Experiment 2, 30 male and female ChAT-Cre/Oprm1$^{fl/fl}$ mice were used. Mice were housed at Miami University in standard shoe box rectangular mouse cages (18.4 cm x 29.2 cm x 12.7 cm) with food (LabDiet 5001) and reverse-osmosis (RO) water provided *ad libitum*. The cage lids were designed to allow for two test bottles to be present at the same time, with food placed on the floor of the cage. The animals resided in a temperature-controlled room with a 12:12 hour reverse light/dark cycle. For drinking experiments, mice habituated to the testing room and single housing conditions for at least one week before testing. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Miami University Institutional Animal Care and Use Committee.

2.2. Drugs

EtOH (15% or 20%) was made volume/volume in RO water. Quinine hemisulfate (Q1250-50G, Millipore-Sigma, St. Louis, MO) was prepared weight/volume in 15% EtOH or RO water. Nicotine tartrate salt (30 µg/mL) was prepared weight/volume in 20% EtOH solution or RO water. Nicotine concentrations are reported as free base. For DID, solutions were made fresh every day. For intermittent access, solutions were prepared on a weekly basis.

2.3. Fluorescent in situ hybridization

Validation of *Oprm1* deletion in the ChAT-Cre/Oprm1$^{fl/fl}$ line was conducted using RNAScope (ACD Biosystems) (**Figure 1B**). Brains from adult mice were extracted and flash frozen. A cryostat was used to collect sections (15 µM) containing the dorsal and ventral striatum. Sections were mounted on slides and fixed in chilled formalin and processed using probes for the cholinergic cell marker Mm-*Chat* (channel 1, cat#408731-C1) and the Mm-*Oprm1* gene (channel 2, cat#315841-C2). Slides were counterstained with DAPI and visualized using a Leica AX70 microscope.

2.4. Experiment 1: Effects of cholinergic MOR deletion on binge-like EtOH consumption and aversion-resistance.

2.4.1. EtOH drinking in the dark

Mice self-administered EtOH in a two-bottle DID paradigm with access to EtOH for two hours a day, five days a week (Monday-Friday) for the duration of three weeks. One of the bottles contained 15% EtOH and the other bottle contained RO water. The side the bottles were presented on was alternated each day to prevent a side preference. Bottles were weighed before and after each drinking session and two "dummy" cages were positioned on the same rack as the mouse cages to account for any evaporation or spillage. During sessions 11-14, quinine hemisulfate was added to the 15% EtOH in increasing concentrations (0 µM, 100 µM, 250 µM, 500 µM) to test for aversion-resistant drinking.

2.4.2. Water drinking in the dark

Following EtOH drinking, mice consumed water only for one week before testing of quinine sensitivity commenced. The aversive effects of quinine in the absence of a drug were tested in two-hour drinking sessions identical to the previous sessions with EtOH. During these sessions, mice had access to

two bottles, one with unadulterated RO water and the other with RO water and increasing concentrations of quinine presented over a series of 4 days (0 µM, 100 µM, 250 µM, 500 µM).

2.5. Experiment 2: Effects of cholinergic MOR deletion on homecage self-administration of EtOH and nicotine.

2.5.1. Intermittent access to EtOH and nicotine

Mice were split into two cohorts roughly accounting for sex and genotype. Cohort one included 15 mice with 6 Cre+ mice (5 females and 1 male) and 9 Cre- mice (4 females and 5 males). Cohort two included 15 mice with 9 Cre+ mice (6 females and 3 males) and 6 Cre- (2 females and 4 males). Treatment and care was identical between cohorts but varied in order. Mice self-administered 20% EtOH, 30 µg/mL nicotine, or EtOH and nicotine in combination in a 24-hour two-bottle intermittent access paradigm with access to the solutions every other day, three days a week (Mon/Wed/Fri) for the duration of two weeks per solution. Drinking bottles were refilled on a daily basis 6 days/week at 12:30 p.m. On intervening sessions (T/Th), mice had access to two bottles of RO water. The bottle positions were alternated each day to prevent a side preference. Bottles were weighed before and after each drinking session and four "dummy" cages were positioned on the same rack as the mouse cages to track any evaporation or spillage. Animal body weights were recorded every day. Cohort one received 20% EtOH, followed by nicotine, and then the combined solution, each for two weeks. Cohort two began with the nicotine solution, followed by EtOH and then the combined solution.

2.6. Data analysis

All analyses were performed using GraphPad Prism (v 10) software. Bottle weights were expressed as grams of EtOH, ug of nicotine, or mL of water consumed per kg of bodyweight. The amount of solution consumed was measured by calculating *=(Initial Bottle Weight - Final Bottle Weight) - Average Dummy Bottle Weight*. Preference was measured by calculating *=(Volume of solution/Volume of solution + Water consumption))*100*. Total fluid consumption on test sessions and intervening sessions was calculated by summing consumption from the two bottles.

Consumption (g/kg) and % preference were analyzed using three-way repeated measures analysis of variance (RM-ANOVA) and mixed factor analyses with factors of genotype, sex, and session/concentration. Experiment 1 was fully powered to detect sex differences and sex was included as a factor in all analyses. Because sex did not interact with genotype in Experiment 1 or in preliminary analyses for Experiment 2, the latter experiment was not fully powered to detect sex differences and data were collapsed across sex for final analyses and presentation. For Experiment 1, separate analyses were conducted for the EtOH/RO water, EtOH/quinine, and RO water/quinine sessions. Aversion-resistance was defined *a priori* as a change from baseline and *post-hoc* Dunnett's tests were conducted to analyze changes in consumption based on sex and genotype during the quinine sessions. For Experiment 2, preliminary analyses determined that the order of presentation of the nicotine and EtOH solutions did not affect the results, so data were combined for each solution and separate analyses were conducted for the EtOH, nicotine, and combined solutions. Body weights and water consumption on intervening sessions were analyzed using two-way ANOVA.

In Experiment 2, a technological error during the nicotine + EtOH portion of the study resulted in data from session 2 being lost so it is not included in the figures or analyses.

3. Results

3.1. Experiment 1: Cholinergic MOR deletion does not affect binge-like EtOH consumption.

3.1.1. Binge-like consumption.

A 3-way RM-ANOVA yielded a main effect of both session ($F (3.138, 185.2) = 18.080$, $p < 0.0001$) and sex ($F (1, 59) = 11.910$, $p = 0.001$) for consumption by body weight (g/kg) (**Figure 2A**), demonstrating greater consumption in female ChAT-Cre/Oprm1$^{fl/fl}$ mice vs. males. EtOH preference exhibited a significant main effect of session ($F (3.654, 215.6) = 27$, $p < 0.0001$) and a session x sex interaction ($F (9, 531) = 2.320$, $p = 0.015$) (**Figure 2B**). There were no main effects or interactions involving genotype for either measure. Consumption is calculated per body weight for each animal, so we analyzed body weights on the first day of the study, and Cre+ and Cre- mice did not differ in weight (Cre- = 23.29 ± 0.62 g; Cre+ = 22.68 ± 0.53 g) (unpaired t-test, $p = 0.455$).

3.1.2. Aversion-resistant drinking.

For quinine-resistant drinking, 3-way RM-ANOVA revealed a main effect of quinine concentration on EtOH consumption ($F(3, 153) = 35.670$, $p < 0.0001$) (**Figure 2C**) and a significant main effect of sex ($F(1, 51) = 9.299$, $p = 0.004$). There was also a concentration x sex x genotype interaction ($F(3, 153) = 2.933$, $p = 0.0354$). To follow up on this interaction, separate 2-way RM ANOVAs were performed in male and female animals. In males, there was a significant main effect of concentration on consumption ($F(3, 77) = 15.300$, $p < 0.0001$) and a significant interaction between concentration and genotype ($F(3, 77) = 3.938$, $p = 0.011$). In females, there was a significant main effect of concentration ($F(3, 75) = 21.130$, $p < 0.0001$). Dunnett's test were used to test aversion-resistance at each quinine concentration by comparing to the 0-µM session. 100-µM quinine reduced consumption in Cre- males ($p = 0.037$) only. For the 250-µM concentration, reductions were observed in Cre+ males ($p = 0.022$) and females ($p = 0.006$). Finally, consumption was reduced at the 500-µM quinine concentration in Cre- males and females ($p < 0.0001$ for both) and in Cre+ males ($p = 0.013$) and females ($p < 0.0001$).

A 3-way RM-ANOVA on EtOH preference during the quinine sessions found a significant main effect of session ($F(3, 129) = 8.850$, $p < 0.0001$) but no effects of sex or genotype (**Figure 2D**). Separate 2-way RM-ANOVAs found significant main effects of quinine concentration in male ($F(3, 78) = 4.722$, $p = 0.004$) and female ($F(3, 75) = 5.747$, $p = 0.001$) animals. There were no significant main effects or interactions with genotype. Preference for quinine-adulterated EtOH was significantly reduced in male Cre- mice at the 500-µM concentration ($p = 0.037$). In Cre+ males, preference was significantly reduced at the 250-µM concentration ($p = 0.033$) and the 500-µM concentration approached the threshold of significance ($p = 0.063$). In females, preference was significantly reduced in Cre- mice at the 250- ($p = 0.013$) and 500-µM ($p = 0.027$) quinine concentrations. The Dunnett's test approached the threshold of significance in female Cre+ mice at the 250-µM concentration ($p = 0.054$).

To further explore and visualize the 3-way interaction between concentration, sex and genotype found for EtOH consumption, g/kg intake on each quinine session (100, 250, and 500 µM) was expressed as a percent of consumption on the 0-µM session and 2-way ANOVAs were performed for each

concentration. For the 100-μM session, there was a significant interaction between sex and genotype ($F(1, 51) = 9.343$, $p = 0.004$) and follow-up Holm-Sidak's tests revealed a significant difference between Cre- and Cre+ consumption in male ($p = 0.008$) but not female ($p = 0.185$) mice (**Figure 2E**). No significant effects were observed at the 250-μM concentration (**Figure 2F**). At 500 μM, there was a significant main effect of genotype ($F(1, 51) = 5.252$, $p = 0.026$) and follow-up Holm-Sidak's tests revealed a significant difference between Cre- and Cre+ consumption in male ($p = 0.029$) but not female ($p = 0.710$) mice (**Figure 2G**).

3.1.3. Quinine sensitivity.

Following the EtOH phase of the study, mice underwent a quinine sensitivity assessment during which they were exposed to increasing concentrations of quinine (0 μM, 100 μM, 250 μM, 500 μM) in water. A mixed effects analysis found a significant main effect of quinine concentration on consumption ($F(3, 152) = 11.070$, $p < 0.0001$) but no main effect of sex ($F(1, 51) = 0.270$, $p = 0.606$) or genotype ($F(1, 51) = 1.220$, $p = 0.275$) (**Figure 3A**). A mixed effects analysis of preference identified a main effect of quinine concentration ($F(3, 152) = 10.320$, $p < 0.0001$) but no main effect of sex ($F(1, 51) = 1.362$, $p = 0.249$) or genotype ($F(1, 51) = 0.555$, $p = 0.460$) (**Figure 3B**). The interaction between concentration, sex, and genotype approached the threshold for significance ($F(3, 152) = 2.401$, $p = 0.070$).

3.2. Experiment 2: Cholinergic MOR deletion reduces nicotine preference.

ChAT-Cre/Oprm1$^{fl/fl}$ mice were next tested for EtOH and nicotine consumption using the Intermittent Access model. As with binge-like consumption in Experiment 1, there were no effects of MOR deletion on EtOH consumption, although a 2-way mixed effects model revealed a main effect of session ($F(3.414, 94.900) = 3.601$, $p = 0.013$) (**Figure 4A**). There were similarly no effects of genotype on consumption of nicotine or the nicotine + EtOH solution when analyzed using 2-way RM-ANOVA (**Figure 4B-C**). The main effect of session approached the threshold for significance for the nicotine solution ($F(3.532, 98.890 = 2.252$, $p = 0.077$) and was significant for nicotine + EtOH ($F(2.924, 81.860) = 6.639$, $p = 0.001$).

A 2-way mixed effects model on 24-hour EtOH preference revealed a main effect of session ($F(3.405, 94.650) = 2.928$ $p = 0.032$) but no effect of genotype (**Figure 4D**). Preference for the nicotine solution was significantly lower in Cre+ mice with MOR deletion, as suggested by a main effect of genotype ($F(1, 28) = 7.347$, $p = .0113$) (**Figure 4E**). 2-way ANOVA found no significant main effect of session or interaction for this solution. Finally, for the combined solution, preference was not affected by genotype but did vary with session ($F(3.028, 84.790) = 4.544$, $p = .005$) (**Figure 4F**).

Effects of genotype on nicotine preference but not consumption suggested that the MOR deletion in cholinergic cells was influencing consumption from the H_2O bottle. Analyses of total fluid and H_2O consumption suggested increased H_2O intake in Cre+ mice. For EtOH, a 2-way mixed effects analysis of total fluid consumption revealed main effects of session ($F(3.680, 103.000) = 3.174$, $p = 0.020$) and genotype ($F(1, 28) = 4.370$, $p = 0.046$) but no interaction (**Figure 5A**). There was a main effect of session for the nicotine solution ($F(2.618, 72.250) = 5.233$, $p = 0.004$) and the combined nicotine + EtOH solution ($F(2.589, 72.490) = 8.678$, $p < 0.001$) (**Figure 5B-C**). The main effect of genotype approached the threshold of significance for both nicotine ($F(1, 28) = 3.990$, $p = 0.056$) and the combined solution ($F(1, 28) = 3.553$, $p = 0.070$). A 2-way ANOVA of H_2O consumption on intervening days (i.e., T/Th) found a main effect of solution ($F(1.533, 42.920) = 35.350$, $p < 0.0001$) and the main effect of genotype approached the threshold for significance ($F(1, 28) = 3.553$, $p = 0.070$) (**Figure 5D**). Because consumption is calculated as a function of the animal's bodyweight, we also analyzed bodyweights on the first day of the study and found that Cre+ mice weighed less than Cre- mice (unpaired t-test, $p < 0.0001$) (**Figure 5E**).

4. Discussion

Here, we investigated the effects of the deletion of cholinergic MORs on nicotine consumption and binge- and compulsive-like EtOH consumption. Male and female ChAT-Cre/Oprm1$^{fl/fl}$ mice demonstrated robust levels of alcohol consumption in the DID task, with females consuming more than males. These levels of consumption are similar to those typically observed in C57BL/6J mice using the same parameters (Schuh et al., 2022; Sneddon et al., 2021, 2019). However, Cre+ mice with MOR

deletion did not differ in their consumption or preference for 15% EtOH. Similarly, ChAT-Cre/Oprm1$^{fl/fl}$ mice consumed 20% EtOH readily in the intermittent access experiment but there were no differences between genotypes. When testing aversion-resistant drinking, we observed that Cre+ male mice demonstrated a tendency to consume more quinine-adulterated 15% EtOH, specifically at the 100-µM and 500-µM concentrations. The deletion of cholinergic MORs did not alter 24-h consumption of the nicotine or nicotine + 15% EtOH solutions, but Cre+ mice did exhibit a lower preference for nicotine than Cre- mice. Overall, these findings demonstrate that cholinergic MORs are not required for EtOH self-administration behaviors and suggest that CINs may be involved in aversion resistance.

Acetylcholine signaling has an established role in mediating alcohol reinforcement. This relationship is demonstrated by the ability of nicotine to alter EtOH self-administration in animal models and alcohol consumption in humans, although the direction of the effect is variable and dependent on a number of experimental factors (for review see Hendrickson et al., 2013). EtOH consumption is also reduced by the nAChR partial agonist varenicline (Blomqvist et al., 1996; Ericson et al., 2009; Hendrickson et al., 2010; Steensland et al., 2007; Wouda et al., 2011) and these effects rely at least in part on cholinergic regulation of dopamine release in the NAc core (Feduccia et al., 2014; Nadal et al., 1998; Schilaty et al., 2014). CINs are the primary source of ACh in the striatum and ablation of these neurons reduced EtOH-induced increases in NAc dopamine (Loftén et al., 2021). Targeted excitation of NAc CINs has also been shown to increase EtOH consumption in mice (Kolpakova et al., 2022). Although the cholinergic system clearly plays an important role in mediating alcohol drinking behaviors, our results in ChAT-Cre/Oprm1$^{fl/fl}$ mice demonstrate that cholinergic MOR expression is not a necessary component of this process. Thus, endogenous opioid release in the striatum following alcohol exposure likely exerts its behavioral effects through MORs expressed on D1 and/or D2 receptor-expressing MSNs or through delta opioid receptors (DOR), which are also found on CINs (Arttamangkul et al., 2021; Le Moine et al., 1994). The latter possibility is supported by findings demonstrating that a DOR, but not MOR, agonist stimulated EtOH intake when infused into the NAc (Barson et al., 2009) and that DOR antagonism in the NAc prevents EtOH-induced release of DA (Acquas et al., 1993).

Although cholinergic MOR deletion did not affect EtOH consumption or preference in the limited access DID or intermittent access tasks, intriguing sex-dependent effects were observed on quinine-resistant drinking. At both the lowest and highest concentrations of quinine, mice with MOR deletion were more resistant to aversion than their Cre- counterparts and this effect was stronger in males. It is unclear at present whether sex differences in this effect represent sex differences in the cholinergic system or a ceiling effect resulting from higher consumption in female mice. Because CINs are known to mediate other types of behavioral flexibility (Aoki et al., 2018, 2015; Okada et al., 2018), including following chronic alcohol exposure (Galaj et al., 2019; Ma et al., 2022), it is interesting to speculate that functional differences in CINs induced by the MOR deletion resulted in a reduced capacity for flexibility in ChAT-Cre/Oprm1$^{fl/fl}$ Cre+ mice. The finding that CINs may participate in aversion-resistant alcohol drinking is also interesting in light of studies demonstrating a role for NAc core neurons in quinine-resistant consumption in rats and mice (Seif et al., 2013; Sneddon et al., 2021). After demonstrating that global inhibition of NAc core neurons reduces aversion-resistant drinking while targeted inhibition of D1 and D2 receptor-expressing neurons in this region does not, we previously speculated that CINs may be important regulators of this behavior (Sneddon et al., 2021).

Similar to EtOH, nicotine induces ACh release from striatal CINs (Mansvelder et al., 2003; Rada et al., 2001) and endogenous opioid release in the striatum (Dhatt et al., 1995; Houdi et al., 1991; Marty et al., 1985). Indeed, multiple behavioral and cellular effects of nicotine are absent in transgenic mice missing MOR or endogenous opioid peptides (Berrendero et al., 2010; Trigo et al., 2009; Walters et al., 2005; Yoo et al., 2004). Further, inhibition of CINs was shown to reduce cue-induced reinstatement of nicotine seeking (Leyrer-Jackson et al., 2021). Based on these lines of evidence, we hypothesized that similar results would be seen for nicotine and EtOH drinking in mice with cholinergic MOR deletion. Instead, we observed that nicotine preference, but not consumption, was attenuated in Cre+ vs. Cre- mice. We speculate that the trend toward increased water consumption in the Cre+ (vs. Cre-) mice explains changes in nicotine preference but not consumption. Whether resulting directly from the lack of MORs or adaptations in the cholinergic system brought on by the genetic manipulation (e.g., alterations in CIN

excitability or nAChR expression), the discrepant results between EtOH and nicotine preference suggest that the cholinergic system plays a stronger role in nicotine than EtOH drinking.

While the current results support the conclusion that cholinergic MORs are not involved in EtOH consumption or preference, there are some caveats to the approach. First, MOR deletion was targeted to all cholinergic cells, including the ChAT+ neurons clustered in the pons and basal forebrain. It is thought that cholinergic neurons in the pedunculopontine tegmentum (PPTg) express MOR (Serafin et al., 1990) and nuclei in the pons provide cholinergic input to the VTA and striatum that influences drug-induced reward and locomotion (Corrigall et al., 1999; Steidl et al., 2017). As such, we cannot isolate the impacts of the current genetic deletion to striatal CINs. Additionally, genetic deletion of MOR was not restricted in time and we have not investigated how this manipulation alters functional properties of cholinergic neurons. It is possible that the observed effects are the result of adaptations in cholinergic neurons or in downstream circuits. Such compensatory adaptations could also mask the function of MORs in behavior, meaning that, despite observing negative effects, we also cannot completely rule out a role for cholinergic MORs in EtOH drinking. Another point of interest is that CIN MOR expression fluctuates significantly throughout the day (Jabourian et al., 2005), which may be responsible for diurnal rhythms in CIN regulation of dopamine release and conditioned responses to reward-paired cues (Stowe et al., 2022). The role of cholinergic MORs in EtOH and nicotine drinking behaviors may consequently be time dependent and not fully captured by the methods used here. Finally, we observed behavioral differences in the Cre- and Cre+ mice that may have confounded some of the results. Cre+ mice tended to consume more fluids than controls and had lower bodyweight in the intermittent access experiment. Although we have not yet tested locomotor activity in these animals, anecdotal evidence suggests that the cholinergic MOR deletion may have induced hyperactivity, possibly explaining the increases in fluid consumption and lower bodyweights. It will be important to perform additional characterization of ChAT-Cre/Oprm1$^{fl/fl}$ line to address these issues.

5. Conclusions

Deletion of *Oprm1* on cholinergic neurons did not alter EtOH consumption or preference using two models of alcohol intake but nicotine preference was reduced in mice with the deletion. Quinine-resistant EtOH consumption was greater in male mice following cholinergic MOR deletion. These results suggest that cholinergic MORs are not required for consumption of alcohol but may influence the tendency to drink despite negative consequences.

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Figure Legends

Figure 1. Knockout of mu opioid receptors on cholinergic neurons. A) Heterozygous ChAT-Cre males (B6.129S-Chattm1(cre)Lowl/MwarJ, strain #031661) were bred with homozygous *Oprm1* floxed females (B6;129-Oprm1tm1.1Cgrf/KffJ, strain #030074) to produce a Cre-mediated deletion of exons 2-3 of the MOR gene (*Oprm1*) on choline acetyltransferase (ChAT)-expressing neurons. ChAT-Cre/Oprm1$^{fl/fl}$ Cre- offspring had intact mu opioid receptors (MOR) but Cre+ offspring were missing MORs on ChAT-expressing neurons. **B)** Fluorescent *in situ* hybridization confirmed *Oprm1* deletion in *Chat* neurons. Green = *Chat*, red = *Oprm1*, blue = DAPI nuclear stain.

Figure 2. Cholinergic mu opioid receptor deletion does not affect ethanol consumption but does impact aversion-resistant drinking. A) Mice reliably drank 15% EtOH across sessions and females consumed more than males. MOR deletion did not lead to differences in 15% ethanol consumption or **B)** preference. **C)** Females drank more 15% ethanol with quinine than males and consumption decreased with increasing concentrations of quinine. The effects of quinine were dependent on sex, genotype, and quinine concentration (three-way interaction). # $p < 0.05$, ## $p < 0.01$ vs. 0-µm session (Dunnett's test). **D)** Cholinergic MOR deletion did not affect preference during the quinine sessions. **E)** Cre+ males consumed more quinine adulterated 15% EtOH than Cre- males at the 100-µM concentration (calculated as a % of consumption on the 0-µM session). ## $p < 0.01$ (Holm-Sidak test). **F)** Genotype did not affect consumption at the 250-µM concentration. **G)** Cre+ male mice consumed more quinine adulterated 15% EtOH at the 500-µM concentration than Cre- males. There were no differences in the female mice at any concentration. # $p < 0.05$ (Holm-Sidak test). * $p < 0.05$, ** $p < 0.01$ main effect (3-way or 2-way ANOVA).

Figure 3. Cholinergic mu opioid receptor deletion does not affect quinine sensitivity. A) Consumption and **B)** preference for quinine-adulterated water decreased with increasing concentrations of quinine. * $p < 0.05$, ** $p < 0.01$ main effect (3-way or 2-way ANOVA).

Figure 4. Cholinergic mu opioid receptor deletion decreased preference for nicotine during 24 hour intermittent access. Deletion of MOR did not impact **A)** 20% EtOH consumption, **B)** nicotine consumption, **C)** or 20% EtOH + nicotine consumption. **D)** Genotype did not impact 20% EtOH preference, **E)** decreased preference for nicotine, and **F)** did not affect 20% EtOH + nicotine preference. * $p < 0.05$ main effect (2-way ANOVA).

Figure 5. Cholinergic neuron mu opioid receptor deletion increases fluid consumption. A) MOR deletion significantly increased total fluid consumption during EtOH sessions but not during **B)** nicotine or **C)** combined EtOH + nicotine sessions. **D)** There was a trend toward increased H_2O in Cre+ mice across drinking solutions ($p = 0.070$). **E)** Cre+ mice weighed significantly less than Cre- controls on the first day of the experiment. * $p < 0.05$, ** $p < 0.01$ main effect (2-way ANOVA). ## $p < 0.01$ (unpaired t-test).

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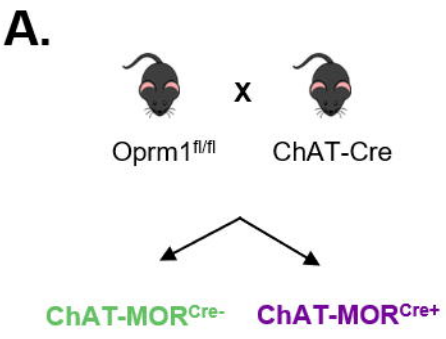
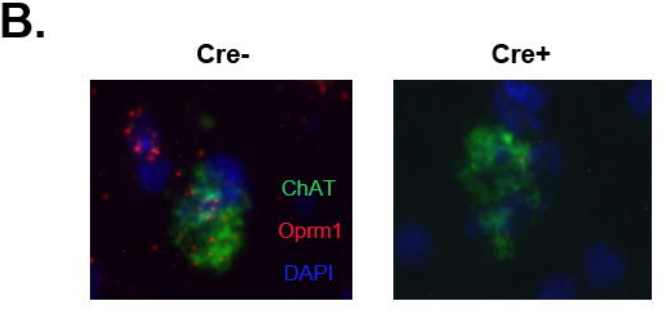
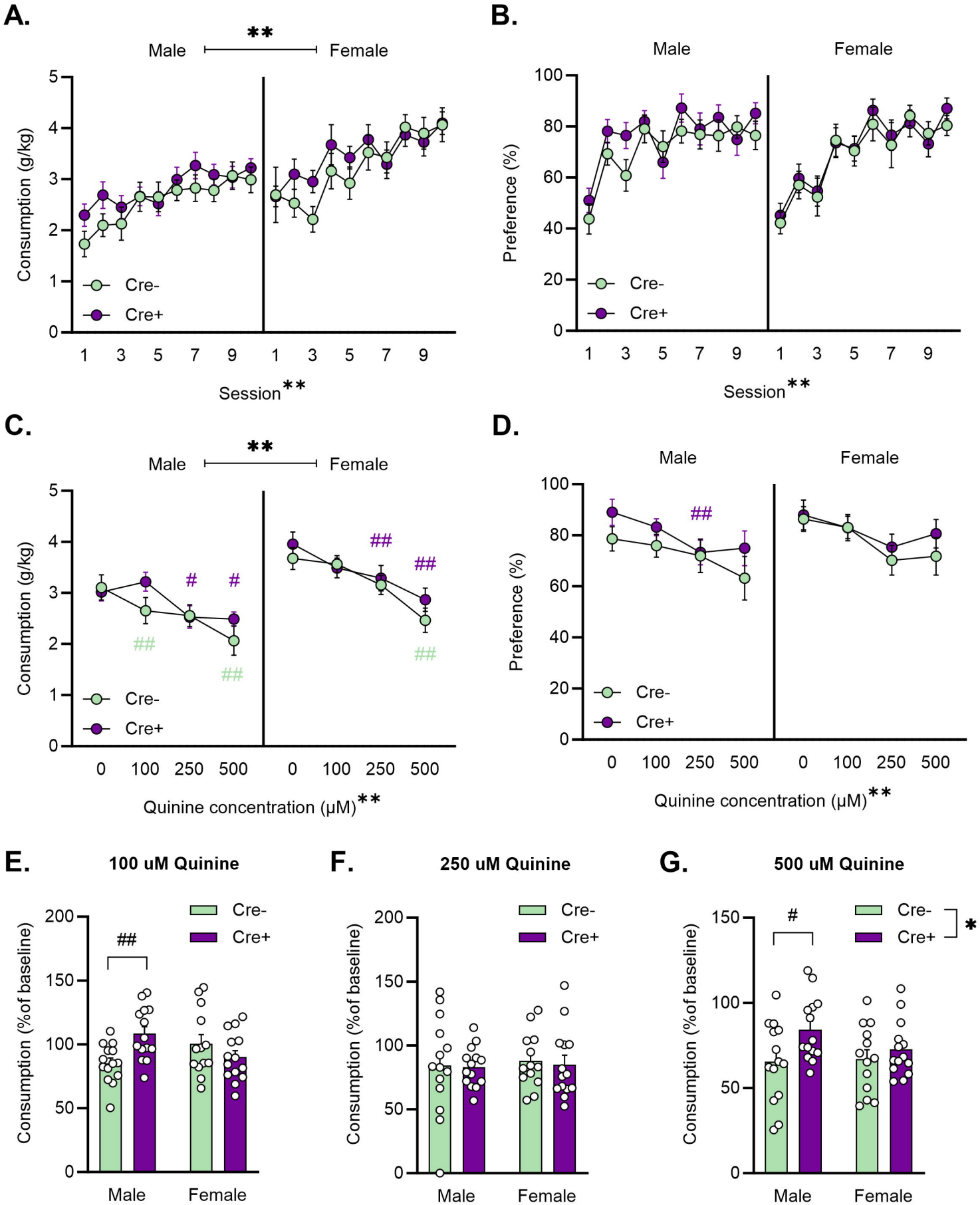
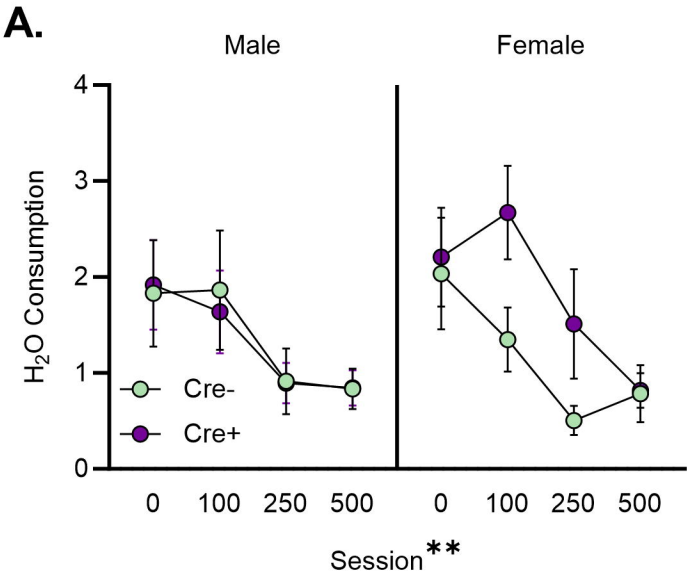
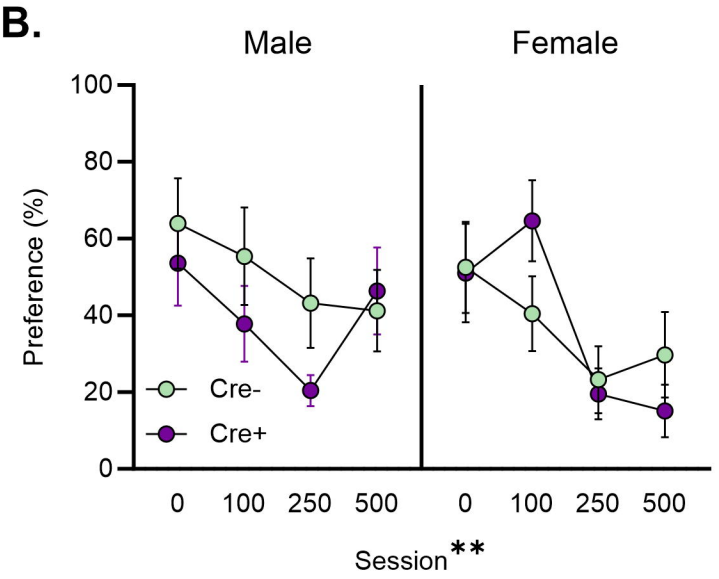
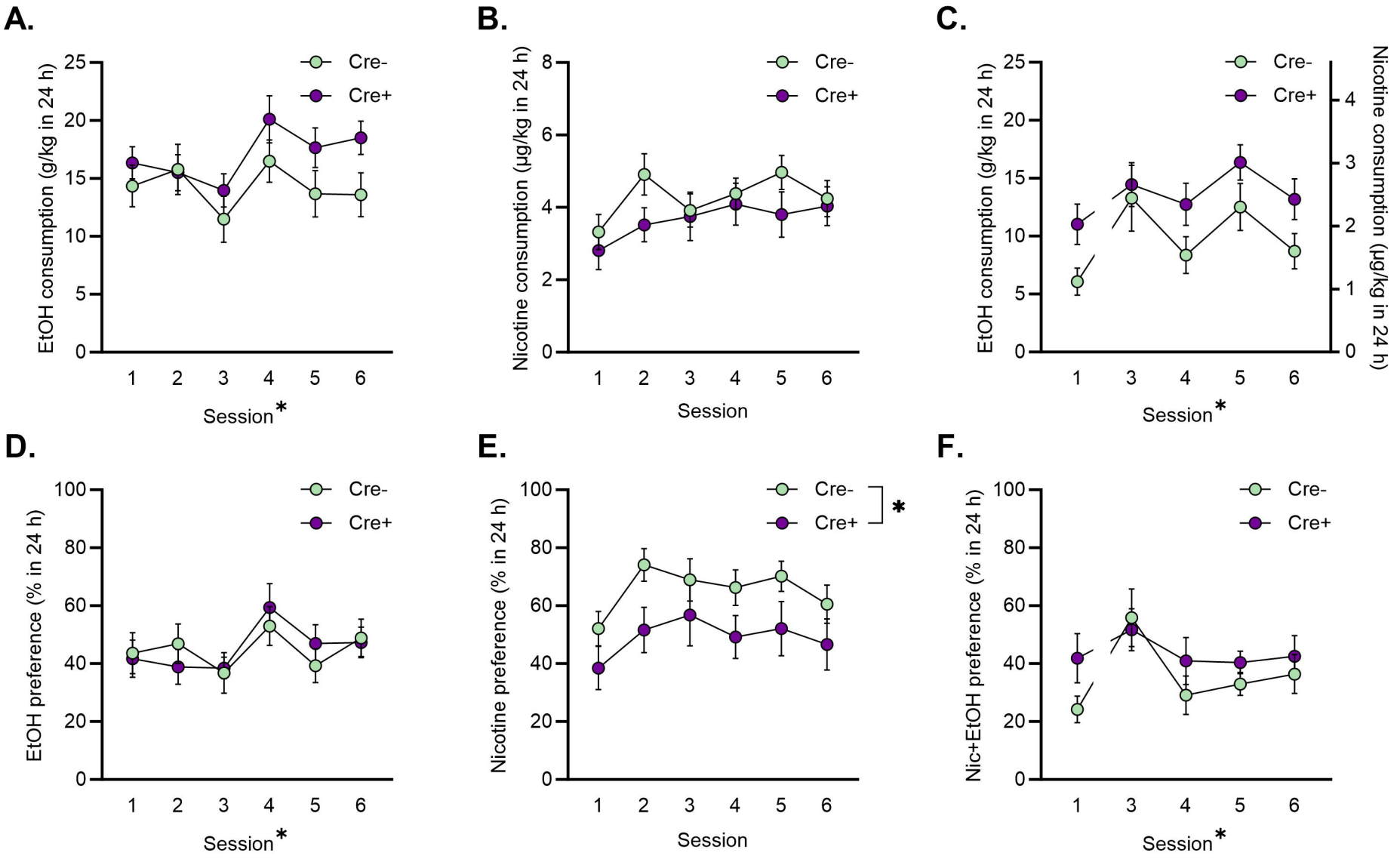

A.
x
Oprm1fl/fl
ChAT-Cre
ChAT-MORCre-
ChAT-MORCre+

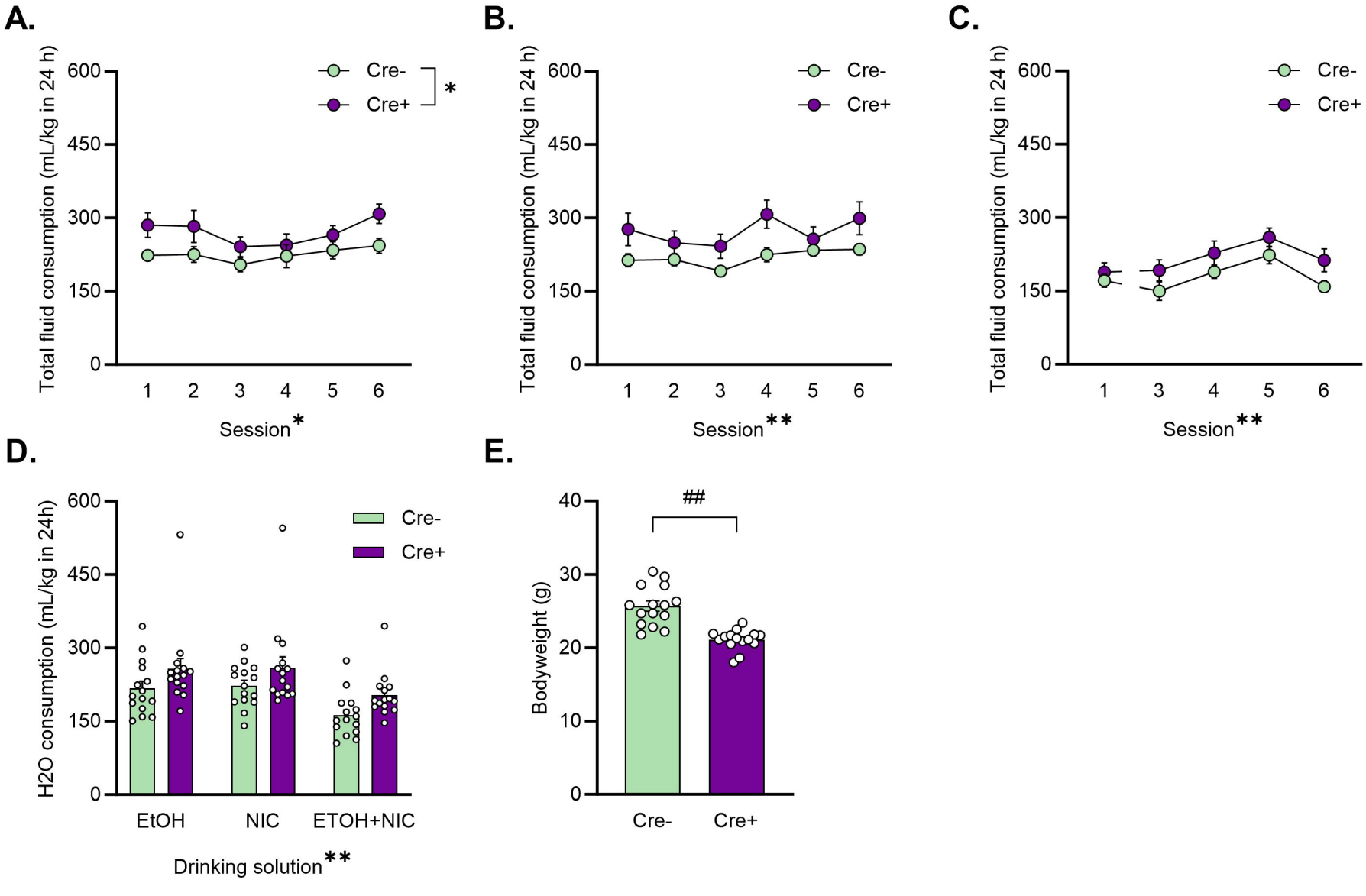

B.
Cre-
Cre+
ChAT
Oprm1
DAPI


A.
**
Male
Female
Consumption (g/kg)
Cre-
Cre+
Session**
B.
Male
Female
Preference (%)
Cre-
Cre+
Session**
C.
**
Male
Female
Consumption (g/kg)
#
#
##
##
##
##
Cre-
Cre+
Quinine concentration (μM)**
D.
Male
Female
Preference (%)
##
Cre-
Cre+
Quinine concentration (μM)**
E.
100 uM Quinine
Consumption (%of baseline)
##
Cre-
Cre+
Male
Female
F.
250 uM Quinine
Consumption (%of baseline)
Cre-
Cre+
Male
Female
G.
500 uM Quinine
Consumption (%of baseline)
#
Cre-
Cre+
*
Male
Female


A.
Male
Female
H_2O Consumption
4
3
2
1
0
Cre-
Cre+
0
100
250
500
0
100
250
500
Session**


B.
Male
Female
Preference (%)
100
80
60
40
20
0
Cre-
Cre+
0
100
250
500
0
100
250
500
Session**


A.
EtOH consumption (g/kg in 24 h)
Cre-
Cre+
Session*
B.
Nicotine consumption (µg/kg in 24 h)
Cre-
Cre+
Session
C.
EtOH consumption (g/kg in 24 h)
Nicotine consumption (µg/kg in 24 h)
Cre-
Cre+
Session*
D.
EtOH preference (% in 24 h)
Cre-
Cre+
Session*
E.
Nicotine preference (% in 24 h)
Cre-
Cre+
*
Session
F.
Nic+EtOH preference (% in 24 h)
Cre-
Cre+
Session*


A.
Total fluid consumption (mL/kg in 24 h)
600
450
300
150
0
Cre-
Cre+
*
1 2 3 4 5 6
Session*
B.
Total fluid consumption (mL/kg in 24 h)
Cre-
Cre+
Session**
C.
Total fluid consumption (mL/kg in 24 h)
Cre-
Cre+
1 3 4 5 6
Session**
D.
H2O consumption (mL/kg in 24h)
Cre-
Cre+
EtOH
NIC
ETOH+NIC
Drinking solution**
E.
Bodyweight (g)
40
30
20
10
0
##
Cre-
Cre+