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TITLE: Melanocortin and Opioid Peptide Interactions in the Modulation of Binge Alcohol Drinking

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| 14. ABSTRACT Frequent binge drinking is associated with numerous negative short- and long-term consequences, including an increased risk of accidental injury, violent behavior, depression, heart disease, and type 2 diabetes. While illicit drug use and cigarette smoking both decreased significantly in the US military between the period of 1980 to 2002, heavy alcohol use increased. In fact, heavy alcohol use and binge drinking are observed in 27% of the military population. Identifying neurochemical pathways in the brain that modulate binge drinking may provide insight into pharmaceutical treatments that could protect against this dangerous behavior. Recently identified candidates for modulating binge drinking are the melanocortin (MC) peptides, such as α -melanocyte stimulating hormone (α -MSH), and the opioid peptide β -endorphin which are produced in the same brain neurons. The specific aims proposed below will test the guiding hypothesis that stimulation of MC receptor and blockade of opioid receptor protect against excessive binge-like alcohol drinking and intoxicating blood alcohol levels (BALs) in an animal model of binge drinking. The aims will also determine if MC receptor (MCR) agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in a synergistic manner. | | | | | |
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INTRODUCTION: Frequent binge drinking is associated with numerous negative short- and long-term consequences, including an increased risk of accidental injury, violent behavior, depression, heart disease, and type 2 diabetes. While illicit drug use and cigarette smoking both decreased significantly in the US military between the period of 1980 to 2002, heavy alcohol use increased. In fact, heavy alcohol use and binge drinking are observed in 27% of the military population. Given that the rate of binge drinking by the civilian population is only 15%, individuals in the military are at an increased risk of regular binge drinking and thus all the health risks that are associated with this disorder. Identifying neurochemical pathways in the brain that modulate binge drinking may provide insight into pharmaceutical treatments that could protect against this dangerous behavior. Recently identified candidates for modulating binge drinking are the melanocortin (MC) peptides, such as α -melanocyte stimulating hormone (α -MSH), and the opioid peptide β -endorphin which are both cleaved from the polypeptide precursor proopiomelanocortin (POMC). The specific aims of this grant will test the guiding hypothesis that MC receptor signaling protects against excessive binge-like alcohol drinking and intoxicating blood ethanol concentrations (BECs) in an animal model of binge drinking. The aims will also determine if MC receptor (MCR) agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in a supraadditive (synergistic) manner. **Specific Aims:** **Specific Aim 1** will test the hypothesis that binge-like alcohol drinking will be associated with a significant reduction of α -MSH levels in candidate brain regions of C57BL/6J mice, and if this reduction of α -MSH will become more robust following repeated binge episodes. This aim will also determine if repeated binge episodes promote increases of binge-like drinking in mice. **Specific Aim 2** will test the hypothesis that central infusion of MCR agonist will protect against, and MCR antagonist will augment, binge-like alcohol drinking in C57BL/6J mice via the MC-4 receptor (MC4R). Mutant mice lacking MC-3 receptor (MC3R) or MC4R will be used to determine the receptor(s) that are involved. **Specific Aim 3** will test the hypothesis MCR agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in a supraadditive manner.

BODY: Experiments during the last funding cycle began work associated with Tasks 3-5, which involved assessing the effects of MCR agonist and antagonist on binge-like ethanol drinking in mutant mice lacking either the MC3R or MC4R. We also completed Tasks 7 and 8, which determine if MCR agonist MTII and opioid receptor antagonist naltrexone (Nal), when administered together, interact synergistically in block binge-like ethanol drinking in mice. During the final year of this project, we will complete Tasks 3-5, as well as Task 6 (Tasks 1,2, 7, and 8 have been completed).

Task 3: Will determine if the MC3R modulates the effects of MCR agonist on binge-like ethanol drinking in C57BL/6J mice. To address this question, we assessed binge-like ethanol drinking in mutant mice lacking the MC3R (MC3R^{-/-}) and littermate wild-type mice (MC3R^{+/+}) that were maintained on a C57BL/6J genetic background. Mice were first implanted with brain cannulae that were aimed at the left lateral ventricle for intracerebroventricular (i.c.v.) infusion of compounds. After mice recovered from surgery, they were given access to ethanol using the “drinking in the dark” (DID) procedure in which 20% ethanol was given in place of water beginning 3 hours into the dark cycle (mice were maintained on a 12 hour light, 12 hour dark schedule). On days 1-3 of this procedure, ethanol was left on the cage for 2 hours. On day 4 (the critical test day), mice were given i.c.v. infusion (in a 0.5 μ l volume) of 0.9% saline or a 0.25 or 0.5 μ g dose of the MCR agonist, melanotan II (MTII; n = 6 to 10 per group) just before ethanol access. Data from this initial experiment are presented in **Figure 1** below. Relative to the saline vehicle treatment, neither dose of MTII significantly altered binge-like ethanol drinking.

This was surprising since we have previously found that these doses of MTII significantly blunt voluntary ethanol drinking and binge-like ethanol drinking in MC4R+/+ mice (see below). We are in the process of testing higher doses of MTII in these mice.

Task 4: Will determine if the MC3R modulates the effects of MCR antagonist on binge-like ethanol drinking in C57BL/6J mice. MC3R^{-/-} and MC3R^{+/+} mice were cannulated for testing with DID procedures as described above. On the test day, mice were given i.c.v. infusion of vehicle or a 0.05 or 0.1 µg dose of the MCR antagonist, agouti-related protein (AgRP; n = 3 to 5/group). Data from this experiment are presented in **Figure 2**. Relative to the vehicle control infusion, the 0.05 dose of AgRP significantly increased ethanol drinking in both MC3R^{-/-} and MC3R^{+/+} mice. Since AgRP increased ethanol drinking normally in MC3R^{-/-} mice, these data suggest that AgRP-induced increases of ethanol drinking do not require the MC3R. We continue to work on this study to increase sample sizes as mice become available from our breeding colony.

Task 5: Will determine if the MC4R modulates the effects of MCR agonist on binge-like ethanol drinking in C57BL/6J mice. MC4R^{-/-} and MC4R^{+/+} mice were cannulated for testing with DID procedures as described above. On the test day, mice were given infusion of vehicle or a 0.25 or 0.5 µg dose of MTII just before ethanol access (n = 3 to 12 per group). Data from this experiment are presented in **Figure 3**. Relative to the vehicle infusion, the 0.25 µg dose of MTII significantly blunted binge-like ethanol drinking, but only in MC4R^{+/+} mice. These observations suggest that MTII-induced blunting of binge-like ethanol drinking requires normal MC4R expression, since this drug was ineffective in MC4R^{-/-} mice. We continue to work on this study to increase sample sizes as mice become available from our breeding colony.

Task 7: Will determine if MCR agonist and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in an additive, supraadditive (synergistic), or infraadditive interaction. The central polypeptide precursor POMC gives rise to β-endorphin, an endogenous opioid peptide, and the melanocortin (MC) peptides including α-MSH. Opioid receptor antagonists, such as naltrexone (NAL), have been demonstrated to reduce ethanol consumption in rodents, and a growing body of evidence indicates that MC receptor agonists blunt ethanol intake. Interestingly, central opioid and MC pathways have been demonstrated to interact in their modulation of nociception and feeding behavior. Since opioids and MC peptides modulate ethanol consumption, the goal of the present work was to determine if compounds aimed at opioid or MC receptors, when presented in combination, interact additively or synergistically in the modulation of binge-like ethanol drinking in C57BL/6J mice. We used DID procedures and first established dose-response effects of intraperitoneally (i.p.) injected NAL or the MC agonist MTII (0, 0.3, 3.0, and 10 mg/kg for each drug) on binge-like drinking (data were presented in the previous progress report). Based on these data, we established the ED₂₀ and ED₃₀ for each drug, and then combined the low (ED₂₀) and high (ED₃₀) dose of each drug with the dose-response range of the other drug. Results showed that MTII was 3.4-fold more potent than NAL in blunting binge-like ethanol drinking (based on ED₅₀ values). MTII was also more effective, as the 10 mg/kg dose of MTII produced a 72% reduction of binge-like ethanol drinking while this same dose of NAL reduced drinking by only 49%. When administered in combination, the low ED₂₀ (but not the ED₃₀) dose of MTII (0.26 mg/kg) shifted the NAL dose-response curve to the left by a factor of 7 (i.e., NAL was 7-fold more potent when administered in combination with MTII relative to when it was administered alone; see **Figure 4**). Subsequent isobolographic analyses (n = 10 to 12 group) of these data showed that MTII synergistically augmented the ability of NAL to blunt binge-like ethanol drinking (**Figure 5**). In this figure, the perpendicular line intersecting the naltrexone ED₅₀ and the MTII ED₅₀ represents

the theoretical line of additivity. Vertical lines represent the 95% confidence level (C.L.) of the ED₅₀ values for either NAL or MTII: when the ED₅₀ was to the left of the theoretical line of additivity and the C.L. lines did not overlap the area encompassed by the dotted lines, the interaction was considered to be supra-additive, whereas when the C.L. lines did overlap with that area, the interaction was considered additive. As seen in Figure 5, the 0.26 mg/kg dose of MTII significantly shifted the NAL ED₅₀ to the left (beyond the C.L. region), indicating that MTII synergistically potentiated the ability of NAL to blunt binge-like ethanol drinking. These observations suggest that MC receptor agonists may improve the therapeutic effectiveness of NAL in the treatment of alcohol abuse disorders when these drugs are given in combination.

Task 8: Will determine if combining maximally effective doses of MTII and naltrexone will produce effects greater than those observed for either drug alone. For this experiment, mice were tested with DID procedures as described above, and first given i.p. injection of MTII or Nal alone. The left panel of **Figure 6** shows selected doses of MTII and Nal presented alone, and the right panel of **Figure 6** shows data when MTII and Nal were presented in combination. The analysis revealed that the combination of 3.0 mg/kg Nal and 3.0 mg/kg MTII produced a 75% decrease in ethanol consumption, which was only slightly larger than the 62% decrease associated the 3.0 mg/kg dose of MTII alone. Similar effects were obtained with the other combinations tested, suggesting that the maximal effects produced by the combination of high doses of naltrexone and MTII were not greater than those produced by the highest dose (10 mg/kg) of these drugs when administered alone

KEY RESEARCH ACCOMPLISHMENTS: Key research accomplishments achieved during the 3rd budget year of this grant are as follows:

- Established that the ability of the MCR agonist, MTII, to blunt binge-like ethanol drinking requires normal expression of MC4R in the brain. Thus, MCR agonist protect against binge-like ethanol drinking by signaling through the MC4R.
- Established the MCR agonist may not be required for normal MC3R to modulate ethanol drinking, though this work continues to be in progress.
- Established that MCR antagonist-induced increases of ethanol drinking do not require normal expression of the MC3R in the brain.
- Established that a low dose of the MCR agonist MTII synergistically increased the ability of naltrexone to blunt binge-like ethanol drinking in mice. Thus, the inclusion of MCR agonists may improve the effectiveness of naltrexone-based therapies in the treatment of alcohol abuse disorders.
- Established that combining the maximally effective doses of MTII and naltrexone did not produced effects on binge-like ethanol drinking greater than those observed for either drug alone.

REPORTABLE OUTCOMES: The following is a list of publications and a published abstract that have been supported by this grant during the 3rd budget year:

PUBLICATONS

1. Navarro, M., Lerma-Cabrera, J. M., Carvajal, F., Lowery, E. G., Cubero, I., & Thiele, T. E. (2011). Assessment of voluntary ethanol consumption and the effects of a melanocortin (MC) receptor agonist on ethanol intake in mutant C57BL/6J mice lacking the MC-4 receptor. *Alcoholism: Clinical & Experimental Research*, 35, 1058-1066.

CONFERENCE PRESENTATION

1. G.M. Sprow; E.G. Lowery; A.M. Lyons; M. Navarro; T.E. Thiele (2011). Repeated Binge-Like Ethanol Intake in C57BL/6J Mice Leads to Decreased α -MSH Immunoreactivity (IR) and Increased AgRP IR in Key Brain Regions *Alcoholism: Clinical & Experimental Research*, 35, 246A.
2. M. Navarro; M. J. Picker; T. E. Thiele (in press). A Low Dose of the Melanocortin Agonist MTII Synergistically Augments Naltrexone-Induced Attenuation of Binge-like Ethanol Drinking in C57BL/6J Mice. To be presented at the 2012 annual meeting of the Research Society on Alcoholism.

CONCLUSIONS: We have made significant progress towards the goals of this research proposal during the third year of funding. Consistent with our hypothesis, we have found that the MCR agonist MTII blunted binge-like ethanol drinking by signaling through the MC4R. In the final year of this project we will continue to work with MC3R and MC4R knockout mice to determine if the MCR antagonist increases binge-like ethanol drinking by signaling through the MC4R. We have established that the opioid receptor antagonist naltrexone and MCR agonist MTII protect against binge-like ethanol drinking in a dose-dependent manner. Importantly, consistent with our hypothesis, we have found that the MCR agonist MTII synergistically augments the ability of the opioid receptor antagonist naltrexone to protect against binge-like drinking in mice. Since naltrexone is currently an FDA approved treatment for alcohol abuse disorders, our results suggest that combining a MCR agonist with naltrexone may be a way to significantly improve an existing pharmacotherapy. So what does this mean? These results have important implications for possible pharmacological medical treatment of binge drinking in the human population. Specifically, melanocortin receptor agonists aimed at the MC4R, as well a opioid receptor antagonist, may prevent binge drinking in at-risk individuals, and thus protect these people from the negative behavioral and biological consequences of regular binge drinking. Importantly, preventing frequent binge drinking will reduce the risk of future alcohol abuse disorders and dependence. These findings may be considered of high relevance to the U.S. military given the high prevalence of binge drinking in the military population.

APPENDICES:

- Figures 1-6. In figures, * indicates significant differences from the control group condition at the $p < 0.05$ level.
- 1 published paper that was supported by this grant. This paper was in press during the last progress report and is now in print.
- 1 published abstract of work supported by this grant that was presented at the 2011 annual meeting of the Research Society on Alcoholism.
- 1 in press abstract of work supported by this grant that will be presented at the 2012 annual meeting of the Research Society on Alcoholism.

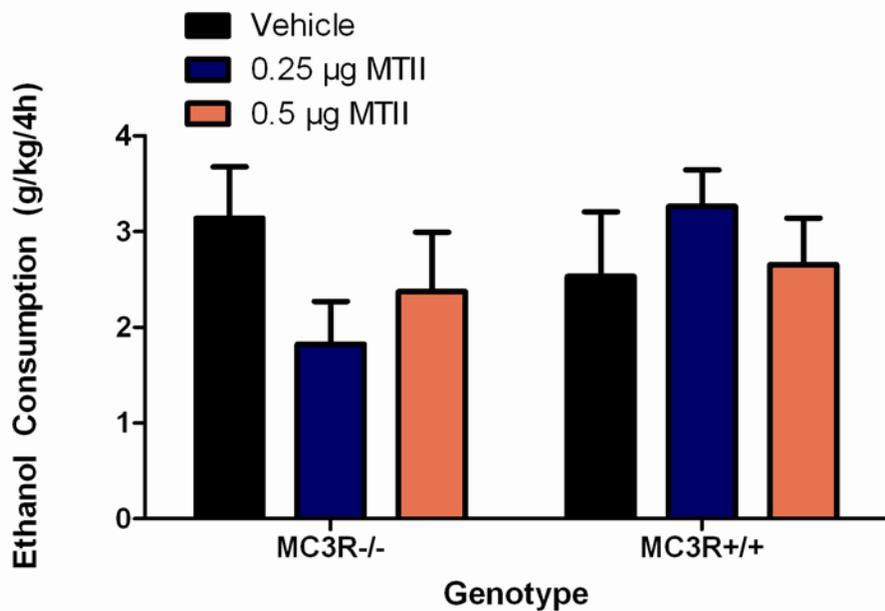


Figure 1: Binge-like ethanol consumption in mutant mice lacking the MC3R (MC3R^{-/-}) or wild-type mice (MC3R^{+/+}) following i.c.v. infusion of vehicle or the non-selective MCR agonist MTII. Neither dose of MTII significantly reduced binge-like ethanol drinking in mice regardless of genotype. Data are presented as mean \pm SEM.

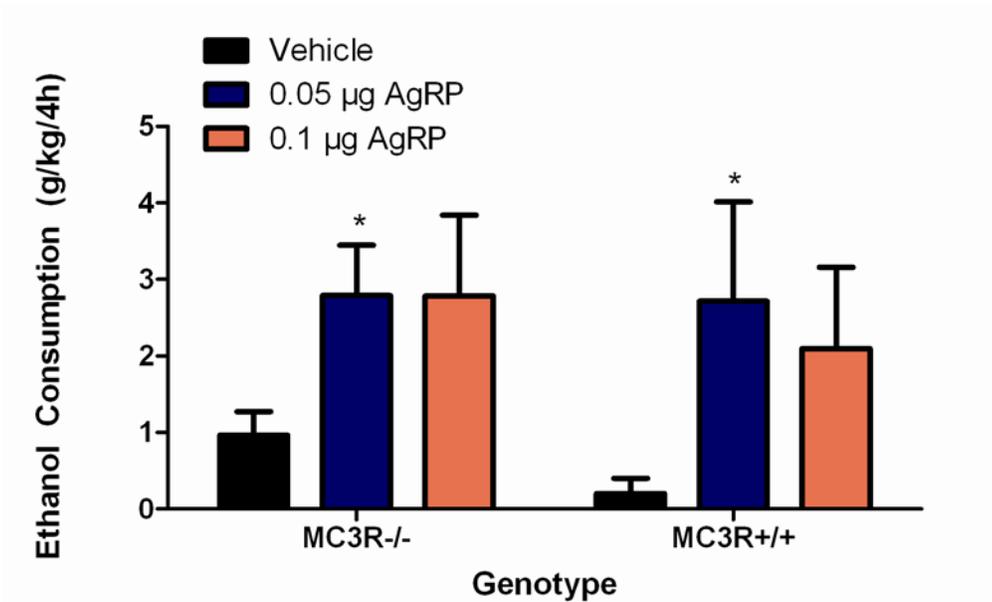


Figure 2 : Binge-like ethanol consumption in mutant mice lacking the MC3R (MC3R^{-/-}) or wild-type mice (MC3R^{+/+}) following i.c.v. infusion of vehicle or the non-selective MCR antagonist AgRP. Administration of AgRP significantly increased binge-like ethanol drinking in both genotypes, suggesting that AgRP-induced increases of binge-like ethanol drinking does not require the MC3R. Data are presented as mean \pm SEM. * $p < 0.05$ relative to vehicle.

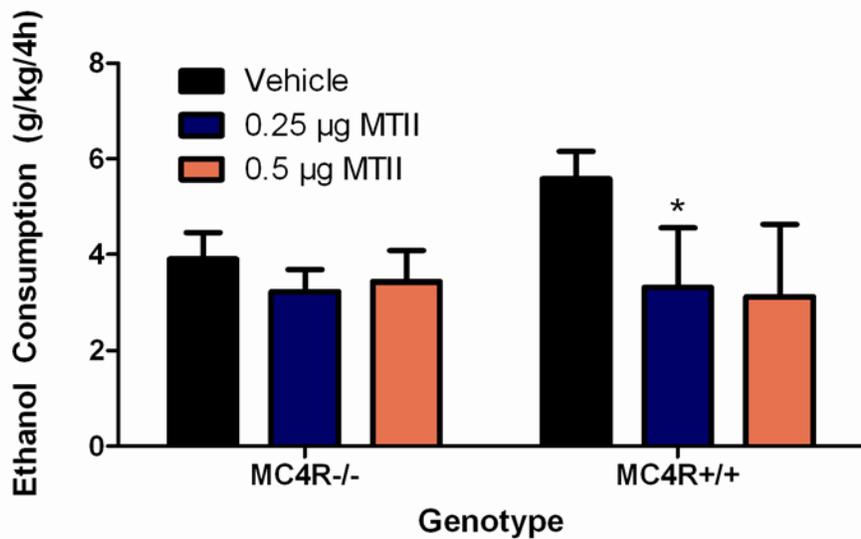


Figure 3: Binge-like ethanol consumption in mutant mice lacking the MC4R (MC4R^{-/-}) or wild-type mice (MC4R^{+/+}) following i.c.v. infusion of vehicle or the non-selective MCR agonist MTII. MTII significantly attenuated binge-like ethanol drinking in wild-type MC4R^{+/+} mice but failed to alter ethanol drinking in MC4R^{-/-} mice, indicating that MTII-induced blunting of binge-like ethanol drinking requires the MC4R. Data are presented as mean \pm SEM. * $p < 0.05$ relative to vehicle.

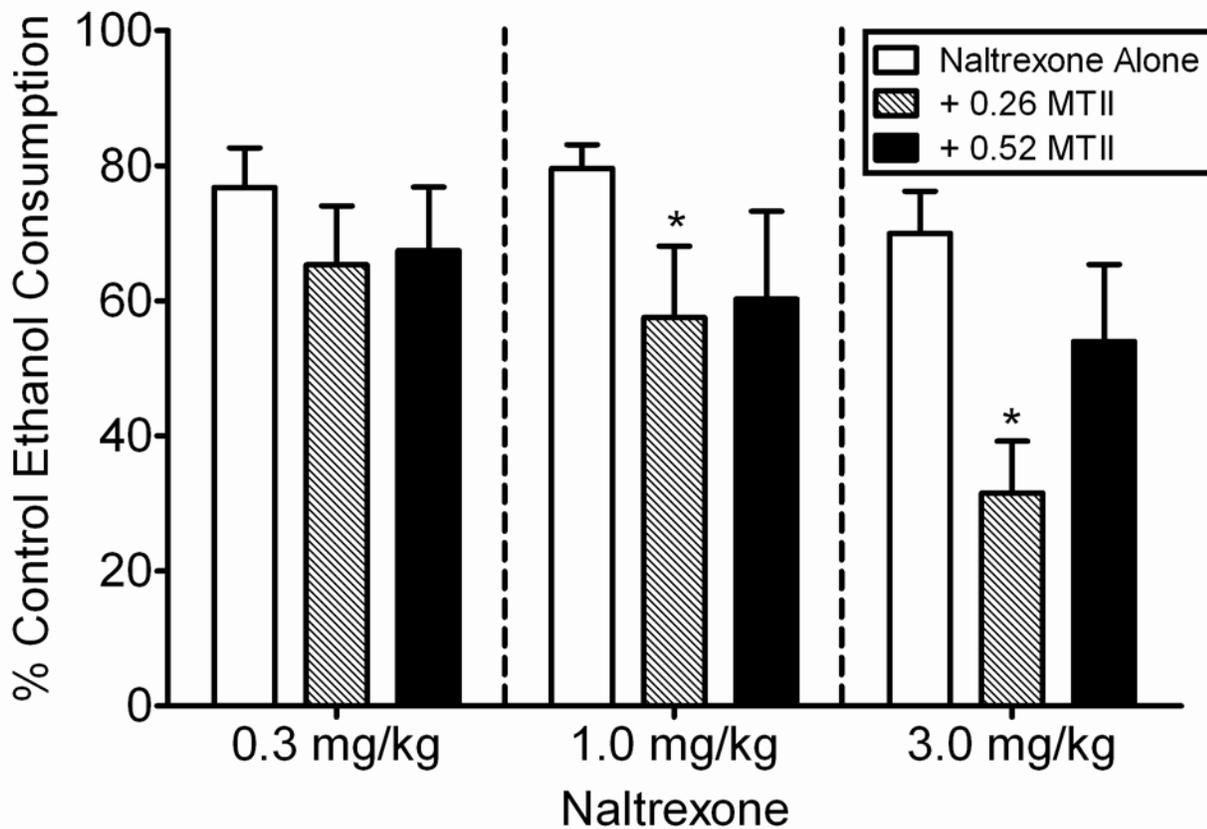


Figure 4: Effects of naltrexone alone and in combination with the approximate ED₂₀ (0.26 mg/kg) and ED₃₀ (0.52 mg/kg) doses of MTII. These doses of MTII were based on a preliminary assessment of its relative potency. Doses of naltrexone were tested in 9-14 mice and the combination with MTII in 8-11 mice. Ordinate: % ethanol consumption expressed as % mean levels of ethanol consumption obtained during the 3 baseline days prior to testing. Abscissa: dose of naltrexone alone or in combination with MTII expressed in mg/kg. Vertical bars represent the standard error of the mean. * p < 0.05 relative to naltrexone alone.

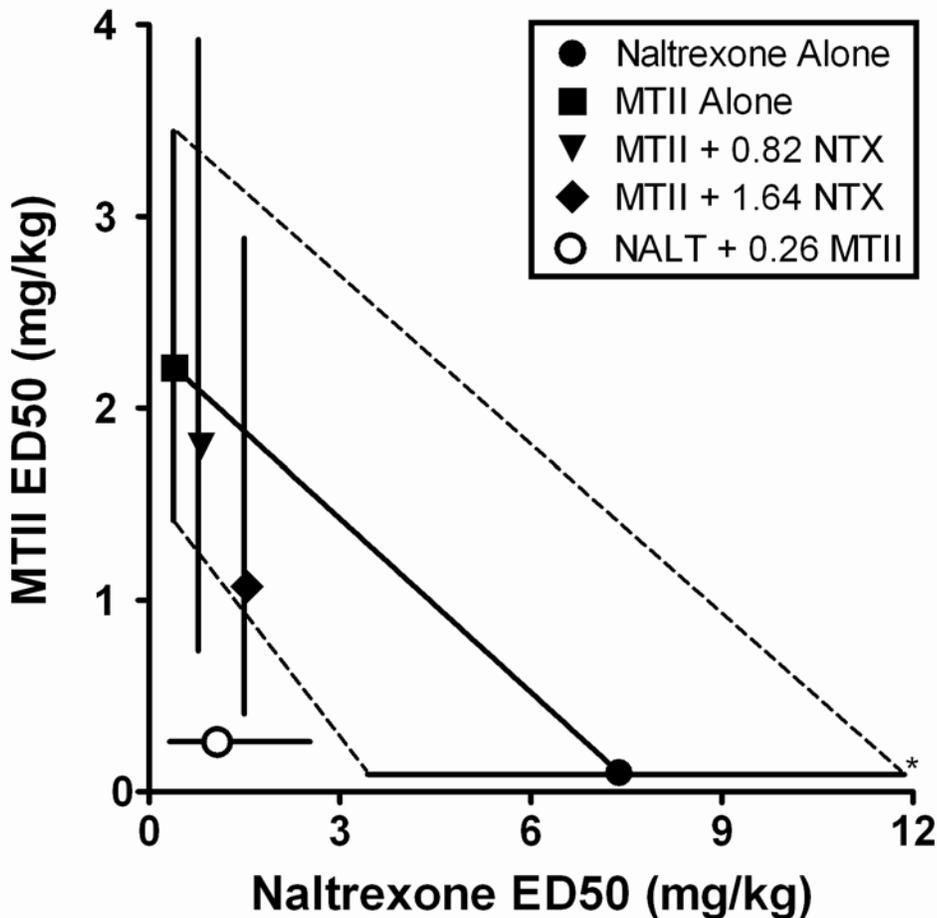


Figure 5: Isobolograms for naltrexone and MTII in combination on ethanol consumption. Abscissa: ED₅₀ value (95% C.L.) for naltrexone expressed in mg/kg. Ordinate: ED₅₀ value (95% C.L.) for MTII expressed in mg/kg. The perpendicular line intersecting the naltrexone ED₅₀ and the MTII ED₅₀ represent the theoretical line of additivity. Vertical lines represent the 95% C.L. of the ED₅₀ values for either naltrexone or MTII. For drug combinations, when the ED₅₀ was to the left of the theoretical line of additivity and the C.L. lines did not overlap the area encompassed by the dotted lines, the interaction was considered to be supra-additive, whereas when the C.L. lines did overlap with that area, the interaction was considered additive. Thus, the 0.26 mg/kg dose of MTII shifted the ED₅₀ of naltrexone to the left to a point considered to be supra-additive (synergistic).

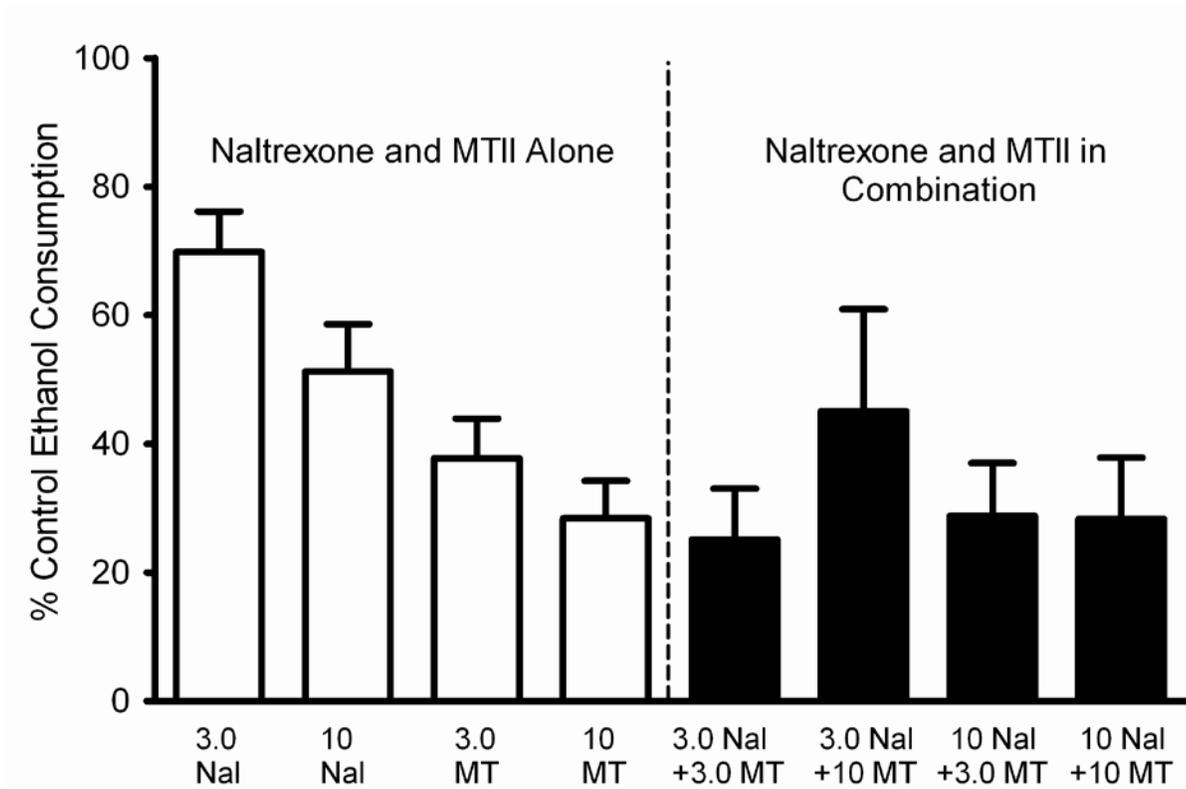


Figure 6: Effects of selected doses of naltrexone and MTII administered alone (left) and in selected combinations (right) on ethanol consumption. The two doses (3.0 and 10 mg/kg) of naltrexone and MTII selected for analysis produced the largest decreases in ethanol consumption when administered alone. When administered alone, doses of naltrexone and MTII were tested in 9-14 mice, whereas the combination data are based on 7-10 mice. Ordinate: % ethanol consumption expressed as % mean levels of ethanol consumption obtained during the 3 baseline days prior to testing. Abscissa: doses of naltrexone and MTII alone and in combination expressed in mg/kg. Vertical bars represent the standard error of the mean.

Assessment of Voluntary Ethanol Consumption and the Effects of a Melanocortin (MC) Receptor Agonist on Ethanol Intake in Mutant C57BL/6J Mice Lacking the MC-4 Receptor

Montserrat Navarro, Jose M. Lerma-Cabrera, Francisca Carvajal, Emily G. Lowery, Inmaculada Cubero, and Todd E. Thiele

Background: The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Recent evidence shows that chronic exposure to ethanol significantly blunts central MC peptide immunoreactivity and MC receptor (MCR) agonists protect against high ethanol intake characteristic of C57BL/6J mice. Here, we assessed the role of the MC-4 receptor (MC4R) in voluntary ethanol intake and in modulating the effects of the nonselective MCR agonist melanotan-II (MTII) on ethanol consumption.

Methods: To assess the role of the MC4R, MC4R knockout ($Mc4r^{-/-}$) and littermate wild-type ($Mc4r^{+/+}$) mice on a C57BL/6J background were used. Voluntary ethanol (3, 5, 8, 10, 15, and 20%, v/v) and water intake were assessed using standard two-bottle procedures. In separate experiments, $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice were given intracerebroventricular (i.c.v.) infusion of MTII (0, 0.5, or 1.0 $\mu\text{g}/1 \mu\text{l}$) or intraperitoneal (i.p.) injection of MTII (0 or 5 mg/kg/5 ml). The effects of MTII (0 or 0.5 $\mu\text{g}/1 \mu\text{l}$, i.c.v.) on 10% sucrose and 0.15% saccharin intake were assessed in C57BL/6J mice.

Results: $Mc4r^{-/-}$ mice showed normal consumption of ethanol over all concentrations tested. I.c.v. infusion of MTII significantly reduced ethanol drinking in $Mc4r^{+/+}$ mice, but failed to influence ethanol intake in $Mc4r^{-/-}$ mice. When administered in an i.p. injection, MTII significantly reduced ethanol drinking in both $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice. MTII attenuated consumption of caloric (ethanol, sucrose, and food) and noncaloric (saccharin) reinforcers.

Conclusions: When given centrally, the MCR agonist MTII reduced ethanol drinking by signaling through the MC4R. On the other hand, MTII-induced reduction of ethanol drinking did not require the MC4R when administered peripherally. Together, the present observations show that the MC4R is necessary for the central actions of MCR agonists on ethanol drinking and that MTII blunts the consumption natural reinforcers, regardless of caloric content, in addition to ethanol.

Key Words: Ethanol Consumption, Melanocortin, MC-3 Receptor, MC-4 Receptor, C57BL/6J, Food Intake.

THE MELANOCORTIN (MC) SYSTEM is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Central MC peptides are produced by neurons within the hypothalamic arcuate nucleus, the nucleus of the solitary tract, and the medulla (Crine et al., 1978; Dores et al., 1986; Hadley and Haskell-Luevano, 1999; Jacobowitz and O'Donohue, 1978;

O'Donohue and Dorsa, 1982) and include adrenocorticotrophic hormone, α -melanocyte-stimulating hormone (α -MSH), β -MSH, and γ -MSH (Hadley and Haskell-Luevano, 1999). Because of a lack of critical dibasic site, β -MSH is not processed in rodent brain (Pritchard et al., 2002). MC neuropeptides act through at least five receptor subtypes, namely MC-1 receptor (MC1R), MC2R, MC3R, MC4R, and MC5R, all of which couple to heterotrimeric Gs-proteins that stimulate adenylyl cyclase activity (Hadley and Haskell-Luevano, 1999). MC receptors (MCRs) in the rodent brain are primarily comprised of the MC3R and MC4R subtypes (Adan and Gispen, 1997), whereas MC1R and MC5R are detected at low levels and only in limited brain regions while the MC2R is expressed primarily in the adrenal cortex (Adan and Gispen, 1997; Barrett et al., 1994; Xia et al., 1995).

It is well established that MCR signaling is involved in the regulation of appetite and energy homeostasis (Gao and Horvath, 2008). A growing literature suggests that there is

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overlapping peptide control of ethanol consumption and feeding behavior (Thiele et al., 2003, 2004), which includes recent evidence that MCR signaling modulates neurobiological responses to ethanol. MCR and α -MSH expression have been identified in brain regions that modulate the reinforcing properties of ethanol, including the nucleus accumbens (NAc), ventral tegmental area, the bed nucleus of the stria terminalis, and amygdala (Bloch et al., 1979; Dube et al., 1978; Jacobowitz and O'Donohue, 1978; O'Donohue and Jacobowitz, 1980; O'Donohue et al., 1979; Yamazoe et al., 1984). Genetic evidence and pharmacological evidence implicate the MC system in the control of voluntary ethanol consumption. Relative to ANA (Alko, Nonalcohol) rats, AA (Alko, Ethanol) rats, selectively bred for high ethanol intake, have significantly lower levels of MC3R in the shell of the NAc, and significantly higher levels of MC3R in the paraventricular, arcuate, and ventromedial nuclei of the hypothalamus. AA rats also have high levels of MC4R in the ventromedial nucleus of the hypothalamus (Lindblom et al., 2002). These data suggest that the high ethanol drinking by AA rats may be mediated, in part, by alterations in central MCR signaling. Consistent with this hypothesis, intracerebroventricular (i.c.v.) infusion of the potent nonselective MCR agonist melanotan-II (MTII) significantly reduced voluntary ethanol drinking by AA rats (Ploj et al., 2002). Similarly, we have found that i.c.v. infusion of MTII and a selective MC4R agonist reduced ethanol drinking (Navarro et al., 2003, 2005), while ventricular infusion of the nonselective MCR antagonist agouti-related protein (AgRP) significantly increased ethanol drinking (Navarro et al., 2005), by high-ethanol-drinking C57BL/6J mice. Consistent with pharmacological data, genetic deletion of endogenous AgRP reduced ethanol-reinforced lever pressing and binge-like ethanol drinking in C57BL/6J (Navarro et al., 2009). Ethanol also has direct effects of central MC and AgRP activity. Thus, chronic exposure to ethanol significantly reduced α -MSH immunoreactivity in specific regions of the rat brain (Navarro et al., 2008), and acute administration of ethanol significantly increased AgRP immunoreactivity in the arcuate nucleus of the hypothalamus of C57BL/6J mice (Cubero et al., 2010).

The MCRs that modulate neurobiological responses to ethanol remain unclear. With respect to ethanol consumption, we found that MTII was similarly effective at reducing ethanol intake in both MC3R knock-out (Mc3r^{-/-}) and littermate wild-type (Mc3r^{+/+}) mice (Navarro et al., 2005). Furthermore, i.c.v. infusion of the highly selective MC4R agonist, cyclo(NH-CH₂-CH₂-CO-His-D-Phe-Arg-Trp-Glu)-NH₂, dose-dependently reduced ethanol drinking by C57BL/6J mice (Navarro et al., 2005). These data suggest that the MC3R does not modulate MCR agonist-induced reductions of ethanol consumption and that the MC4R is a likely candidate. The first goal of the present report was to directly assess the role of the MC4R. To this end, we examined voluntary ethanol consumption and the effects of centrally and peripherally administered MTII on ethanol intake, in Mc4r^{-/-} and littermate Mc4r^{+/+} mice. The second goal was to further

characterize the effects of MTII on consumption of other caloric (food and sucrose) and noncaloric (saccharin) reinforcers.

MATERIALS AND METHODS

Animals

The generation of Mc4r^{-/-} mice has been described elsewhere (Huszar et al., 1997). The Mc4r^{-/-} mice were originally derived on a mixed 129/SvJ \times C57BL/6J genetic background and show increased body weight and feeding behavior beginning at about 3-4 months of age (Huszar et al., 1997; Ste Marie et al., 2000). For the present work, we backcrossed Mc4r^{-/-} mice to a C57BL/6J genetic background for 8 generations. Despite the lack of the MC4R, Mc4r^{-/-} mice show normal brain expression of MC3R mRNA (Rowland et al., 2010). Littermate knockout and wild-type mice were used, and approximately equal numbers of male and female mice were used in each treatment condition. Because we have previously found no sex differences in the effect of MTII on ethanol consumption in C57BL/6J mice (Navarro et al., 2005), and because of low numbers of male and female mice within each treatment condition, sex was not included as a factor in analyses described later. The genetic status of all mice was determined using polymerase chain reaction (PCR) procedures, and mice were approximately 6 weeks of age at the beginning of experiments. We also used male C57BL/6J mice that were purchased at 6 weeks of age from Jackson Laboratory (Bar Harbor, ME). Mice were individually housed in polypropylene cages with corncob bedding and had ad libitum access to water and standard rodent chow (Tekland, Madison, WI) throughout each experiment. The colony room was maintained at approximately 22°C with a reverse 12:12 hours light:dark cycle with lights off at 10:00 AM. All procedures used in this study were in compliance with the National Institute of Health guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Experiment 1: Two-Bottle Consumption of Ethanol, Sucrose, Saccharin, and Water

Mc4r^{-/-} ($n = 8$) and Mc4r^{+/+} ($n = 9$) mice were tested for voluntary ethanol consumption using a homecage 2-bottle choice procedure. Over 4 days, mice were given 24-hour access to 2-bottles on their homecage, one containing tap water and the other containing a 3% (v/v) ethanol solution. The concentrations of ethanol were then increased to 5, 8, 10, 15, and 20% every 4 days. The positions of the bottles were alternated every 2 days to control for position preferences. Each drinking bottle was weighed every 2 days, and body weights were recorded every 4 days. An empty cage was used for the placement of dummy bottles (1 ethanol and 1 water), and fluid lost from each of these bottles was subtracted off the consumption totals as a control for fluid spillage. A separate set of Mc4r^{-/-} ($n = 12$) and Mc4r^{+/+} ($n = 12$) mice were tested for voluntary consumption of 0.15% (w/v) saccharin solution versus water followed by 10% (w/v) sucrose solution versus water in a two-bottle test. Mice were given access to each sweet solution for 2 days.

Experiment 2: Ethanol Consumption Following Central Infusion of the MCR Agonist MTII

Mice were anesthetized with a cocktail of ketamine (117 mg/kg) and xylazine (7.92 mg/kg) and surgically implanted with a 26-gauge guide cannula (Plastic One, Roanoke, VA) aimed at the left lateral ventricle, with the following stereotaxic coordinates: 0.2 mm posterior to bregma, 1.0 mm lateral to the midline, and 2.3 mm ventral to the surface. Mice were allowed to recover for approximately 2 weeks before experimental procedures were initiated. After experimental

procedures, cannula placement was verified histologically. I.c.v. infusions were given in a 1.0 μ l volume over a 1-minute period using a 33-gauge injector needle that extended 0.5 mm beyond that guide cannula. Compounds were administered manually with a 1- μ l Hamilton syringe. The injectors were left in place for an additional 1 minute to allow for drug diffusion and to minimize vertical capillary action along the injector tract when it was removed.

After recovery from surgery, animals received 5 days of habituation to 24 hour, 2-bottle consumption with one bottle containing water and the second bottle containing a solution of 10% (v/v) ethanol. Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on ethanol consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and ethanol, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30- to 15-minutes before the beginning of the dark cycle, mice were given i.c.v. infusions of a 0.5 (Mc4r^{-/-} mice, $n = 11$; Mc4r^{+/+} mice, $n = 8$)- or 1 (Mc4r^{-/-} mice, $n = 6$; Mc4r^{+/+} mice, $n = 5$)- μ g dose of MTII (Bachem, Torrance, CA) dissolved 0.9% saline, or an equal volume of 0.9% saline (Mc4r^{-/-} mice, $n = 8$; Mc4r^{+/+} mice, $n = 7$). We have previously found that the 1- μ g dose of MTII was effective in reducing ethanol intake in C57BL/6J mice (Navarro et al., 2003, 2005). We chose MTII as we previously assessed the effects of MTII in Mc3r^{-/-} mice (Navarro et al., 2005) and could thus make direct comparisons between studies. The 10% ethanol solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Experiment 3: Ethanol Consumption Following Peripheral Administration of the MCR Agonist MTII

Mc4r^{-/-} and Mc4r^{+/+} mice received 5 days of habituation to 24 hour, 2-bottle consumption with 1 bottle containing water and the second bottle containing a solution of 10% (v/v) ethanol. Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on ethanol consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and ethanol, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30- to 15-minutes before the beginning of the dark cycle, mice were given an intraperitoneal (i.p.) injection of a 5 mg/kg dose of MTII dissolved in 0.9% saline (Mc4r^{-/-} mice, $n = 15$; Mc4r^{+/+} mice, $n = 14$) or an equal volume of 0.9% saline given in a 5 ml/kg volume (Mc4r^{-/-} mice, $n = 15$; Mc4r^{+/+} mice, $n = 15$). We chose the 5 mg/kg dose of MTII because it falls between doses (2 and 10 mg/kg) that have been shown to effectively attenuate feeding behavior (Chen et al., 2000; Choi et al., 2003). The 10% ethanol solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Experiments 4 and 5: Sucrose and Saccharin Solution Consumption Following Central Infusion of the MCR Agonist MTII

Surgery for cannula placement and i.c.v. infusion procedures were the same as described in Experiment 2. After recovery from surgery, animals received 5 days of habituation to 24 hour, 2-bottle consumption with one bottle containing water and the second bottle containing a solution of 10% (w/v) sucrose (Experiment 4) or 0.15% (w/v) saccharin (Experiment 5). Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on sweet solution consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and sweet solution, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30 to 15 minutes before the beginning of the dark cycle, mice were given i.c.v. infusions of a 0.5- μ g dose of MTII dissolved 0.9% saline

(Experiment 4, $n = 8$; Experiment 5, $n = 12$) or an equal volume of 0.9% saline (Experiment 4, $n = 7$; Experiment 5, $n = 12$). The sweet solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Data Analyses

To obtain a measure that corrected for individual differences in body weight, grams of ethanol or food and milliliters of water or sweet solution consumed per kilogram of body weight were calculated. Ethanol preference ratios were also calculated by dividing the volume of ethanol consumed by total fluid (ethanol + water) consumption. Ethanol consumption data from Experiment 1 were analyzed with a 2 x 6 (genotype x ethanol concentration) repeated-measures analysis of variance (ANOVA), and saccharin and sucrose consumption data were analyzed with 2 x 2 (genotype x days) repeated-measures ANOVAs. Data from Experiments 2 and 3 were analyzed with two-way 2 x 3 (genotype x MTII dose) mixed-factor ANOVAs. Finally, data from Experiments 4 and 5 were analyzed using one-way (dose) ANOVAs. Tukey's tests were used for post hoc analyses. All data are presented as means \pm SEM, and the level of significance was set at $p < 0.05$ in all cases.

RESULTS

Experiment 1: Two-Bottle Consumption of Ethanol and Water

Data showing 24-hour voluntary consumption of ethanol and water and ethanol preference ratios in Mc4r^{-/-} and Mc4r^{+/+} mice during 2-bottle testing are presented in Fig. 1. A repeated-measures ANOVA performed on ethanol consumption data revealed a significant main effect of ethanol concentration [$F(5,75) = 59.149$; $p = 0.001$], reflecting the increase in g/kg of ethanol consumed as the concentration of ethanol was increased over the course of the experiment (Fig. 1A). No other effects were statistically significant. A repeated-measures ANOVA performed on water consumption data revealed a significant main effect of ethanol concentration phase [$F(5,75) = 23.685$; $p = 0.001$], reflecting the greater consumption of water as the concentration of ethanol was increased. Interestingly, there was a significant main effect of genotype [$F(1,15) = 5.473$; $p = 0.034$], as Mc4r^{+/+} mice (71.98 ± 5.24 ml/kg/24-h) drank significantly more water than Mc4r^{-/-} mice (54.11 ± 5.56 ml/kg/24-h) over the course of the experiment (Fig. 1B). No other effects related to the water data were statistically significant. A repeated-measures ANOVA performed on ethanol preference ratio data revealed a significant main effect of ethanol concentration phase [$F(5,75) = 26.831$; $p = 0.001$], reflecting the reduced preference for ethanol solution relative to water as the concentration of ethanol was increased (Fig. 1C). Finally, a repeated-measures ANOVA comparing body weight data at each phase of the experiment revealed that there were no significant differences in body weight between Mc4r^{+/+} mice (20.40 ± 1.09 g average over the course of the experiment) and Mc4r^{-/-} mice (23.23 ± 1.11 g average over the course of the experiment).

A repeated-measures ANOVAs performed on saccharin consumption data revealed a significant effects of days [$F(1,22) = 8.627$; $p = 0.008$], reflecting increased consumption of

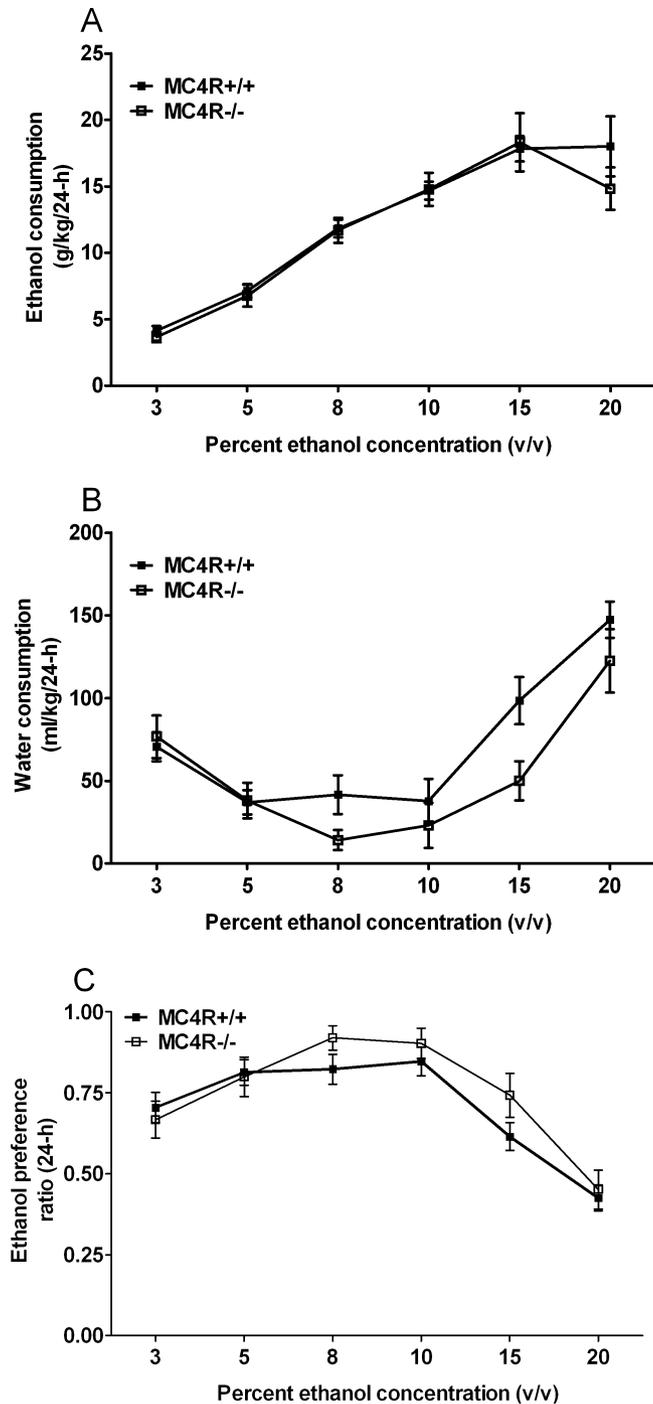


Fig. 1. Voluntary consumption of 3, 5, 8, 10, 15, and 20% (v/v) ethanol (panel A), water during access to different concentrations of ethanol (panel B), and ethanol preference ratios at each concentration of ethanol (panel C) in Mc4r^{-/-} and Mc4r^{+/+} mice in the two-bottle testing study (Experiment 1). All values are means \pm SEM. Mc4r^{+/+} mice drank significantly more water than Mc4r^{-/-} mice over the course of the experiment as revealed by a significant main effect of genotype ($p = 0.001$).

0.15% saccharin over days. However, Mc4r^{-/-} mice (289.66 ± 41.32 ml/kg/d) and Mc4r^{+/+} mice (302.51 ± 35.24 ml/kg/d) did not differ significantly in the volume of saccharin solution consumed, nor were there any genotype differences in water intake during access to saccharin.

Similarly, a repeated-measures ANOVAs performed on sucrose consumption data revealed a significant effect of days [$F(1,22) = 80.103$; $p = 0.001$], reflecting increased consumption of 10% sucrose over days. Mc4r^{-/-} mice (390.50 ± 13.29 ml/kg/d) and Mc4r^{+/+} mice (388.00 ± 26.44 ml/kg/d) did not differ significantly in the volume of sucrose solution consumed, nor were there any genotype differences in water intake during access to sucrose.

Experiment 2: Ethanol Consumption Following Central Infusion of the MCR Agonist MTII

Data showing 6-hour consumption measures following i.c.v. infusion of MTII in the Mc4r^{-/-} and Mc4r^{+/+} mice are presented in Fig. 2A–C. A two-way ANOVA performed on ethanol consumption data revealed a significant interaction effect between genotype and MTII dose [$F(2,39) = 3.739$; $p = 0.033$], but the genotype and MTII dose main effects were not significant. Post hoc tests showed that while each dose of MTII significantly reduced ethanol intake relative to control infusion in Mc4r^{+/+} mice, neither dose tested altered ethanol intake in the Mc4r^{-/-} mice (Fig. 2A). A two-way ANOVA performed on food intake data revealed a main effect of genotype [$F(1,39) = 6.854$; $p = 0.013$] and a significant interaction between genotype and MTII dose [$F(2,39) = 6.747$; $p = 0.003$] (Fig. 2B). Post hoc tests showed that while MTII was ineffective in Mc4r^{-/-} mice, each dose of the agonist tested significantly reduced food intake (relative to vehicle treatment) in the Mc4r^{+/+} mice. A two-way ANOVA performed on water intake data showed a significant interaction between genotype and MTII dose [$F(2,39) = 4.147$; $p = 0.023$], but the main effects were not statistically significant (Fig. 2C). Despite the significant interaction effect, post hoc tests revealed that MTII did not significantly alter water drinking relative to the vehicle treatment in either Mc4r^{-/-} or Mc4r^{+/+} mice. A two-way ANOVA performed on ethanol preference ratio data failed to show any significant effects (data not shown). Finally, a two-way ANOVA performed to compare body weight of mice in each treatment condition revealed that while there was a main effect of genotype [$F(1,39) = 10.020$; $p = 0.003$] such that Mc4r^{+/+} mice (22.71 ± 0.93 g) weighed less than Mc4r^{-/-} mice (26.667 ± 0.84 g), there was no significant interaction between genotype and MTII dose, suggesting that body weight did not likely contribute to the genotype \times MTII dose interaction effects observed with ethanol consumption and food intake data. Increased body weight in Mc4r^{-/-} mice has previously been reported (Huszar et al., 1997; Marsh et al., 1999).

Experiment 3: Ethanol Consumption Following Peripheral Administration of the MCR Agonist MTII

Data showing 6-hour consumption measures following i.p. injection of MTII in the Mc4r^{-/-} and Mc4r^{+/+} mice are presented in Fig. 2D–F. A two-way ANOVA performed on ethanol consumption data revealed a main effect of MTII

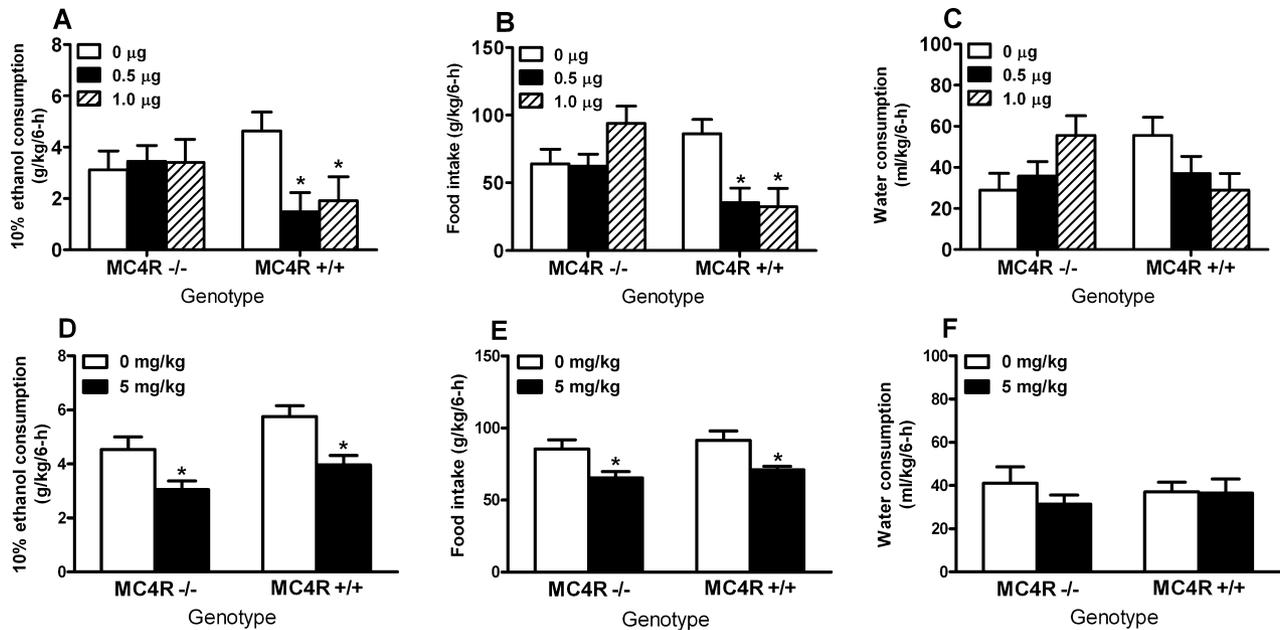


Fig. 2. Consumption of 10% (v/v) ethanol (g/kg/6-h), food (g/kg/6-h), and water (ml/kg/6-h) in *Mc4r*^{-/-} and *Mc4r*^{+/+} mice given intracerebroventricular infusion of saline (0 µg) or melanotan-II (MTII) (0.5 or 1.0 µg) are presented in panels **A**, **B**, and **C**, respectively (Experiment 2). Similarly, consumption of ethanol, food, and water in *Mc4r*^{-/-} and *Mc4r*^{+/+} mice given intraperitoneal injection of 0.9% saline (0 mg/kg) or MTII (5 mg/kg) are presented in panels **D**, **E**, and **F**, respectively (Experiment 3). All values are means ± SEM. **p* < 0.05 relative to 0 µg or 0 mg/kg dose.

dose [$F(1,55) = 17.22$; $p = 0.001$]. Neither the genotype main effect nor the interaction effect was significant (Fig. 2D). A two-way ANOVA performed on food intake data revealed a main effect of MTII dose [$F(1,55) = 14.423$; $p = 0.001$], but the genotype main effect and interaction effect did not achieve statistical significance (Fig. 2E). A two-way ANOVA performed on water intake data failed to show any statistically significant effects (Fig. 2F). Similarly, a two-way ANOVA performed on ethanol preference ratio data failed to show any significant effects (data not shown). Finally, a two-way ANOVA performed to compare body weight of mice in each treatment condition revealed a main effect of genotype [$F(1,55) = 7.023$; $p = 0.011$] such that *Mc4r*^{+/+} mice (23.38 ± 0.67 g) weighed less than the *Mc4r*^{-/-} mice (25.89 ± 0.66 g). No other effects were significant.

Experiments 4 and 5: Sucrose and Saccharin Solution Consumption Following Central Infusion of the MCR Agonist MTII

Figure 3 shows data representing 6-h consumption measures during sucrose testing in Experiment 4 (Fig. 3A–C) and saccharin testing in Experiment 5 (Fig. 3D–F) in C57BL/6J mice that were given i.c.v. infusion of vehicle or a 0.5-µg dose of MTII. One-way ANOVAs performed on sucrose, food, and water intake data from Experiment 4 revealed that the 0.5-µg dose of MTII significantly reduced sucrose [$F(1,13) = 8.477$; $p = 0.012$] and food [$F(1,13) = 6.456$; $p = 0.025$] intake but did not significantly alter water drinking relative to the control condition. One-way ANOVAs performed on saccharin, food, and water intake data from

Experiment 5 revealed that the 0.5-µg dose of MTII significantly reduced saccharin intake relative to the control injection [$F(1,22) = 7.622$; $p = 0.011$], but did not significantly alter food or water intake.

DISCUSSION

Constitutive deletion of the MC4R was not associated with significant alterations of voluntary ethanol consumption or consumption of saccharin or sucrose solutions (Experiment 1). An initial conclusion might be that endogenous MC4R signaling does not play a critical role in modulating ethanol self-administration. However, developmental compensation in constitutive knockout mice may mask the contribution of the deleted gene (Gerlai, 1996, 2001); thus, a role for endogenous MC4R signaling in modulating ethanol drinking cannot be ruled out by null data. Interestingly, consistent with a recent report implicating MCR signaling in the modulation of water intake (Yosten and Samson, 2010), the present data suggest that endogenous MC4R signaling may play a role in the modulation of water intake as *Mc4r*^{+/+} mice drank more water than *Mc4r*^{-/-} mice over the course of Experiment 1. Importantly, i.c.v. infusion of MTII (0.5- and 1.0-µg doses) significantly reduced 6-hour ethanol consumption and food intake in *Mc4r*^{+/+} mice without significantly altering water drinking, but failed to influence ethanol drinking or feeding in *Mc4r*^{-/-} mice (Experiment 2). These observations support previous findings showing that MTII significantly reduces ethanol intake in C57BL/6J mice (Navarro et al., 2003, 2005) and extend the literature by showing that the MC4R is the primary receptor involved in

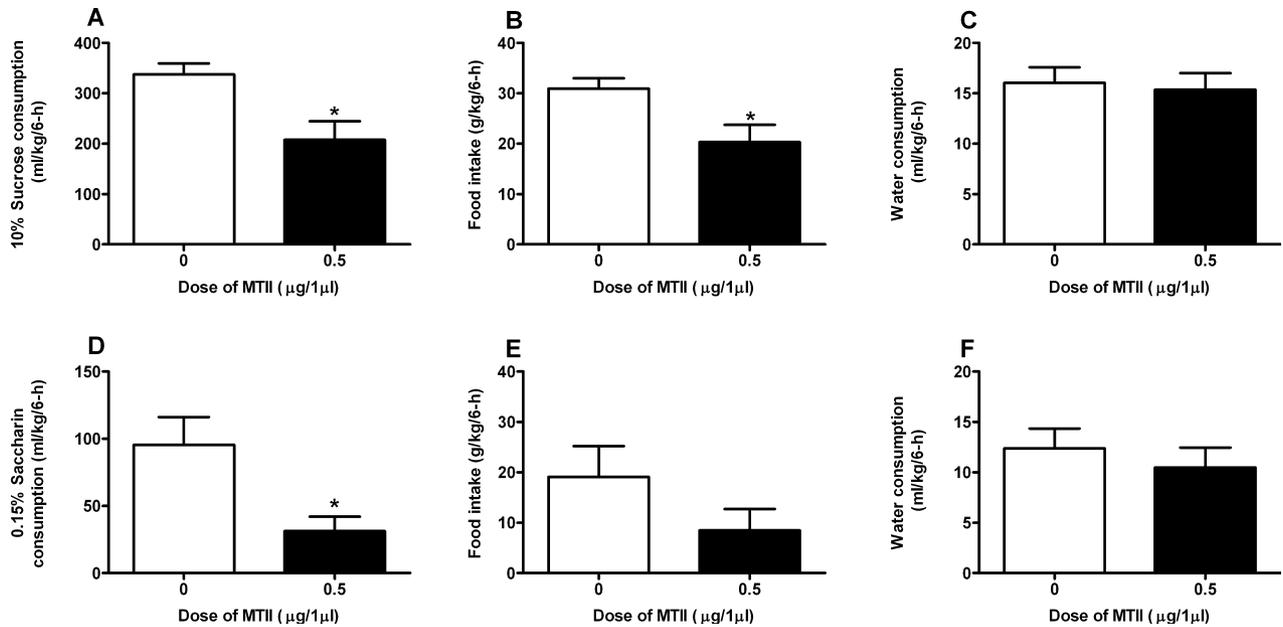


Fig. 3. Consumption of sweet solution (ml/kg/6-h; panels **A** and **D**), food (g/kg/6-h; panels **B** and **E**), and water (ml/kg/6-h; panels **C** and **F**) in C57BL/6J mice given intracerebroventricular infusion of saline (0 μ g) or melanotan-II (0.5 μ g). Sweet solution was made from 10% sucrose (Experiment 4; panels **A**, **B**, and **C**) or 0.15% saccharin (Experiment 4; panels **D**, **E**, and **F**). All values are means \pm SEM. * p < 0.05 relative to 0- μ g dose.

modulating the protective effects of centrally infused MTII on excessive ethanol intake. The present findings also replicate previous work demonstrating that central administration of MTII attenuates food intake (Grill et al., 1998; Hollopeter et al., 1998; Marsh et al., 1999; Navarro et al., 2003, 2005; Pierroz et al., 2002) and requires the MC4R (Marsh et al., 1999). Together, the present work highlights the critical role of the MC4R in modulating the central pharmacological effects of the MCR agonist MTII on ethanol intake and feeding. On the other hand, as *Mc3r*^{-/-} mice showed normal ethanol drinking and food intake when MTII was centrally infused (Navarro et al., 2005), the MC3R does not appear to be involved.

Consistent with previous reports (Cettour-Rose and Rohner-Jeanrenaud, 2002; Navarro et al., 2003, 2005; Pierroz et al., 2002), here we show that the peripherally administered MTII (5 mg/kg) reduced ethanol drinking and food intake. However, unlike central administration, when administered peripherally, MTII did not require normal MC4R expression to suppress feeding or ethanol intake. This conclusion is supported by the observations that i.p. injection of MTII significantly reduced 6-hour ethanol consumption and food intake (but not water drinking) with similar effectiveness in *Mc4r*^{-/-} and *Mc4r*^{+/+} mice (Experiment 3). Because the MC4R is necessary for the central actions of MTII, the present data suggest that the effects of peripherally administered MTII on ethanol drinking and food intake may be modulated by other MCRs. A possibility is that peripheral MCRs (other than the MC4R) are involved. In fact, radiolabeled MTII, when given in an intravenous injection at a dose that attenuated food intake, was evident in the circumventricular organs but did not readily penetrate the blood-brain barrier in rats (Trivedi

et al., 2003), and a more recent study showed low penetration of peripherally administered MTII into mouse brain (Hatzieremia et al., 2007). MC immunoreactivity and MC receptor binding have been observed in peripheral tissues, including the gastrointestinal tract and the adrenal glands (Dhillon et al., 2005; Saito et al., 1983; Tatro and Reichlin, 1987), and it is therefore possible that peripherally administered MTII attenuated ethanol consumption and food intake by actions within these peripheral regions. It should be noted that while a previous report showed that an i.p. injection of a 10 mg/kg dose of MTII reduced food intake in both *Mc4r*^{-/-} and *Mc4r*^{+/+} mice (Chen et al., 2000), a more recent finding showed that an i.p. injection of a 100- μ g dose of MTII failed to alter feeding in *Mc4r*^{-/-} mice but was effective in *Mc4r*^{+/+} mice (Balthasar et al., 2005). Thus, it is also possible that lower doses of peripherally administered MTII require the MC4R to reduce food (and ethanol) intake, while higher doses (such as the 5 mg/kg dose used here) influence ingestive behaviors by acting on other MCRs. A more comprehensive assessment of the effects of peripherally administered MCR agonists, over a range of doses, on ethanol intake (as well as possible nonspecific effects) will be the focus of future research.

One goal of the present report was to assess the effects of MTII on the consumption of various reinforcing stimuli, in addition ethanol and food. I.c.v. infusion of a 0.5- μ g dose of MTII, which significantly reduced 6-hour ethanol drinking and food intake in wild-type mice, also attenuated 6-hour consumption of a 10% sucrose solution and a 0.15% saccharin solution without altering water drinking. Thus, the MCR agonist MTII blunts the consumption of both caloric (ethanol, food, and sucrose) and noncaloric (saccharin) reinforcers, observations that are consistent with the hypothesis

that overlapping MC pathways modulate ethanol consumption and the consumption of natural reinforcers, regardless of caloric content. In fact, this should not come as a surprise in light of electrophysiological evidence demonstrating that both drugs of abuse and “natural” reinforcers (food and water) produce similar cell firing in the NAc (Carelli et al., 2000; Hollander et al., 2002; Roitman et al., 2004, 2005, 2008; Roop et al., 2002), and the observation that a growing list of peptides and proteins modulate both ethanol consumption and food intake (Thiele et al., 2003). For example, opioid receptor antagonists, which are approved for treating alcoholism, reduce both ethanol consumption and food intake (Gonzales and Weiss, 1998; Kamdar et al., 2007; Kotz et al., 1997; Middaugh et al., 2000; Yeomans and Gray, 2002). Interestingly, it has been proposed that cannabinoid receptor (CB1) agonists may be useful therapeutic agents for treating obesity (Cota et al., 2003) and alcoholism (Racz et al., 2003), and we suggest that MCR agonists may also provide a dual therapeutic role.

Given that administration of MTII was associated with reduced consumption of each of the reinforcing stimuli examined here, one potential concern is that administration of MTII produces nonspecific, and potentially aversive, effects. However, contrary to this hypothesis is the observation that MTII failed to significantly alter water intake relative to vehicle treatment in each of the experiments reported here, and we have previously observed MTII-induced attenuation of ethanol drinking that was not associated with altered water intake (Navarro et al., 2003, 2005). Another potential concern is that the effects of MCR agonists on ethanol drinking may be secondary to alterations of ethanol metabolism. However, this is unlikely because we have previously shown that peripheral administration and central administration of MTII do not alter blood ethanol clearance (Navarro et al., 2003, 2005).

Interestingly, while not significant when compared to the vehicle condition, there was a trend for the 1.0- μ g dose of MTII to increase food intake in *Mc4r*^{-/-} mice in Experiment 2. MCR agonist-induced increase in food intake has previously been reported in *Mc4r*^{-/-} mice and was hypothesized to reflect a compensatory increase in MC3R signaling (Kumar et al., 2009). Consistent with this idea, a selective MC3R agonist was found to increase food intake, suggesting that the MC3R functions as a presynaptic autoreceptor in brain regions that modulate food intake (Cone, 2006; Marks et al., 2006).

In conclusion, the present work provides new insight into the mechanism by which MCR signaling influences ethanol consumption and feeding by demonstrating the essential role of central MC4R in modulating MCR agonist-induced reductions of ethanol intake and food intake. On the other hand, the MC4R does not modulate the effects of peripherally administered MCR agonist (MTII) on ethanol and food intake, suggesting that different populations of MCRs modulate the actions of centrally versus peripherally administered MTII. Centrally administered MTII also attenuated the consumption of sucrose and saccharin solutions at a dose that

did not alter water drinking, consistent with the hypothesis that overlapping central MC pathways modulate the reinforcing properties of ethanol and natural reinforcers, independent of caloric content. Taken together, the present observations and previous work suggest that MC4R agonists, in addition to being attractive targets for treating obesity, may have therapeutic value for treating excessive ethanol consumption in individuals afflicted with alcohol abuse disorders or that are ethanol dependent.

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REPEATED BINGE-LIKE ETHANOL INTAKE IN C57BL/6J MICE LEADS TO DECREASED α -MSH IMMUNOREACTIVITY (IR) AND INCREASED AGRP IN KEY BRAIN REGIONS
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Recent data has shown that the melanocortin (MC) system modulates neurobiological responses to ethanol. MC receptor (MCR) agonists decrease, while antagonists increase, ethanol consumption. Chronic exposure via an EtOH-containing diet has been shown to cause a significant decrease in α -MSH immunoreactivity (IR) in thalamic and amygdalar regions. Additionally, mice lacking the endogenous MCR antagonist agouti-related protein (AgRP) show a decrease in binge-like ethanol consumption. The purpose of this study was to examine α -MSH and AgRP IR following cycles of binge-like EtOH exposure. Eighty male C57BL/6J mice were randomly assigned to one of eight groups: mice received 1, 3, or 6 four-day binge-like drinking sessions with EtOH (20%, v/v) or sucrose (thus: 1-EtOH, 1-Suc, 3-EtOH, 3-Suc, 6-EtOH, and 6-Suc), continuous water (CW) or continuous ethanol (CE). Immediately after bottles were removed following the final drinking session, tail bloods were collected to measure blood ethanol concentrations (BECs). Brains were collected and processed for DAB IR. EtOH consumption in 1-EtOH and 3-EtOH mice was significantly lower than that of CE mice on the final binge day; there were no differences between sucrose groups. Mice in the CE group also achieved significantly lower BECs than mice in either the 3-EtOH or 6-EtOH groups. All EtOH-treated groups showed attenuated α -MSH IR in the lateral hypothalamus (LH) relative to the CW group. The dorsomedial (DMH) and arcuate nuclei (ARC) of the hypothalamus showed treatment-dependent decreases in α -MSH IR. In these three regions, binge-like ethanol drinking-induced attenuation of α -MSH IR was not associated with alterations of α -MSH IR related to sucrose drinking. Ethanol-induced changes were not evident in the nucleus accumbens (NAc), central medial thalamus (CMTH), or central amygdala (CeA). AgRP IR was significantly increased in the ARC in both CE and the 6-EtOH mice. No changes in AgRP IR were detected in the NAc, CMTH, or DMTH. Together, these results suggest that ethanol consumption induces alterations of α -MSH and AgRP, changes that become more robust with greater ethanol exposure. Interestingly, reduction of α -MSH was evident after one binge-like drinking cycle, suggesting that endogenous α -MSH modulates neurobiological responses associated with binge-like ethanol drinking. (Supported by NIH grants AA013573, AA015148 and AA019839 and the Department of Defense grants W81XWH-06-1-0158 and W81XWH-09-1-0293).

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BINGE DRINKING IN ADOLESCENCE INCREASES DELTA OPIOID RECEPTOR FUNCTION IN THE DORSAL STRIATUM THAT CONTRIBUTES TO ETHANOL CONSUMPTION IN ADULTHOOD

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Binge drinking during adolescence increases the propensity for alcohol use disorders (AUDs) later in adult life, however, the mechanisms that drive this are incompletely understood. The delta opioid peptide receptor (DOP-R) has been shown to play a role in the reinforcing effects of ethanol and administration of DOP-R ligands to rodents modulates ethanol intake. The objective of this study was to investigate the role and function of the DOP-R with long-term high ethanol intake from adolescence into adulthood. Groups of rats (P28, P56, P154) were given intermittent access to 20% ethanol or water only for different lengths of time (0-6 months). Tail-flick DOP-R-mediated analgesia and [³⁵S]GTP_γS binding in rat brains were measured in each group. We show that DOP-R activity in the dorsal striatum and DOP-R-mediated analgesia changes during development, being highest during early adolescence and significantly reduced in middle and late adulthood. We show that intermittent and heavy ethanol intake in peri-adolescent and young adult rats leads to an increase in the function of the DOP-R in late adulthood rats. We show that chronic intermittent ethanol but not continuous ethanol or water consumption increases DOP-R [³⁵S]GTP_γS binding specifically in the dorsal striatum. Multiple administrations of the DOP-R antagonist, naltrindole, for 28 days produces selective, long-lasting and permanent reductions of voluntary ethanol consumption such that when naltrindole treatment was terminated, ethanol consumption was maintained at a reduced level for the next 28 days, compared to post-vehicle-treated rats. Rats given multiple administrations of naltrindole for 28 days have attenuated DOP-R function in the dorsal striatum after naltrindole treatment was terminated for a further 28 days, compared to vehicle-pretreated rats. These findings show that long-term high ethanol intake from adolescence increases DOP-R activity into adulthood and that the DOP-R function in the dorsal striatum contributes to the escalation and maintenance of ethanol consumption. These studies suggest the DOP-R plays a significant role in ethanol-mediated behaviors and suggest that targeting the DOP-R is an alternative strategy for the treatment of AUDs. This work was supported by funding from the State of California for Medical Research through UCSF to S.E.B and Department of Defense Grant W81XWH-06-1-0240 to S.E.B. S.E.B. and C.K.N. were supported in part by the NARSAD Young Investigator Award.

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DISTURBANCES IN BEHAVIOR AND ENKEPHALIN GENE EXPRESSION DURING ANTICIPATION OF ETHANOL IN HIGH-DRINKING RATS
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The anticipation of ethanol can activate specific brain circuits involved in ethanol-seeking behavior. Although past studies have focused on the dopamine reward system in drinking behaviors, more recent studies point to an important role of the opioid peptide, enke (ENK), in promoting enhanced ethanol drinking behavior by acting within several hypothalamic and mesocorticolimbic regions. The goal of the current study was to determine whether a subpopulation of Sprague-Dawley rats that overconsume ethanol exhibit disturbance behavior during the anticipation of ethanol and also in the expression of ENK within the paraventricular nucleus of the hypothalamus (PVN) and medial prefrontal cortex (mPFC) regions known to promote consummatory behavior and impulsivity, respectively. To rats were trained for 2 weeks in a limited access (3h/d) paradigm to consume 20% ethanol solution right at dark onset. With ethanol intake being positively correlated from day 1 ($r = +0.76, p < 0.01$), two distinct subgroups of animals ($n = 8/\text{group}$) were formed, a low-drinker (LD) group, which consumed an average of 0.6 g/kg/3 h, and a high-drinker (HD), which consumed considerably more ethanol (1.7 g/kg/3 h) and showed increased ethanol content (65 ± 8.9 vs. 21 ± 5.6 mg/dl, $p < 0.05$). In order to measure ethanol-seeking behavior in these animals during the anticipation of the ethanol, locomotor (ambulatory distance) and exploratory (vertical counts) behaviors in an open field activity chamber were measured for 10 minutes just prior to daily ethanol access. The results showed that LD rats a significant increase in ambulatory distance traveled (+43%, $p < 0.05$) and vertical counts (+47%, $p < 0.05$) during the anticipation of ethanol. Next, in order to examine disturbances in ENK expression during this anticipation period, animals were characterized as HD and LD in an identical manner as above and sacrificed just before scheduled access to ethanol. Using quantitative real-time PCR to measure gene expression the data showed increased ENK mRNA in the PVN (+46%, $p < 0.05$) and mPFC (+46%, $p < 0.05$) of HD compared to LD rats as they were anticipating their daily bouts of ethanol. Together these data suggest that, in animals prone to drinking excess amounts of ethanol, anticipatory behaviors related to ethanol-seeking may be driven by enhanced ENK expression within the PVN and mPFC, regions known to stimulate consummatory and impulsive behaviors.

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LONG-TERM ETHANOL BINGE DRINKING ALTERS DENDRITIC MORPHOLOGY IN LIMBIC BRAIN REGIONS OF LONG-EVANS RATS

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The early onset of ethanol intake often develops into high levels of episodic binge drinking, increasing the likelihood of chronic intake or alcoholism. Evidence suggests that chronic ethanol intake produces changes in the structure of dendrites and spines in the nucleus accumbens of rodents. Other studies have determined that chronic human alcohol consumption has cortical neuronal alterations in the terminal branches of dendrites, in opposing directions on proximal versus distal branches. Our objective was to determine whether moderate-term binge drinking produces alterations in dendritic branching and complexity in the nucleus accumbens, a brain region involved in motivational aspects of ethanol self-administration. Male Long-Evans rats self-administered 2% sucrose/10% ethanol (2S10E) or 2% sucrose/10% water (2S) by performing a daily fixed number of lever presses (response requirement, RR 20) for 1 year. Mean ethanol intake was 1.3 g/kg/day and was consumed in a daily 20-minute session. At the end of the study fresh brains were collected and impregnated using the rapid staining method, and sectioned on a cryostat microtome. Dendritic morphological analysis was performed on medium spiny neurons in the nucleus accumbens using stereology. These results indicate that there were significant increases in the 2S10E versus 2S groups: 40% increase in the number of branch points ($p < 0.001$); 45% in the total number of primary branches ($p < 0.001$); 38% in the total number of branch tips ($p < 0.001$); 30% in the mean number of terminal branches ($p < 0.01$). The total dendritic arbor and average segment length were not significantly altered. These results indicate that a moderate dose of long-term binge drinking of 2S10E versus 2S produced an increase in dendritic branching possibly due to lack of pruning or the result of a beneficial environmental interaction while drinking ethanol. The results in this study are consistent with evidence that the number of second and third order terminal branches increases in human chronic ethanol drinkers. There is evidence that ethanol related neuronal damage is regional specific. The current study is the first to demonstrate that repeated moderate ethanol binge drinking in rodents beginning in adolescence and continuing through adulthood for over a year alters dendritic branching in a brain region associated with motivational aspects of alcohol drinking. This work was supported by NIAAA grant K01 AA015194.

A LOW DOSE OF THE MELANOCORTIN AGONIST MTII SYNERGISTICALLY AUGMENTS NALTREXONE-INDUCED ATTENUATION OF BINGE-LIKE ETHANOL DRINKING IN C57BL/6J MICE

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The central polypeptide precursor proopiomelanocortin (POMC) gives rise to beta-endorphin, an endogenous opioid peptide, and the melanocortin (MC) peptides including alpha-melanocyte stimulating hormone. Opioid receptor antagonists, such as naltrexone (NAL), have been demonstrated to reduce ethanol consumption in rodents, and a growing body of evidence indicates that MC receptor agonists blunt ethanol intake. Interestingly, central opioid and MC pathways have been demonstrated to interact in their modulation of nociception and feeding behavior. Since opioids and MC peptides modulate ethanol consumption, the goal of the present work was to determine if these peptides, when presented in combination, interact additively or synergistically in the modulation of binge-like ethanol drinking in C57BL/6J mice. We used drinking in the dark procedures, an established model of binge-like ethanol drinking, to first established dose-response effects of intraperitoneally (i.p.) injected NAL or the MC agonist MTII (0, 0.3, 3.0, and 10 mg/kg for each drug) on binge-like drinking. Based on these data, we established the ED₂₀ and ED₃₀ for each drug, and then combined the low (ED₂₀) and high (ED₃₀) dose of each drug with the dose-response range of the other drug. Results showed that MTII was 3.4-fold more potent than NAL in blunting binge-like ethanol drinking (based on ED₅₀ values). MTII was also more effective, as the 10 mg/kg dose of MTII produced a 72% reduction of binge-like ethanol drinking while this same dose of NAL reduced drinking by only 49%. When administered in combination, the low ED₂₀ (but not the ED₃₀) dose of MTII (0.26 mg/kg) shifted the NAL dose-response curve to the left by a factor of 7 (i.e., NAL was 7-fold more potent when administered in combination with MTII relative to when it was administered alone). Subsequent isobolographic analyses of these data showed that MTII synergistically augmented the ability of NAL to blunt binge-like ethanol drinking. NAL shifted the MTII dose effect curve to the left, but this effect was additive. The present results show that a low dose of MTII synergistically potentiates the ability of NAL to blunt binge-like ethanol drinking. These observations suggest that MC receptor agonists may improve the therapeutic effectiveness of NAL in the treatment of alcohol abuse disorders when these drugs are given in combination. (Supported by NIH grants AA013573 and AA015148, and the Department of Defense grant W81XWH-09-1-0293).