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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 Manuel Lerma-Cabrera**, **Francisca Carvajal**, **Emily G. Lowery**, **Inmaculada Cubero**, and **Todd E. Thiele**
Department of Psychology, University of North Carolina at Chapel Hill, CB#3270, Chapel Hill, NC, 27599-3270, USA (MN, EGL, TET); Department of Neurociencia y Ciencias de la Salud, University of Almería, Almería, 04120, Spain (JML-C, FC, IC)

Abstract

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 non-selective MCR agonist melanotan-II (MTII) on ethanol consumption.

Methods—To assess the role of the MC4R, MC4R knockout ($Mc4r^{-/-}$) and littermate wildtype ($Mc4r^{+/+}$) 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 μg/1 μl) or intraperitoneal (i.p.) injection of MTII (0 or 5 mg/kg/5 ml). The effects of MTII (0 or 0.5 μg/1 μl, 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 non-caloric (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.

Keywords

ethanol consumption; melanocortin; MC-3 receptor; MC-4 receptor; C57BL/6J; food intake

Address for Correspondence: Dr. Todd E. Thiele, Department of Psychology, University of North Carolina, Davie Hall CB# 3270, Chapel Hill, NC 27599-3270, Phone: (919) 962-1519, Fax: (919) 962-2537, thiele@unc.edu.

Introduction

The Melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor pro-opiomelanocortin (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 adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH (Hadley and Haskell-Luevano, 1999). Due to a lack of a 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 overlapping peptide control of ethanol consumption and feeding behavior (Thiele et al., 2003; Thiele et al., 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 and pharmacological evidence implicate the MC system in the control of voluntary ethanol consumption. Relative to ANA (Alko, Non-Alcohol) 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 of central MCR signaling. Consistent with this hypothesis, intracerebroventricular (i.c.v.) infusion of the potent non-selective 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., 2005; Navarro et al., 2003), while ventricular infusion of the non-selective 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-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Glu)-NH_2, 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 non-caloric (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 × 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 below. 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 12h:12h light:dark cycle with lights off at 10:00 a.m. All procedures used in the present 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 (one ethanol and one 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 μl 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 μl 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, $n = 5$) μg 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 μg dose of MTII was effective in reducing ethanol intake in C57BL/6J mice (Navarro et al., 2005; Navarro et al., 2003). 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 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 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 above. 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 μg 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 × 6 (genotype × ethanol concentration) repeated-measures analysis of variance (ANOVA), and saccharin and sucrose consumption data were analyzed with 2 × 2 (genotype × days) repeated-measures ANOVAs. Data from Experiments 2 and 3 were analyzed with two-way 2 × 3 (genotype × 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 ± 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 the $Mc4r^{-/-}$ mice (23.23 ± 1.11 g average over the course of the experiment).

A repeated-measure ANOVAs performed on saccharin consumption data revealed a significant effects of days [$F(1, 22) = 8.627$; $p = 0.008$] reflecting increased consumption of 0.15% saccharin over days. However, $Mc4r^{-/-}$ mice (289.66 ± 41.32 ml/kg/day) and $Mc4r^{+/+}$ mice (302.51 ± 35.24 ml/kg/day) 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-measure ANOVAs performed on sucrose consumption data revealed a significant effects 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/day) and $Mc4r^{+/+}$ mice (388.00 ± 26.44 ml/kg/day) 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 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 × 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 dose [$F(1, 55) = 17.22$; $p = 0.001$]. Neither the genotype main effect nor the interaction effect were 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

Fig. 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; Gerlai, 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., 2005; Navarro et al., 2003), and extend the literature by showing that the MC4R is the primary receptor involved in 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., 2005; Navarro et al., 2003; 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, since $Mc3r^{-/-}$ 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., 2005; Navarro et al., 2003; Pierroz et al., 2002), here we show 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). Since 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 (Hatziieremia et al., 2007). MC immunoreactivity and melanocortin receptor binding have been observed in peripheral tissues, including the gastrointestinal tract and the adrenal glands (Dhillo 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 μg 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 non-specific 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 μg 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 non-caloric (saccharin) reinforcers, observations which 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 nucleus accumbens (Carelli et al., 2000; Hollander et al., 2002; Roitman et al., 2004; Roitman et al., 2005; Roitman et al., 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 non-specific, 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., 2005; Navarro et al., 2003). 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 since we have previously shown that peripheral and central administration of MTII do not alter blood ethanol clearance (Navarro et al., 2005; Navarro et al., 2003).

Interestingly, while not significant when compared to the vehicle condition, there was a trend for the 1.0 μg dose of MTII to increase food intake in $Mc4r^{-/-}$ mice in Experiment 2. MCR agonist-induced increase of food intake has previously been reported in $Mc4r^{-/-}$ mice and was hypothesized to reflect a compensatory increase of 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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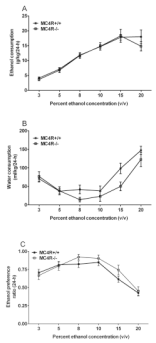

Fig. 1.

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 ± 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 ($p = 0.001$).

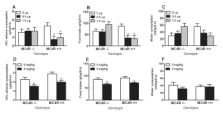

Fig. 2.

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 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.

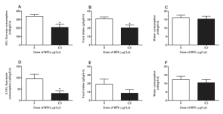

Fig. 3.

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 μg) or MTII (0.5 μg). Sweet solution was made from 10% sucrose (Experiment 4; panels A, B, and C) or 0.15% saccharin (Experiment 5; panels D, E, and F). All values are means ± SEM. * $p < 0.05$ relative to 0 μg dose.