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Short communication

The protective effects of the melanocortin receptor (MCR) agonist, melanotan-II (MTII), against binge-like ethanol drinking are facilitated by deletion of the MC3 receptor in mice

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ABSTRACT

Recent data have implicated the melanocortin (MC) system in modulating voluntary ethanol consumption. Administration of melanotan-II (MTII), a nonselective melanocortin receptor (MCR) agonist, reduces voluntary ethanol consumption in C57BL/6J mice. Previous studies have demonstrated that central infusion of MTII effectively reduced voluntary ethanol drinking in mutant mice lacking normal expression of MC3R (MC3R$^{-/-}$ mice) but failed to alter ethanol drinking in mice lacking expression of MC4R, demonstrating that central MTII administration reduces voluntary ethanol drinking by signaling through the MC4R. However, evidence shows that the neurocircuitry recruited during excessive binge-like ethanol drinking versus moderate ethanol drinking are not identical. Thus the present study sought to investigate the potential role of the MC3R in binge-like ethanol intake. To this end, the "drinking in the dark" (DID) procedure, a commonly used animal model of binge-like ethanol drinking, was employed. Wild-type MC3R$^{+/+}$ and MC3R$^{-/-}$ mice were given intracerebroventricular (i.c.v.) infusion of MTII (0.0, 0.25, 0.50, or 1.0 μg) prior to the onset of a 4-h testing period in which mice were given access to 20% (v/v) ethanol. Immediately after the 4-h testing period, tail blood samples were collected from each animal in order to assess blood ethanol concentrations (BECs). Consistent with previous findings, central administration of MTII blunted binge-like ethanol drinking in both MC3R$^{+/+}$ and MC3R$^{-/-}$ mice. Interestingly, all doses of MTII blunted binge-like ethanol drinking in MC3R$^{-/-}$ mice during the first hour of testing, while only the 1.0 μg dose reduced binge-like drinking in MC3R$^{+/+}$ mice. Thus, MC3R$^{-/-}$ mice were more sensitive to the protective effects of MTII. These data suggest that MC3Rs oppose the protective effects of MTII against binge-like ethanol drinking, and thus selective MC3R antagonists may have potential therapeutic roles in treating excessive ethanol drinking.

1. Introduction

The melanocortin (MC) peptides, α-, β-, γ- melanocyte stimulating hormone (MSH), and adrenocorticotropic hormone (ACTH), are produced centrally by proopiomelanocortin (POMC)-expressing neurons within the arcuate nucleus of the hypothalamus, the nucleus of the solitary tract, and medulla (Jacobowitz and O'Donohue, 1978). These peptides, as well as their endogenous antagonists, agouti and agouti-related protein (AgRP), act through five, seven-transmembrane G-protein coupled melanocortin receptor (MCR) subtypes. The MCRs within the rodent brain are predominantly comprised of the MC3R and MC4R subtypes, while MC1R, MC2R, and MC5R are expressed primarily in the periphery (Adan and Gispen, 1997; Barrett et al., 1994; Xia et al., 1995). Together, these ligands and their associated receptors, collectively referred to as the MC system, regulate a myriad of physiological functions including pigmentation (Robbins et al., 1993), sexual function (Argiolas et al., 2000), and appetite regulation (Fan et al., 1997; Giraudo et al., 1998), among others.

A growing body of literature has also implicated MCR signaling in modulating ethanol consumption. This association was first uncovered when it was reported that ethanol naïve alcohol preferring rats displayed abnormal expression of MC3R and MC4R within the nucleus accumbens and hypothalamus relative to their non-preferring counterparts (Lindblom et al., 2002). Extending on these findings, it was later demonstrated that central infusion of a non-selective MCR agonist, melanotan-II (MTII), attenuated voluntary ethanol consumption in these preferring rats (Ploj et al., 2002). Furthermore, central infusion of MTII reduced voluntary ethanol

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drinking in both wild-type and mutant mice deficient in MC3R, suggesting that the MC3R does not modulate voluntary drinking (Navarro et al., 2005). What is more, Navarro and colleagues also showed that a selective MC4R agonist blunted voluntary ethanol consumption in wild-type C57BL/6J mice, implicating the MC4R in modulating ethanol intake (Navarro et al., 2003). Consistent with these findings, later work revealed that MTII had no effect on voluntary ethanol consumption in mutant mice lacking MC4R (Navarro et al., 2011). Taken together, these data suggest that central MTII reduces voluntary ethanol drinking by signaling through the MC4R, but not the MC3R.

However, previous findings have implicated that the MC3R regulates the transmission of α-MSH from POMC neurons. Specifically, the MC3R has been found to be expressed on POMC neurons (Bagnol et al., 1999), suggesting that it functions as an autoreceptor. Subsequent *in vitro* investigations revealed that activation of these receptors using the selective MC3R agonist, $_{D}$-Trp^{8}-γ-MSH, induced a marked increase in IPSC frequency on POMC neurons (Cowley et al., 2001). Additionally, central infusions of this same compound caused a downregulation of POMC mRNA levels in rats (Lee et al., 2008). What is more, rats receiving intracerebroventricular (i.c.v.) infusions of an MC3R agonist displayed increased food intake, while low doses of an MC3R antagonist reduced food intake (Lee et al., 2008). Together, these studies provide converging evidence that indicate the MC3R serves as an inhibitory autoreceptor on POMC neurons.

Additionally, recent converging evidence has suggested that different neurocircuitry modulates moderate level ethanol drinking versus excessive binge-like ethanol drinking (Lowery et al., 2010; Lowery-Gionta et al., 2012; Sparta et al., 2008). Despite the growing body of literature implicating the MC system in voluntary ethanol consumption, the role of the MC system in binge-like ethanol consumption remains relatively unexplored. We recently showed that mutant mice lacking AgRP exhibited blunted binge-like ethanol drinking, providing initial evidence that MCR signaling modulates binge-like drinking (Navarro et al., 2009). To further characterize the role of MC system in binge-like ethanol drinking, we employed "drinking in the dark" (DID) procedures and used mutant mice lacking the MC3R (MC3R$^{-/-}$ mice) and their wild-type counterparts (MC3R$^{+/+}$ mice) in the current study to determine the potential contribution of the MC3R in modulating the protective effects of MTII against binge-like ethanol drinking. Consistent with the previous data (Navarro et al., 2005), we found that infusions of MTII attenuated binge-like ethanol consumption in both MC3R$^{+/+}$ and MC3R$^{-/-}$ mice. However, we observed that MTII was more effective in reducing binge-like ethanol drinking in MC3R$^{-/-}$ relative to MC3R$^{+/+}$ mice. These data suggest that MC3Rs oppose the protective effects of MTII against binge-like ethanol drinking, and thus selective MC3R antagonists may have potential therapeutic value in treating excessive ethanol drinking.

2. Materials and methods

2.1. Animals

The generation of MC3R$^{-/-}$ has been described previously (Chen et al., 2000). Sixteen littermate knockout (MC3R$^{-/-}$) and ten wild-type (MC3R$^{+/+}$) mice maintained on a C57BL/6J background were bred in-house from heterozygous stock. Genotype was determined via polymerase chain reaction (PCR). All mice were housed in individual home cages located in a vivarium with an ambient temperature of approximately 22 °C and a 12:12 h reverse light/dark cycle with lights off at 7:00 am. Food and water were available *ad libitum* except where indicated below. It has previously been demonstrated that compounds targeting MCRs exhibit similar effects on ethanol intake in male and female mice (Navarro et al., 2005); therefore, both sexes were included in the present study in an effort to increase sample sizes. All procedures 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.

2.2. Cannulation surgery and infusion procedure

Prior to testing, mice underwent cannulation surgery targeting the left lateral ventricle, which has been described previously (Navarro et al., 2011, 2005, 2003). Following surgery, mice were given approximately one week to recover before testing. Following completion of testing, cannula placement was verified histologically. All mice in the current study were found to have accurate placement; thus no animals were excluded from the analysis due to poor cannula placement. The non-selective MCR agonist melanotan-II (MTII; American Peptide Company, Sunnyvale, CA) was dissolved in 0.9% saline to reach the desired concentration (0.0, 0.25, 0.5, or 1.0 μg). A non-selective, rather than selective, MCR compound was chosen for this study in order to elucidate the potential interaction of the MC3R and MC4R in modulating the effects of a MCR agonist on binge-like ethanol drinking behavior. All doses were infused in a 1.0 μl volume using a Hamilton syringe (Hamilton Company USA, Reno, NV), which was administered manually over the course of 1 min. This injector was left in place for an extra 30 s to allow for diffusion and to prevent reflux of the compound up the cannula tract. Following the infusion, mice were returned to their homecages.

2.3. "Drinking in the dark" procedures

A 4 day DID procedure was used to model binge-like ethanol drinking (Rhodes et al., 2005). It has previously been shown that mice in this paradigm are able to achieve blood ethanol concentrations (BECs) that surpass the 80 mg/dl criterion used by the National Institute on Alcohol Abuse and Alcoholism to define an episode of binge drinking (National Institute on Alcohol Abuse and Alcoholism, 2004; Rhodes et al., 2007) and that drinking using this method is likely not motivated by caloric need (Lyons et al., 2008). For the first three days of DID, MC3R$^{+/+}$ and MC3R$^{-/-}$ mice were weighed and given mock infusions at the beginning of the dark cycle in order to acclimate the animals to the infusion procedure. Using standard DID procedures outlined by Rhodes et al. (2005), water bottles were removed three hours into the dark cycle and replaced with a single bottle of ethanol (20% v/v) for two hours. Water bottles were returned to the mice at the end of each day of testing. Treatment groups were equated based on ethanol consumption on days 1–3. On the fourth day of DID, mice were infused with a 0.0, 0.25, 0.5 or 1.0 μg dose of MTII as described above. Although it has previously been reported to have a relatively short half-life (Ugwu et al., 1994) in rats, the detailed pharmacokinetics of MTII have yet to be explored in mice. Nonetheless, previous studies have demonstrated that its effects on ethanol consumption persist for several hours following initial administration. For example, Navarro et al. (2003) observed that a single injection of MTII blunted ethanol drinking as long as eight hours post-injection. Binge-like ethanol consumption was assessed on this fourth day of testing, which followed the same schedule as the first 3 days with the exception that ethanol access was extended to four hours. MC3R$^{+/+}$ and MC3R$^{-/-}$ mice were given i.c.v. infusions of MTII 2 h before ethanol access. Consumption measures were collected at the first hour of ethanol access as well as at the end of the 4 h of testing in order to measure the immediate and prolonged effects of the drug on ethanol intake, respectively. Tail bloods were collected from each animal at the end of the 4-h testing period in