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Lateral Hypothalamic Melanocortin Receptor Signaling Modulates Binge-Like Ethanol Drinking in C57BL/6J Mice

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Abstract

Binge ethanol drinking is a highly pervasive and destructive behavior yet the underlying neurobiological mechanisms remain poorly understood. Recent work suggests that overlapping neurobiological mechanisms modulate feeding disorders and excessive ethanol intake, and converging evidence indicates that the melanocortin (MC) system may be a promising candidate. The aims of the present work were to examine how repeated binge-like ethanol drinking, using the “drinking in the dark” (DID) protocol, impacts key peptides within the MC system and if site-specific manipulation of MC receptor (MCR) signaling modulates binge-like ethanol drinking. Male C57BL/6J mice were exposed to 1, 3 or 6 cycles of binge-like ethanol, sucrose, or water drinking, after which brain tissue was processed via immunohistochemistry (IHC) for analysis of key MC peptides, including alpha-melanocyte stimulating hormone (α -MSH) and agouti-related protein (AgRP). Results indicated that α -MSH expression was selectively decreased, while AgRP expression was selectively increased, within specific hypothalamic subregions following repeated binge-like ethanol drinking. To further explore this relationship, we used site-directed drug delivery techniques to agonize or antagonize MCRs within the lateral hypothalamus (LH). We found that the non-selective MCR agonist melanotan-II (MTII) blunted, while the non-selective MCR antagonist AgRP augmented, binge-like ethanol consumption when delivered into the LH. As these effects were region-specific, the present results suggest that a more thorough understanding of the MC neurocircuitry within the hypothalamus will help provide novel insight into the mechanisms that modulate excessive binge-like ethanol intake and may help uncover new therapeutic targets aimed at treating alcohol abuse disorders.

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Author Contributions

GMS conducted experiments, analyzed data, contributed to the concept and design of experiments, and drafted the manuscript. JAR, EGLG, and AMS conducted experiments, analyzed data, and aided in the writing of portions of the manuscript. TET and MN were responsible for the study concept, overseeing experimental design, and editing the manuscript. All authors approved the final version of the manuscript for publication.

Keywords

agouti-related peptide; α -MSH; binge-like drinking; ethanol; lateral hypothalamus; melanocortin

Introduction

Ethanol dependence and relapse in abstinent alcoholics are major health problems in the United States and neurochemical pathways that modulate these disorders are currently under investigation. However, heavy ethanol use and binge ethanol drinking have received far less attention. A ‘binge’ is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as a pattern of drinking that produces blood ethanol concentrations (BECs) greater than 80 mg/dL, or 0.08% (NIAAA, 2004). Interestingly, while about 90% of the ethanol consumed by individuals under the age of 21 in the United States is in the form of binge drinking, 75% of ethanol consumed by adults in the US is also in the form of binge drinking (SAMHSA, 2002). Further, 70% of binge drinking episodes in the US involve adults age 26 years and older (Naimi et al., 2003). Thus, binge drinking is not restricted to the young but is a risky behavior prevalent in adults. As with all patterns of ethanol abuse, frequent binge drinking is associated with numerous health consequences including heart disease, high blood pressure, and type 2 diabetes (Fan et al., 2008). Perhaps most alarming is the finding of increased risk for developing ethanol dependence in individuals that binge drink early in life (Hingson et al., 2006). Thus, it is of paramount importance to identify neurochemical pathways in the brain that modulate binge drinking as such knowledge will provide insight into novel pharmaceutical treatments that will protect against this dangerous behavior.

An expanding body of research suggests that overlapping neural pathways modulate both eating disorders and drug and ethanol addiction (Volkow et al., 2013b). Both palatable foods and drugs of abuse (including ethanol) impact dopamine signaling in a number of neural pathways involved in reward, habit formation, and executive function (Volkow et al., 2013a). Excessive food intake has also been shown to elicit addictive-like behaviors, including compulsive food seeking and withdrawal (Avena et al., 2008; Johnson and Kenny, 2010). One example of an overlapping neural substrate potentially involved in the modulation of both eating disorders and drug addiction is the lateral hypothalamus (LH), known for key involvement in modulating feeding behavior, as its many projections innervate regions implicated in drug- and reward-related behaviors (Fadel and Deutch, 2002; Kampe et al., 2009). Hypothalamic peptides with known roles in feeding behavior, including melanocortin (MC)-related peptides, have also been implicated in controlling neurobiological responses to drugs of abuse (Thiele et al., 2003).

Post-translational cleavage of pro-opiomelanocortin (POMC) yields the MC receptor agonist α -melanocyte stimulating hormone (MSH), as well as β -MSH, γ -MSH, adrenocorticotrophic hormone (ACTH), and β -endorphin (O’Donohue and Jacobowitz, 1980; Yeo and Heisler, 2012). These α -MSH-producing neurons, mainly originating in the lateral portion of the arcuate nucleus (ARC), innervate a wide distribution of regions, particularly within the hypothalamus (Nahon, 2006). Agouti-related protein (AgRP) also originates from ARC neurons and functions as an endogenous MC receptor (MCR) antagonist (Shutter et al.,

1997). MC-related peptides exert their actions onto five MC receptors (MCRs) subtypes, which are coupled through Gas signaling pathways (Dores et al., 2014). There is an expanding literature that demonstrates a role for the MC system in modulating neurobiological responses to ethanol and ethanol consumption, the subject of a recent review (Olney et al., 2014a).

For example, ethanol exposure significantly reduces expression of α -MSH (Navarro et al., 2008; Rainero et al., 1990; Wilkinson et al., 1986) and POMC (Navarro et al., 2013), and following a period of abstinence after chronic ethanol exposure region-specific increases in α -MSH immunoreactivity (IR) have been observed (Kokare et al., 2008). Further, acute administration of ethanol increases AgRP IR in the hypothalamus (Cubero et al., 2010). Importantly, central and peripheral administration of a non-selective MCR agonist or central administration of a selective MC-4 receptor (MC4R) agonist reduces ethanol consumption and preference (Navarro et al., 2005; Navarro et al., 2003; Navarro et al., 2011; Ploj et al., 2002), and recent data indicate that MC-3 receptors (MC3Rs) oppose the protective effects of MC4R agonism against binge-like ethanol drinking (Olney et al., 2014b). Finally, genetic deletion of AgRP is associated with reduced ethanol consumption, self-administration, and binge-like ethanol drinking (Navarro et al., 2009)

The goal of the present project was to further study the role of MCR signaling in modulating binge-like ethanol drinking, and to characterize the specific brain regions in which MCR signaling modulates excessive ethanol intake. To this end, we used the pre-clinical animal model of binge ethanol drinking commonly referred to as “drinking in the dark” (DID), which promotes high levels of ethanol consumption over a 2- to 4-h period of time in C57BL/6J mice, associated with BECs that typically reach 100 mg/dL or greater (Sprow and Thiele, 2012; Thiele et al., 2014; Thiele and Navarro, 2014). Here, we demonstrate that binge-like ethanol drinking is associated with significant alterations in α -MSH and AgRP IR in a number of hypothalamic subregions, and that MCR agonism or antagonism within the LH significantly blunts or augments binge-like ethanol drinking, respectively.

Materials and Methods

Animals

Male C57BL/6J mice (Jackson Laboratories) were 6–8 weeks of age and weighed approximately 23 g at the beginning of experimental manipulations. Mice were individually housed in polypropylene cages with corncob bedding and had *ad libitum* access to food (IsoPro RMH 3000, Purina LabDiet, St. Louis, MO) and water except where noted. Animal colonies were maintained at approximately 22° Celsius with a 12h:12h light:dark cycle. All procedures used in the present study were in accordance with the National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drugs

Ethanol (20% v/v) solutions were prepared using tap water and 95% ethyl alcohol (Decon Laboratories Inc., King of Prussia, PA). Sucrose (3% w/v or 10% w/v) solutions were

prepared using tap water and D-Sucrose (Fisher Scientific, Fair Lawn, NJ). All compounds for site-directed infusions were dissolved in sterile isotonic saline. The nonselective MCR agonist melanotan-II (MTII; 0.5nmol/0.5µl/side; American Peptide, Sunnyvale, CA), the nonselective MCR antagonist agouti-related protein (AgRP; 0.1nmol/0.5µl/side; Phoenix Pharmaceuticals, Inc., Belmont, CA), or vehicle were used in the experiments described below. In an attempt to reduce the effects of stress associated with brain infusions, and because all of the compounds used here have previously been shown to exert long-lasting effects on food and/or ethanol intake (Navarro et al., 2005; Navarro et al., 2003; Navarro et al., 2011; Ploj et al., 2002), infusion were given 90 min before ethanol or sucrose access.

Drinking in the Dark (DID) Procedures

To model binge-like drinking, animals underwent a 4-day DID procedure (Thiele et al., 2014). Briefly, on days 1–3, water bottles were removed from cages 3 hr into the dark cycle. Bottles containing 20% ethanol, 3% sucrose (Experiment 1; a lower concentration was used to avoid potential weight gain stemming from longer-term sucrose access), or 10% sucrose (Experiments 2–3) were placed on the cage for 2 hr. On day 4, the ethanol/sucrose access was extended to 4 hr. In experiments where mice received more than one 4-day DID cycle, each cycle was separated by at least 3 days.

Surgery and Micro-infusion Procedures

For experiments 2 and 3, approximately 2 weeks after arrival, mice were anesthetized with a ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail and underwent stereotaxic surgery using the Leica Angle Two (Leica Biosystems, Buffalo Grove, IL). Bilateral 26-gauge cannulae (Plastic One, Roanoke, VA) were implanted into the LH (in reference to bregma, AP: –1.10mm; ML: ± 1.10mm, and DV: – 5.10mm), the bed nucleus of the stria terminalis (BNST; AP: + 0.25mm; ML: ± 1.10mm; DV: – 4.10mm), or the dorsal striatum (DS; AP: + 0.30mm, ML: ± 1.70mm, DV: – 4.10mm). Mice were allowed to recover for a minimum of one week before the initiation of experimental procedures. Cannulae placements were verified histologically at the end of the experiments, and only animals with bilateral hits were included in analyses. Site-directed infusions were delivered using a 1.0 µl Hamilton micro-syringe controlled by a Harvard Apparatus PHD 2000 micro-infusion pump. Post-infusion, the injector was left in place for at least 1 min to allow for drug diffusion away from the injector.

Perfusions, Brain Preparation, and Immunohistochemistry (IHC)

Perfusion and IR procedures were based on those routinely used in our laboratory (Hayes et al., 2005). Immediately following the final cycle of DID, brains were collected and processed for IHC using antibodies raised against α-MSH (Millipore, Temecula, Ca; 1:20000), AgRP (Phoenix Pharmaceuticals, Inc., Burlingame, CA; 1:4000), or NeuN, a marker for the presence of neurons (Millipore, Temecula, CA; 1:1000). In the absence of either the primary or the secondary antibodies, staining was not evident; indicating that staining in the tissue was not nonspecific binding and required the presence of both antibodies. Tissue was processed with Vectastain Elite kits (Vector Laboratories, Burlingame, CA) as per the manufacturer's instructions for standard ABC/HRP/diaminobenzadine-based IHC. The staining was visualized by reacting the sections with a

3,3'-diamino-benzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, PA) solution containing 0.05% DAB, 0.005% cobalt chloride, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. Tissue sections were mounted on glass slides, air-dried, and cover slipped for viewing.

Digital images of α -MSH, AgRP, and NeuN IR were obtained on a Nikon E400 Microscope equipped with a Nikon Digital Sight DS-U1 digital camera (Nikon Instruments Inc., Melville, NY). Photos were taken on software set at autoexposure, and the light intensity was set approximately in the middle between the minimum and maximum adjustments. Images were taken in JPEG format and were not converted or altered in any way, and all photos were taken using these same parameters. Using ImageJ (NIH, Bethesda, MD) an experimenter blind to treatment conditions analyzed the density of the IR in each region by calculating the percent of the total area showing staining relative to a subthreshold background. The size of each analyzed region was kept constant between animals and groups. Anatomically matched pictures of a slice from the left and right sides of the brain were used to produce an average density for each brain region and great care was taken to match sections through the same region and at the same level with the aid of a mouse stereotaxic atlas (Franklin and Paxinos, 1997).

Blood Ethanol Concentration (BEC) Analysis

Tail-blood samples were collected immediately following bottle removal on the final day of binge-like drinking (Experiment 1) or on day 4 of each DID cycle (Experiments 2–3). Approximately 10–15 μ l of blood was collected in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA). Blood samples were centrifuged and 5 μ l of the separated plasma was analyzed using the Analox Blood Ethanol Analyzer (Analox Instruments USA, Lunenburg, MA). Sucrose and water drinking animals received an identical tail nick but no blood was collected.

Experiment 1: The effect of repeated DID cycles on MC peptide IR—Separate groups of C57BL/6J mice experienced 1, 3, or 6 cycles of binge-like ethanol (20%) or sucrose (3%) drinking as described above. Mice were placed into one of seven groups matched for body weight ($n = 10/\text{group}$): 1-ethanol (EtOH), 1-sucrose (SUC), 3-EtOH, 3-SUC, 6-EtOH, 6-SUC, and continuous water control (WAT). Mice in the WAT group were handled exactly the same as mice in the experimental groups and received bottles containing fresh tap water during the 2-hr and 4-hr experimental sessions. The initiation of binge-like drinking was staggered such that all animals began the final 4-day binge-like drinking cycle at approximately the same age. Immediately following the final 4-hr session, tail-blood samples were collected and mice were transcardially perfused with 4% paraformaldehyde (w/v) in 0.1M phosphate buffer and brain tissue was collected for IHC analysis.

Experiment 2: The effect of intra-LH infusion of MCR agonist or antagonist on binge-like ethanol consumption—Because the most robust binge-induced reduction of α -MSH IR was observed in the LH, we chose to assess the effects of manipulating MCR signaling in the LH on binge-like ethanol drinking. Mice were randomly placed into one of two groups ($n = 10/\text{group}$): MTII (Experiment 2A) or AgRP (Experiment 2B). Following

day 3 of the DID cycle, mice in each group were rank ordered based on average ethanol consumption on days 1–3 and assigned to receive either drug or vehicle treatment, such that the average ethanol (20%) consumption between the two groups was similar. On day 4 mice received a bilateral infusion of the designated compound or vehicle into the LH prior to ethanol bottle presentation. Ethanol consumption was monitored hourly for 4 hr. After three rest days, all mice underwent a second cycle of DID in which they received the opposite drug treatment in a Latin-square design. Approximately one week later, animals underwent two cycles of sucrose (10%) using DID procedures identical to those described above.

Experiment 3: The effect of intra-BNST or dorsal striatum (DS) infusion of MCR agonist or antagonist on binge-like ethanol consumption—A separate group of mice was surgically implanted with bilateral cannulae targeting the BNST ($n = 10/\text{group}$) or DS ($n = 11/\text{group}$) to examine site-specificity of the effects observed following the LH manipulations. Mice with cannulae targeting the BNST were placed into one of two groups: MTII (Experiment 3A) or AgRP (Experiment 3B). Mice with cannulae targeting the DS received MTII or vehicle only (Experiment 3C). The design of these experiments were identical to that of the Experiment 2 with the exception that animals in Experiments 3B and 3C only received DID with ethanol (sucrose intake was not assessed). It should be noted that animals in experiment 3C also received micro-injections of the neuropeptide Y Y1 receptor agonist, Leu,Pro-NPY, 2 weeks prior to this experiment.

Data Analysis

Experiment 1: One-way analyses of variance (ANOVAs) were used to assess the differences between groups (1-EtOH, 3-EtOH, 6-EtOH) in total ethanol consumed and BECs following the final 4-h DID session. Identical analyses were used to assess group differences with sucrose-drinking animals. ANOVAs were also used to assess group differences in α -MSH, AgRP, and NeuN IR as a function of binge-like ethanol consumption versus water intake or of binge-like sucrose consumption versus water intake. LSD post-hoc tests were used to determine specific differences in IR and behavior between the continuous water and all other groups. For BEC measurements, as well as IR data, some animals were excluded from analysis (due to insufficient blood samples or unclear IR staining in a particular region), which accounts for differing degrees of freedom between similar analyses. *Experiment 2–3:* Only animals with bilateral cannulae targeting the LH, BNST or DS were included in any analyses described herein (Experiment 2A & B $n = 18$, Experiment 3A, B & C $n = 29$). As no drug by treatment order interaction effects were detected in any experiment, data were collapsed across groups and paired t-tests were performed to examine between-treatment differences in ethanol consumption and BECs achieved. All data are presented as mean $\pm$ SEM, and significance was accepted at $p < 0.05$ (two-tailed).

Results

Experiment 1: The effect of repeated DID cycles on MC peptide IR

Mice in 1-EtOH, 3-EtOH, and 6-EtOH groups consumed similar amounts of ethanol ($F(2, 27) = 0.811$, $p = 0.455$), and achieved similar BECs ($F(2, 24) = 2.958$, $p = 0.07$) during the final binge-like drinking day (Figure 1A–B). Sucrose consumption and body weight data

from the IHC study can be seen in supplemental Figure S1. IR of α -MSH, AgRP, and NeuN were examined following 1, 3, or 6 DID cycles with ethanol or sucrose, or 6 weeks of continuous water access. Representative photomicrographs of α -MSH and AgRP IR from brain slices of animals in either the water (WAT) or 6-EtOH groups can be seen in Figure 2 (right column). Images were taken from the LH (Figure 2B; AP: ~ -1.3 mm from Bregma), dorsomedial hypothalamus (DMH; Figure 2D; AP: ~ -1.6 mm from Bregma), arcuate nucleus (ARC; Figure 2F; AP: ~ -1.7 mm from Bregma), and paraventricular nucleus of the hypothalamus (PVN; Figure 2H; AP: ~ -0.9 mm from Bregma). Figure S2 depicts a brain atlas map showing the location in which IR was quantified in the LH. Relative to water drinking controls, animals in all ethanol-drinking groups displayed a significant decrease in α -MSH IR in the LH ($F(3, 27) = 5.481, p = 0.004$; Figure 2A). A significant decrease in α -MSH IR was also observed in the DMH in ethanol-drinking animals ($F(3, 27) = 3.561, p = 0.027$), with post hoc analyses indicating that only mice in the 3-EtOH and 6-EtOH groups showed a significant reduction relative to the water drinking control group (Figure 2C). Likewise, α -MSH IR was reduced following 6 cycles of binge-like ethanol drinking in the ARC ($F(3, 24) = 3.258, p = 0.039$; Figure 2E) and the PVN ($F(3, 30) = 3.109, p = 0.041$; Figure 2G). Identical analyses in sucrose-drinking groups showed that none of the sucrose drinking control groups exhibited significant alterations of α -MSH IR in any brain region examined relative to water drinking controls (see supplemental section, Table S1), indicating the altered α -MSH IR, relative to water drinking controls, was specific to binge-like ethanol drinking. In accordance with previous literature indicating an increase in AgRP IR following chronic ethanol consumption in rats (Navarro et al., 2008), AgRP IR was significantly increased in the PVN ($F(3, 26) = 19.842, p < 0.001$), specifically in the 6-EtOH group (Figure 2I; photomicrographs presented in Figure 2J; AP: ~ -0.9 mm from Bregma). However, a history of sucrose drinking did not significantly alter AgRP IR (see supplemental section, Table S2). Importantly, none of the regions that displayed decreases in α -MSH or increases in AgRP IR displayed significant changes in NeuN IR following either ethanol or sucrose drinking (see supplemental section, Table S3).

Experiment 2: The effect of intra-LH infusion of MCR agonist or antagonist on binge-like ethanol consumption

Mice were implanted with bilateral cannulae aimed at the LH region (Figure 3G shows the map of cannulae termination sites). Bilateral infusion of the non-selective MCR agonist MTII prior to ethanol access significantly blunted binge-like ethanol consumption relative to vehicle-treated animals ($t(8) = 2.684, p = 0.028$; Figure 3A). The effect of MTII was most apparent during the first hour of the test session ($t(8) = 4.994, p = 0.001$), and faded by the second hour of testing ($t(8) = 1.189, p = 0.268$; Figure 3B). MTII-treated mice showed a corresponding, albeit non-significant, trend in the same direction with regards to BECs achieved during the binge test ($t(8) = 1.909, p = 0.093$; Figure 3C). Bilateral infusion of MTII into the LH did not have a significant effect on sucrose consumption under an identical protocol ($t(7) = 1.929, p = 0.095$; Figure 3D). Importantly, the effects of MTII on ethanol drinking did not depend on food availability (see supplemental Figure S4).

In contrast, bilateral administration of the non-selective MCR antagonist AgRP into the LH significantly increased binge-like ethanol consumption relative to vehicle-treated animals

($t(8) = -3.343$; $p = 0.010$; Figure 3E), although BECs were not significantly altered (vehicle = 146.27 ± 19.89 mg/dl; AgRP = 147.91 ± 17.65 mg/dl; $t(8) = -0.068$; $p = 0.948$). The observation that binge-like ethanol drinking did not alter endogenous AgRP IR in the LH (see supplemental Table S2) suggests that the dose of exogenous AgRP used in the present study, which increased ethanol drinking, may have been supra-physiological, an explanation for the seemingly inconsistent results between IHC and pharmacological studies. Further, AgRP-infusion into the LH did not alter sucrose consumption under an identical DID protocol ($t(8)=2.1$; $p = 0.069$; Figure 3F).

Experiment 3: The effect of intra-BNST or DS infusion of MCR agonist or antagonist on binge-like ethanol consumption

A separate group of mice was implanted with bilateral cannulae targeting the BNST (Figure 4D shows the map of cannulae termination sites), as IHC analysis (Experiment 1) indicated robust levels of α -MSH IR in this region that were unaffected by a history of ethanol or sucrose consumption (see supplemental section, Table S1). Intra-BNST infusions of MTII significantly reduced ethanol drinking relative to vehicle treatment ($t(5) = 3.158$; $p = 0.025$, Figure 4A), although these animals did not display a similar change in BEC achieved (vehicle = 148.59 ± 27.5 mg/dl; MTII = 190.86 ± 47.30 mg/dl; $t(4) = -1.372$, $p = 0.242$). Similar to above, the observation that binge-like ethanol drinking did not alter endogenous α -MSH IR in the BNST suggests that the dose of MTII used in the present study, which decreased ethanol drinking, may have been supra-physiological, potentially explaining the seemingly inconsistent results between IHC and pharmacological studies. Consumption of a sucrose solution under an identical design was unaffected by MTII-treated ($t(6) = 2.319$, $p = 0.06$; Figure 4B). Interestingly, intra-BNST infusion of AgRP did not significantly alter binge-like ethanol consumption compared to vehicle treated animals ($t(8) = 0.535$, $p = 0.607$; Figure 4C) or BECs achieved (vehicle = 121.19 ± 21.76 mg/dl; AgRP = 110.87 ± 22.10 mg/dl; $t(8) = 0.642$, $p = 0.539$). As antagonism of MCRs within the BNST did not have a significant effect on binge-like ethanol consumption (suggesting that endogenous MCR signaling in this region does not modulate binge-like ethanol drinking), we did not run a control sucrose consumption study.

Since cannulae that target the LH pass through other dorsal structures, we determined if MTII infusion into the DS would alter binge-like ethanol drinking, as the MCRs are strongly expressed in the striatum (Alvaro et al., 1996). Mice treated with bilateral infusions of MTII into the DS prior to ethanol access did not show significant alterations in ethanol consumption relative to vehicle treated animals ($t(20) = 1.406$, $p = 0.1752$; see supplemental section, Figure S3A). Additionally, there was no significant difference in BECs achieved by between MTII-treated or vehicle-treated groups (Figure S3B).

Discussion

In the present studies, we provide compelling evidence for the role of hypothalamic MCR signaling in modulating binge-like ethanol consumption in C57BL/6J mice. These data are particularly interesting in light of a growing body of literature suggesting that similar mechanisms may underlie feeding disorders and drug addiction (Volkow et al., 2013a, b),

particularly given the known role of the MC system in modulating feeding behavior and energy homeostasis (Cansell et al., 2012; Shimizu et al., 2007).

Mice maintained stable levels of ethanol drinking and BECs after up to 6 cycles of binge-like drinking, in accordance with previous reports (Lowery-Gionta et al., 2012; Sparrow et al., 2012). Additionally, as total intake and BECs were similar between all ethanol-drinking groups, it is unlikely that the observed IR changes reflect between-group differences in BECs at the time of sacrifice. Ethanol-drinking mice showed a significant reduction in α -MSH IR in several hypothalamic regions, including the LH, PVN, DMH, and ARC, with the most striking reduction occurring after just one cycle in the LH. Consistent with evidence that the LH modulates appetitive and drug-seeking behaviors (Dayas et al., 2008; Marchant et al., 2012), the present results strongly implicated α -MSH signaling in the LH in modulating neurobiological responses associated with excessive binge-like ethanol drinking.

In addition to the observed decreases in α -MSH IR following repeated cycles of binge-like drinking, ethanol drinking mice demonstrated an increase in AgRP IR, specifically within the PVN, and no changes in NeuN expression in any region examined. As NeuN is a marker for the presence of neurons, these latter results suggest that the observed decreases in α -MSH may reflect disruptions in normal neuropeptide function rather than general neuronal loss. Such results are in line with previous work (Cubero et al., 2010; Navarro et al., 2008) suggesting that blunted α -MSH signaling, and increased AgRP activity, may emerge over the course of repeated binge-like drinking cycles, weakening the protective effects exerted by the MC system and leading to continued binge-like drinking.

Because the most robust effects of binge-like ethanol drinking on α -MSH IR were observed in the LH, we chose to first manipulate MCR signaling in this region and assess subsequent effects on binge-like ethanol drinking. The MCR agonist MTII significantly blunted binge-like ethanol drinking when injected into this region, while blockade of MCR signaling with AgRP significantly increased binge-like ethanol consumption. These observations reveal that MCR signaling in the LH modulates binge-like ethanol drinking, and the effects of AgRP (i.e., MCR blockade) suggest that endogenous α -MSH signaling is involved. Because we noted robust α -MSH IR in the BNST, but no ethanol-induced changes in α -MSH IR in this region, we assessed the effects of infusing MCR agonist and antagonist into the BNST on binge-like ethanol drinking, with the logic that the BNST could serve as a control for site-specificity. Interestingly, while infusion of MTII into the BNST significantly blunted binge-like ethanol drinking, infusion of the MCR antagonist was without effects. These results suggest that endogenous α -MSH signaling in the BNST does not modulate binge-like ethanol drinking, results consistent with the absence of changes in α -MSH IR in the BNST of mice with a history of binge-like ethanol drinking. Further, we found that infusion of MTII into the DS failed to alter binge-like ethanol drinking. Thus, when taken together the current results suggest that endogenous α -MSH signaling modulates binge-like ethanol drinking specifically in the LH. Other hypothalamic regions exhibiting alterations of α -MSH or AgRP IR in mice with a history of binge-like ethanol drinking will be the targets of future studies.

Importantly, the effects of activating or blocking MCR signaling appear to be specific to binge-like ethanol drinking using DID procedures, as bilateral infusion of MTII or AgRP into the LH (or MTII into the BNST) failed to alter sucrose solution drinking in control experiments that also employed DID procedures. However, specificity to ethanol consumption appears to only apply under conditions employing DID procedures as we also found, as noted below, that bilateral LH-infusion of a 0.5 nmol/0.5 μ l/side dose of MTII significantly blunted food intake (15.53 ± 5.3 g/kg/4-h) relative to the vehicle condition (37.33 ± 5.2 g/kg/4-h). Since site-directed infusion of a MC4R agonist was also found to reduce, and a MC4R antagonist was found to increase, food intake when injected into the LH of Sprague-Dawley rats (Lerma-Cabrera et al., 2012), the absence of MCR manipulations on sucrose consumption in the present study appear to be at odds with previous results as sucrose, like food, is a caloric compound. Furthermore, we have previously reported that central administration of MTII significantly attenuated food intake and sucrose drinking in mice (Navarro et al., 2011). Since we found that LH infusion of MTII reduced food intake (Figure S4, panel D), that lack of an effect of LH-infused MTII (or AgRP) on sucrose drinking may be related to the relatively high reward value of the 10% sucrose solution. It is possible that higher doses of MTII and AgRP are necessary to alter drinking of this highly salient reinforcer.

We found that alterations of α -MSH and AgRP IR became more robust in several hypothalamic regions as the number of binge-like drinking cycles increased. These observations suggest that as the number of binge-like ethanol drinking episodes increase, perturbations in the MC system become more robust. It will be important for future studies to examine the duration of observed changes in α -MSH and AgRP following a history of binge-like ethanol drinking. In line with the allostasis model of drug dependence (Koob and Le Moal, 2001), it may be expected that the decrease in α -MSH IR (and increase in AgRP IR) may initially be a transient phenomenon, becoming more long-lasting with repeated exposures to binge-like ethanol drinking. Thus, protracted dysregulation of α -MSH signaling may contribute to the development of a dependent state, though future research will be necessary to confirm this hypothesis.

Given the role of the LH in both appetitive behaviors and in the modulation of the reinforcing properties of natural rewards and drugs of abuse (Marchant *et al.*, 2009), it is tempting to speculate that the robust impact of a history of binge-like ethanol drinking on α -MSH IR in the LH indicates that α -MSH signaling in this region is involved in modulating the reinforcing properties of ethanol. It is possible that MC signaling within the LH alters ethanol drinking via local interactions with other systems that project to key brain regions involved in reward and appetitive behaviors, such as orexin (Balcita-Pedicino and Sesack, 2007) and neurotensin (Kempadoo et al., 2013) which project to the ventral tegmental area (VTA) from the LH. In fact, MC4R are expressed on neurotensin neurons in the LH (Cui et al., 2012), and MC neurons regulate the activity of orexin neurons in the LH (Lopez et al., 2007). Indeed, manipulation of the MC system has been shown to alter neuronal activity in critical regions of the mesolimbic dopaminergic pathway (Lindblom et al., 2002; Lindblom et al., 2001), and evidence suggests that interactions between endogenous MC and opioid signaling may be important for ethanol drinking behavior (Ploj et al., 2002). Interestingly, a recent report showed that infusion of a selective MC4R agonist into the either the VTA or

nucleus accumbens (NAc) blunted ethanol drinking in rats (Lerma-Cabrera et al., 2012), evidence that the MC system interacts directly with the mesolimbic dopaminergic reward pathway.

The present experiments extend the current literature by indicating that MC signaling, particularly within the LH, modulates binge-like ethanol drinking. These results suggest that a more thorough understanding of MC neurocircuitry within the hypothalamus will help provide novel insight into the mechanisms that modulates excessive binge-like ethanol intake and may help uncover new therapeutic targets aimed at treating alcohol abuse disorders.

Supplementary Material

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Figure 1.

Male C57BL/6J mice ($n = 10/\text{group}$) experienced 1, 3, or 6 cycles of DID with access to 20% ethanol (EtOH), while control animals had continuous access to tap water. Mice in all EtOH-drinking groups consumed approximately the same amount of EtOH (A) and achieved similar blood ethanol concentrations (BECs) (B) during the final binge-like drinking session. The dashed line indicates 80 mg/dl, typically accepted as a binge-like level. All data is expressed as mean + SEM.

Figure 2.

Immunohistochemistry analysis of α-MSH or AgRP IR showed that, compared to water drinking controls (WAT), binge-like ethanol consumption was associated with a decrease in α-MSH IR in the LH (A), the DMH (C), the ARC (E), and the PVN (G). Interestingly, although this decrease in α-MSH IR in the LH was apparent after just one cycle of drinking, other hypothalamic regions did not show similar decreases until 3 or 6 cycles of ethanol drinking. In contrast, binge-like ethanol consumption was associated with an increase in AgRP IR in the PVN, although this increase was only apparent after 6 cycles of drinking (I). Representative photomicrographs of α-MSH and AgRP IR are shown to the right of each bar graph in the left column (B, D, F, H, and J) from a WAT drinking control (left photo) and an

animal that experienced 6 cycles of binge-like ethanol (6-EtOH) consumption (right photo). The LH and ARC photos were taken with a 40x objective (scale bars = 100 μ M) and the DMH and PVN were taken with a 20x objective (scale bars = 300 μ m). All data are expressed as the mean $\pm$ SEM; * $p < 0.05$ vs. WAT, ** $p < 0.01$ vs. WAT.

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Figure 3.

Mice implanted with bilateral cannulae targeting the LH received a bilateral infusion of the nonselective MCR agonist MTII (0.5nmol/0.5µl/side), the nonselective MCR antagonist AgRP (0.1nmol/0.5µl/side), or vehicle (sterile saline; 0.5 µl/side) before they were presented with either 20% EtOH or 10% sucrose for 4 hr. Bilateral infusion of MTII into the LH significantly blunted binge-like EtOH consumption over the 4 hr test session (A), although the effect was most pronounced during the first hour of testing (B). Corresponding BECs in MTII-treated mice showed a non-significant trend in the same direction (C). There was no effect of MTII on sucrose consumption (D). Bilateral infusion of AgRP into the LH, on the

other hand, significantly increased binge-like EtOH consumption relative to vehicle-treated animals (E), but had no effect on sucrose consumption (F). Correct cannulae placements are marked by circles on modified images from the mouse brain atlas (Franklin and Paxinos, 1997) (G). Arabic numerals indicate distance from Bregma (mm). All data is expressed as mean $\pm$ SEM; * $p < 0.05$ vs. vehicle, ** $p < 0.01$ vs. vehicle.

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Figure 4.

Mice implanted with bilateral cannulae targeting the BNST received bilateral infusions of the nonselective MCR agonist MTII (0.5nmol/0.5 μ l/side), the nonselective MCR antagonist AgRP (0.1nmol/0.5 μ l/side), or vehicle (sterile saline; 0.5 μ l/side) before they were presented with 20% EtOH or 10% sucrose for 4 hr. Bilateral infusion of MTII into the BNST significantly attenuated EtOH consumption (A), but not sucrose consumption (B), over the 4 hr test session. Intra-BNST infusion of AgRP did not significantly affect binge-like EtOH consumption (C). Correct cannulae placements are marked by circles on modified images from the mouse brain atlas (Franklin and Paxinos, 1997); incorrect placements (not included in data analysis) are marked with an X (D). Arabic numerals indicate distance from Bregma (mm). All data is expressed as mean $\pm$ SEM; * $p < 0.05$ vs. vehicle.