Adolescent binge-like ethanol exposure reduces basal α-MSH expression in the hypothalamus and the amygdala of adult rats

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Abstract

Melanocortins (MC) are central peptides that have been implicated in the modulation of ethanol consumption. There is experimental evidence that chronic ethanol exposure reduces -MSH expression in limbic and hypothalamic brain regions and alters central pro-opiomelanocortin (POMC) mRNA activity in adult rats. Adolescence is a critical developmental period of high vulnerability in which ethanol exposure alters corticotropin releasing factor, neuropeptide Y, substance P and neurokinin neuropeptide activities, all of which have key roles in ethanol consumption. Given the involvement of MC and the endogenous inverse agonist AgRP in ethanol drinking, here we evaluate whether a binge-like pattern of ethanol treatment during adolescence has a relevant impact on basal and/or ethanol-stimulated -MSH and AgRP activities during adulthood. To this end, adolescent Sprague-Dawley rats (beginning at PND25) were pre-treated with either saline (SP group) or binge-like ethanol exposure (BEP group; 3.0 g/kg given in intraperitoneal (i.p.) injections) of one injection per day over two consecutive days, followed by 2 days without injections, repeated for a total of 8 injections. Following 25 ethanol-free days, we evaluated -MSH and AgRP immunoreactivity (IR) in the limbic and hypothalamic nuclei of adult rats (PND63) in response to ethanol (1.5 or 3.0 g/kg i.p.) and saline. We found that binge-like ethanol exposure during adolescence significantly reduced basal -MSH IR in the central nucleus of the amygdala (CeA), the arcuate nucleus (Arc) and the paraventricular nucleus of the hypothalamus (PVN) during adulthood. Additionally, acute ethanol elicited AgRP IR in the Arc. Rats given the adolescent ethanol treatment required higher doses of ethanol than saline-treated rats to express AgRP. In light of previous evidence that endogenous MC and AgRP regulate ethanol intake through MC-receptor signaling, we speculate that the -MSH and AgRP disturbances induced by binge-like ethanol exposure during adolescence may contribute to excessive ethanol consumption during adulthood.

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Keywords

-MSH; AgRP immunoreactivity; binge-like ethanol; adolescents

1. 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 in the hypothalamic arcuate nucleus (Arc) and nucleus of the solitary tract (Cone, 2005; Dores et al., 1986; Jacobowitz and O'Donohue, 1978; O'Donohue and Dorsa, 1982) and include adrenocorticotropic hormone, -melanocyte stimulating hormone (-MSH), - MSH, and -MSH (Hadley and Haskell-Luevano, 1999). The MC system possesses an inverse agonist, agouti-related peptide (AgRP), that has actions opposed to those of -MSH and acts on the same MC receptors (MC-R) (Chai et al., 2003; Haskell-Luevano and Monck, 2001, Nijenhuis et al., 2001).

Accumulating anatomic, genetic and pharmacological studies have provided evidence that MC and AgRP play key roles in ethanol consumption and neurobiological responses to ethanol. First, -MSH- and AgRP-producing cells project to brain regions that are critically involved in neurobehavioral responses to ethanol, including the nucleus accumbens (NAc), the ventral tegmental area (VTA), the bed nucleus of the stria terminalis (BNST) and the amygdale (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). Second, intracerebroventricular (i.c.v.) infusion of the potent non-selective MC-R agonist melanotan-II (MTII) significantly reduces voluntary ethanol drinking by adult AA (alko, alcohol) rats (Ploj et al., 2002) and C57BL/6J mice (Navarro et al., 2003); i.c.v administration of AgRP increases ethanol drinking (Navarro et al., 2003), and genetic deletion of endogenous AgRP reduces ethanol-reinforced lever pressing and binge-like ethanol drinking (Navarro et al., 2009). Third, relative to ANA (alko, non alcohol) rats, AA adult rats selectively bred for high ethanol intake exhibit lower levels of MC3-R in the shell of the NAc and higher levels of MC3-R in the paraventricular (PVN), Arc, and ventromedial (VMH) nuclei of the hypothalamus, which might be indicative of genetic disturbances in the central MC system that underlie high ethanol consumption in AA rats (Lindblom et al., 2002). Fourth, infusion of a selective MC4R agonist into the nucleus accumbens, but not into the lateral hypothalamus, reduces voluntary ethanol consumption, while infusion of the selective antagonist HS016 increases ethanol consumption without affecting caloric intake in adult Sprague-Dawley rats (Lerma-Cabrera et al., 2012). Moreover, stimulation of MC4R signaling in the accumbens shell region dramatically reduces ethanol palatability (Lerma-Cabrera et al., 2013), which suggests a key role for MC signaling in limbic regions in the hedonic responses to ethanol.

Previous evidence indicates that continued ethanol consumption alters basal -MSH and ethanol-stimulated AgRP regional immunoreactivity (IR). Thus, chronic exposure to an ethanol-containing diet in adult Sprague-Dawley rats reduces -MSHIR in the Arc, the lateral hypothalamus (LH) and the central nucleus of the amygdala (CeA) (Navarro et al., 2008), while abstinence following a chronic ethanol diet increases -MSH IR (Kokare et al., 2008). C57BL/6J mice, which show high rates of voluntary ethanol intake, have higher basal -MSH IR in the Arc, dorsomedial nucleus of the hypothalamus (DMH), and LH and lower -MSH IR in the medial amygdala relative to 129/SvJ mice, which exhibit low rates of spontaneous ethanol drinking (Cubero et al., 2010). Alternatively, acute ethanol injection increases AgRP, but not -MSH IR, at the Arc of adult high ethanol drinking C57BL/6J

mice, while AgRP IR remains unaffected in the moderate ethanol drinking 129/SvJ strain following an ethanol injection (Cubero et al., 2010).

Adolescence is an important period of brain development during which binge-like ethanol exposure causes long-lasting neuroadaptive changes in neural pathways that are critically involved in the neurobehavioral responses to ethanol (Maldonado-Devincci et al., 2010a; Allen et al, 2011) and increases the risk of ethanol consumption and preference during adulthood (Maldonado-Devincci et al., 2010b; Pascual et al., 2009). Notably, there is evidence that ethanol exposure during adolescence might also alter neuropeptide systems that are critically involved in voluntary ethanol intake. Thus, following binge-like ethanol exposure during adolescence, adult animals show increased mRNA expression of basal corticotropin releasing factor (CRF) in the PVN (Przybycien-Szymanska et al., 2011), decreased overall hippocampal neuropeptide Y IR in Wistar rats, and increased substance P and neurokinin IR in the caudate of Sprague-Dawley rats (Slawecki et al., 2005).

MC and AgRP have regulatory roles in ethanol consumption (Navarro et al., 2003; 2005; 2009; Ploj et al., 2002; York et al., 2011), and chronic ethanol diets reduce -MSH IR in hypothalamic and limbic brain regions in adult rats (Navarro et al., 2008). In light of these observations and given the vulnerability of the adolescent brain to ethanol exposure, the present study addresses whether binge-like ethanol exposure during adolescence [postnatal days (PND) 25–38] alters basal and/or ethanol-induced -MSH and AgRP IR later during adulthood (PND 63) in limbic and hypothalamic brain regions known to be involved in neurobiological responses to ethanol and ethanol consumption. To that end, we selected a specific pattern of intermittent binge-like ethanol exposure during adolescence that promotes alcohol intake in the adult rat (Pascual et al., 2009; Guerri and Pascual, 2010), induces neuroinflammatory damage (Pascual et al, 2009; 2011), induces chromatin remodeling, elicits changes in histone acetylation and methylation (Pascual et al., 2012), decreases monoamine levels in adulthood and alters the mesolimbic dopaminergic and glutamatergic systems (Pascual et al., 2009). Additionally, this experimental procedure promotes behavioral changes in social interaction behaviors, locomotor activity, anxiety-like responses, object memory recognition and conditioned taste aversion (Pascual et al., 2010; 2011; Rodriguez-Arias et al., 2011).

2. Materials and methods

2.1. Animals

Eighty-two Sprague-Dawley rat pups(Charles River Laboratories, Spain) were used as subjects in these experiments beginning at postnatal day 25 (PND25). The pups remained housed in groups of four rats per cage and maintained in an environmentally controlled room (22 °C temperature on a 12:12 h light-dark cycle). Standard rodent chow and water were provided *ad libitum* throughout the experiments, and all the manipulations were conducted during the dark phase. Behavioral procedures and pharmacological techniques were in compliance with the animal care guidelines established by the Spanish Royal Decrees 1025/2005 for reducing animal pain and discomfort, and the protocols were approved by the University of Almería Bioethical Animal Care and Use Committee.

2.2. Binge-like ethanol exposure during adolescence

Morning doses of either ethanol (3.0 g/kg, 25% w/v mixed in isotonic saline) given in an intraperitoneal (i.p.) injection (binge-like ethanol pre-treatment group, BEP) or an equal volume of isotonic saline (saline pre-treatment group, SP) were administered beginning on PND25. A second injection was given on PND26, followed by 2 days without injections. This pattern of injections and rest was repeated such that rats received injections on PND 25, 26, 29, 30, 33, 34, 37 and 38 as previously described (Pascual et al., 2007; 2009) (Fig. 1).

2.3. Acute ethanol administration

On PND63, 25 days after the last injection was administered during adolescence, animals in the BEP and SP groups were randomly re-assigned to three sub-groups according to ethanol doses. Because -MSH and AgRP have been implicated in feeding behaviors (Coll and Tung, 2009; Pandit et al., 2011), procedures were carried out during the animals' light cycle, a time of day in which feeding behavior in rats is low, to avoid possible confounding effects of food/water consumption on -MSH and AgRP IRs; additionally, water and food were removed on the test day from all cages 5 minute before the injections. Injections began 2 hours into the light cycle and were staggered (counterbalanced by pretreatment and injection condition), and rats were perfused exactly 2 hours after injection. Rats were given an i.p. injection of either saline or one of two doses of ethanol (1.5 g/kg or 3.0 g/kg; 25% w/v mixed in isotonic saline) and then returned to their home cage immediately after the injection, where they remained until perfusion procedures.

2.4. Blood ethanol concentrations (BECs) in response to acute ethanol in adulthood in the saline (SP) and binge-like ethanol (BEP) pre-treated groups

To estimate whether binge-ethanol pre-treatment during adolescence altered ethanol metabolism later during adulthood, we avoided the induction of confounding effects on the interpretation of molecular data by giving separate sets of binge-like ethanol and saline pre-treated adult rats i.p. injections of 1.5 g/kg (BEP: n=5; SP: n=6) or 3.0 g/kg (BEP: n=5; SP: n=6) doses of ethanol at PND63, and blood samples were collected 2 hours later to assess BECs. Approximately 10 µl of blood was collected from the tail vein of each rat; the samples were centrifuged, and 5 µl of plasma from each sample was analyzed for BECs, which were measured in mg/dl (Analox Instruments, Lunenburg, MA). This study assured that differences found in peptide IRs between experimental conditions were not primarily associated with group differences in peripheral ethanol metabolism.

2.5. Perfusions, Brain Preparation, and Immunohistochemistry

Two hours after ethanol or saline injection, rats were euthanized with an overdose of sodium pentothal (80 mg/kg in 1 ml/kg volume) and transcardially perfused with phosphate buffered saline (PBS) followed by 0.1 M phosphate buffered paraformaldehyde 4% (pH 7.4). We chose a 2 hour post-injection perfusion time because we have previously found treatment-induced differences in proteins at this time point (Cubero et al., 1999; Cubero et al., 2010; Thiele et al., 2000). Brains were removed and post-fixed in paraformaldehyde for 48 h at 4°C, at which point they were transferred to PBS. The cerebrums were cut in 50-µm-thick coronal sections with a motorized vibrotome and stored in PBS until the immunohistochemistry (IHC) assay. The sections were evenly divided into two sets (every other section) for processing with -MSH or AgRP antibodies.

2.5.1. IHC for α-MSH and AgRP expression—After rinsing in fresh PBS 4 times (10 minutes each), the tissue sections were blocked in 10% rabbit serum (for -MSH) or 10% goat serum (for AgRP) and 0.1% triton-X-100 in PBS for 1 hour. The sections were then transferred to fresh PBS containing primary sheep anti- -MSH (Millipore, Billerica, MA; 1:10,000) or primary rabbit anti-AgRP (Phoenix Pharmaceuticals, Inc., Burlingame, CA; 1:4,000) for 3 days at 4°C. After the 3 days of incubation, the sections were rinsed (4X, PBS) and then processed with Vectastain Elite kits (Vector Labs) according to the manufacturer's instructions for standard ABC/HRP/ diaminobenzidine-based IHC. The sections processed for -MSH or AgRP were visualized by reacting the sections with a 3,3-diamino- benzidine tetrahydrochloride (DAB, Polysciences, Inc., Warrington, PA) reaction solution containing 0.05% DAB, 0.005% cobalt, 0.007% nickel ammonium sulfate, and

0.006% hydrogen peroxide. Following proper development, the slices were rinsed (PBS, 10 min), mounted on glass slides, and cover slipped with Permount.

2.5.2. Quantification of regional α-MSH and AgRP immunoreactivity—Digital images of -MSH and AgRP IR were obtained on an Olympus BX50 microscope equipped with a Pixel Link digital camera running Visiopharm software. A total of 4 different brain regions were collected based on Paxinos and Watson's stereotaxic atlas coordinates (Paxinos and Watson, 1998). We quantified -MSH IR in regions of the hypothalamus (Arc: bregma −2.12 to −2.8 mm, PVN: bregma −1.8 to −2.12 mm and LH: bregma −1.8 to −2.56 mm) and the CeA: bregma −3.14 to −2.30 mm; i.e., regions in which we have found robust -MSH staining and which have been implicated in neurobiological responses to ethanol (Barson et al., 2011; Cannella et al., 2009; Chang et al., 1995; Vilpoux et al., 2009). Consistent with our previous findings (Cubero et al., 2010; Navarro et al., 2008), AgRP IR was observed primarily in the Arc. During the analysis, we took great care to match sections through the same region of brain and at the same level using anatomic landmarks with the aid of a rat stereotaxic atlas (Paxinos and Watson, 1998). Densitometric procedures were used to assess protein levels. Flat-field corrected digital pictures (8-bit gray scale) were taken using the Pixel Link digital camera, and the density of staining was analyzed using Image J software (Image J, National Institute of Health, Bethesda, MD) by calculating the percent of the total area examined that showed signal (cell bodies and processes) relative to a sub-threshold background. The sizes of the areas that were analyzed were the same between animals and groups. The sub-threshold level for the images was set such that any area without an experimenter-defined level of staining was given a value of zero. Anatomically matched pictures of the left and right sides of the brain were used to produce an average density for each brain region from each slice. In all cases, quantification of the IHC data was conducted by an experimenter who was blind to the experimental conditions. The procedure for densitometric AgRP IR measurements was validated by manual cell counting. To do this, all visible cell bodies stained within the defined brain region were counted manually by an experimenter blinded to group condition. Data from each brain region from each animal were calculated by taking the average counts from 2 brain slices. Data from each slice were calculated by taking the average counts from the left and right sides of the brain at the specific brain region of interest. Because the densitometric procedure for -MSH measures has previously been validated by manual counting (Navarro et al., 2008), no additional manual cell counting for -MSH IR data was performed in the present study.

Data Analyses: All data collected in this study are presented as the mean ± SEM, and differences between groups were analyzed using analyses of variance (ANOVAs). The assumptions of the ANOVA model for each set of data were assessed before ANOVA analysis in all cases. When the statistical model assumptions were not met, appropriate alternative statistical tests were applied. Because we expected that the ethanol-induced alterations of -MSH and AgRP would be site specific, separate two-way, 2×3 (pre-treatment x dose) ANOVAs were performed on the IHC data collected from each brain region. Additional independent ANOVAs were performed on baseline body weight (BW) data collected during adolescence and adulthood and on BECs. When significant differences were found, post hoc analyses were conducted using DHS-Tukey contrasts because this test controls the type I error rate and is more powerful in small samples. In all cases, $p < 0.05$ (two-tailed) was used as the level of statistical significance. In addition to the statistical significance levels, two effect sizes estimates were calculated: 1) *Cohen's d* for contrasts that implicate 2 means, and 2) ^{2} (*eta-squared*) for those contrasts implicating more than 2 means. These indicators provided estimates of the magnitude of the effects that are independent of the sample size.

3. Results

3.1. BW in saline (SP) and binge-like ethanol (BEP) treated groups

The role of -MSH and AgRP in BW regulation is well established (Seeley et al., 2004). Given that binge-ethanol exposure during adolescence may produce temporary reductions in BW and that early undernutrition and being underweight have been linked to disturbed neuroendocrine parameters, altered activity in the hypothalamic-pituitary-adrenal (HPA) axis (Allen et al, 2011; Bouret, 2010; Ferreti et al., 2011; Grayson et al., 2010) and changes in POMC expression (Ehrlich et al., 2010), we analyzed the BWs of the BEP and SP animals at different time-points: a) during adolescence, immediately before (BW baseline on PND25) and after the conclusion of binge-ethanol pretreatment (on PND39), and b) during adulthood (averaged BWs over PND59-PND63).

3.1.1. BW during adolescence—A 2×2 (pre-treatment x time) mixed factor repeated-measures ANOVA was used to compared BW (g) data collected pre- (BW baseline on PND25) and post-binge-ethanol treatment (on PND39) during adolescence. The ANOVA revealed significant main effects of pre-treatment [$F_{(1, 24)} = 8.85$, $p < 0.05$] and time [$F_{(1, 24)} = 1847.12$, $p < 0.05$] and a significant interaction (pre-treatment x time) effect [$F_{(1, 24)} = 46.08$, $p < 0.05$]. The interaction was analyzed with the simple-effect analysis strategy using DHS-Tukey tests. The results showed that, while BW baseline were similar in the BEP (86.08 ± 2.05 g) and SP (84.62 ± 1.62 g) during adolescence (BW on PND25), BWs were significantly reduced in BEP animals (on PND39) when measured at the conclusion of the binge-ethanol treatment (BEP $= 240.4 \pm 7.09$ g; SP $= 296.81 \pm 5.6$ g). The effect size estimate for the interaction effect was $^2 = 0.66$.

3.1.2. BW during adulthood—A one-way ANOVA (pre-treatment) was conducted on averaged BW (g) data collected daily for 5 days prior to the experimental manipulations during adulthood, beginning on PND59. The ANOVA revealed no significant main effect of pre-treatment [$F_{(1, 20)} = 3.64$, $p > 0.05$], [mean $\pm$ SEM: SP $= 359 \pm 7.47$ g and BEP $= 339 \pm 6.77$ g]; this finding is consistent with previous reports that have employed the same binge-like ethanol exposure procedure (Pascual et al., 2009) and indicates that adult rats that were previously underweight during adolescence due to binge-ethanol exposure completely recovered their BWs.

3.2. Blood ethanol concentration (BEC) in response to acute ethanol in saline (SP) and binge-like ethanol (BEP) pre-treated groups

One key question of our study was whether binge-like ethanol treatment during adolescence causes primary disturbances in ethanol metabolism during adulthood. We measured BECs (mg/dl) in adulthood in response to acute ethanol exposure (1.5 g/kg or 3 g/kg, i.p.) in the BEP (n = 5 per dose condition) and SP (n = 6 per dose condition) rats. Due to non-compliance with the assumptions for parametric tests and the reduced sample size in this study, the Wilcoxon-Mann-Whitney test using exact inference was performed. This analysis revealed no group differences in BECs reached in the SP and BEP adult animals in response to administration of 1.5 g/kg doses of ethanol ($p = 0.53$; mean $\pm$ SEM SP $= 114.36 \pm 9.20$ mg/dl and BEP $= 121.35 \pm 10.15$ mg/dl) or in response to the administration of 3.0 g/kg doses of ethanol ($p = 0.08$; mean $\pm$ SEM SP $= 265.14 \pm 10.44$ mg/dl and BEP $= 289.51 \pm 4.20$ mg/dl). Although our statistical analyses suggest that binge-like ethanol treatment during adolescence does not alter ethanol metabolism during adulthood, it is premature to completely rule out the existence of mild metabolic disturbances in BEP rats.

3.3. α-MSH IHC in regions of the hypothalamus and CeA following saline or ethanol injection in saline (SP) and binge-like ethanol (BEP) pre-treated groups

3.3.1. Arcuate nucleus of the hypothalamus—The average densities of -MSH IR data in the Arc of SP and BEP rats given i.p. injections of saline (n = 6 and 9, respectively) and a 1.5 g/kg dose of ethanol (n = 7 and 10, respectively) or a 3.0 g/kg dose of ethanol (n = 6 and 10, respectively) were analyzed by a two-way (2×3) (pre-treatment x dose) ANOVA, which revealed a significant main effect of pre-treatment [F (1, 42) =3.84, p < 0.05] resulting from lower -MSH IRs in the Arc of BEP animals (figure 2). The Cohen's d effect size was 0.55. The main effects of dose [F (2, 42) = 0.86, p > 0.05] and the pre-treatment x dose interaction [F (2, 42) = 1.07, p > 0.05] did not attain statistical significance.

3.3.2. Paraventricular nucleus of hypothalamus—The average densities of the -MSH IR data in the PVN of SP and BEP rats given i.p. injections of saline (n = 6 and 9, respectively) and a 1.5 g/kg dose of ethanol (n = 8 and 10, respectively) or a 3.0 g/kg dose of ethanol (n = 7 and 10, respectively) were analyzed with a two-way (2×3) (pre-treatment x dose) ANOVA that revealed a statistically significant main effect of pre-treatment [F (1, 44) = 4.814, p < 0.05] resulting from lower -MSH IRs in the PVN of the BEP group (figure 3). The Cohen's *d* effect size was 0.67. The main effect of dose [F (2, 44) = 0.792, p > 0.05] and the interaction effect [F (2, 44) = 1.515, p > 0.05] did not attain statistical significance.

3.3.3. Lateral hypothalamic area—The average densities of the -MSH IR data in the LH of SP and BEP rats given i.p. injections of saline (n = 6 and 9, respectively), a 1.5 g/kg dose of ethanol (n = 8 and 10, respectively) or a 3.0 g/kg dose of ethanol (n = 8 and 10, respectively) were analyzed by a two-way (2×3) (pre-treatment x dose) ANOVA. No statistically significant effects of pre-treatment [F (1, 45) = 0.476, p > 0.05], dose [F (2, 45) = 1.979, p > 0.05], or the pre-treatment x dose interaction [F (2, 45) = 2.775, p > 0.05] (figure 4) were significant.

3.3.4. Central nucleus of amygdala—The average densities of the -MSH IR data from the CeA of SP and BEP rats given i.p. injections of saline (n = 5 and 9, respectively), 1.5 g/kg dose of ethanol (n = 8 and 10, respectively) or a 3.0 g/kg dose of ethanol (n = 7 and 10, respectively) were analyzed by a two-way (2×3) (pre-treatment x dose) ANOVA, which revealed a significant main effect of pre-treatment [F (1, 43) = 4.897, p < 0.05] resulting from lower -MSH IRs in the CeA of the BEP group (figure 5). The Cohen's *d* effect size was 0.7. Neither the main effect of dose [F (2, 43) = 0.514, p > 0.05] nor the interaction effect [F (2, 43) = 0.280, p > 0.05] attained statistical significance.

3.4. AgRP IHC in the arcuate nucleus following saline or ethanol injection in saline (SP) and binge-like ethanol (BEP) pre-treated rats

The average densitometric densities of AgRP IRs in the Arcs of SP and BEP rats given i.p. injections of saline (n = 7 and 5, respectively), a 1.5 g/kg dose of ethanol (n = 8 and 9, respectively) or a 3.0 g/kg dose of ethanol (n = 8/group), were analyzed by a two-way (2×3) (pre-treatment x dose) ANOVA. This analysis revealed a statistically significant main effect of dose [F (2, 39) = 6.65, p < 0.05] and a significant dose x pre-treatment interaction [F (2, 39) = 4.49, p < 0.05]; the pre-treatment main effect was not statistically significant [F (1, 39) = 0.30, p > 0.05] (figure 6A). Additional DHS-Tukey tests conducted to further analyze the interaction revealed no statistically significant differences between pre-treatment groups in terms of AgRP IR in responses to acute saline. Moreover, while the AgRP IR of the SP group was significantly increased in response to the lower ethanol dose of ethanol (1.5 g/kg) relative to saline administration, AgRP IRs were significantly increased in the BEP group only after administration of the highest dose of ethanol (3.0 g/kg). The effect size estimate for the interaction was $^2 = 0.64$. An additional two-way (2×3) (pre-treatment x

dose) ANOVA conducted on the manually scored AgRP IR measures showed the same pattern of statistical results revealed by the densitometric procedures (figure 6B). Thus, this analysis revealed a statistically significant main effect of dose [$F_{(2, 39)} = 12.9$, $p < 0.01$] and a significant dose x pre-treatment interaction [$F_{(2, 39)} = 3.7$, $p < 0.05$] (figure 6B). The effect size estimate for this interaction was $^2 = 0.17$. The pre-treatment main effect was not statistically significant [$F_{(1, 39)} = 1.7$, $p > 0.05$] (figure 6A). Additional DHS-Tukey tests conducted to analyze the interaction revealed the same pattern of results obtained by the densitometric analysis.

4. Discussion

The present study addressed whether a pattern of binge-like ethanol pre-treatment during adolescence alters basal and/or ethanol-stimulated regional -MSH and AgRP activity in brain regions known to be involved in neurobiological responses to ethanol during adulthood. The most important observations were the following: 1) binge-like ethanol pre-treatment during adolescence reduced basal -MSHIR in the Arc, PVN, and CeA during adulthood; 2) acute ethanol triggered AgRP, but not -MSH, IR in the Arc when tested during adulthood in both the SP and BEP groups; 3) while SP animals showed increased AgRP IR in response to the low dose of ethanol employed, BEP animals showed increased AgRP IR in response to the high dose.

We found significant reductions in basal -MSH IR in the Arc, the PVN, and the CeA but not in the LH in animals pre-exposed to a binge-like ethanol exposure pattern during adolescence. These data are consistent with and extend previous molecular evidence showing that continued ethanol exposure in adult rats reduces -MSH IR in some hypothalamic brain regions and, in the CeA (Navarro et al 2008; Rainero et al., 1990), continued alcohol exposure blunts -MSH protein precursor POMC mRNA expression (Rasmussen et al., 2002; Scanlon et al., 1992; Zhou et al., 2000) and POMC IR in the Arc (Navarro et al, 2012). The observation that binge-like ethanol pre-treatment reduced -MSH in some, but not all, regions, suggests that the effects are brain-region specific and limits the possibility that reductions in -MSH IR reflect non-specific ethanol-induced cellular toxicity.

One hypothesis that could explain the present data relies on the observed impact of binge-ethanol on BW during adolescence. BEP animals showed a significant reduction in BW during adolescence, but, nonetheless, they progressively recovered and showed no significant group differences at the time of testing during adulthood (PND63). There is consistent experimental evidence that being underweight and being exposed to under-nutrition are physiological conditions that cause long-term, long-lasting disruption of neuroendocrine parameters and HPA axis activity (Allen et al, 2011; Bouret, 2010; Ferreti et al., 2011; Grayson et al., 2010). Importantly, the roles of endogenous -MSH and AgRP signaling in the PVN, Arc and CeA in BW regulation are well established (Seeley et al., 2004). Together, these results do not resolve whether changes in the basal -MSH activities in the hypothalamus and amygdala are directly caused by the pharmacological properties of ethanol administered during adolescence or if they result from the early and prolonged reduction in BW during the developmental period. Nonetheless, the relevant conclusion of this study is that binge-ethanol during adolescence, either directly or indirectly, is associated with changes in MC function during adulthood.

Stress early in life significantly modifies the neural activity of the stress circuitry, which includes the amygdala and the hypothalamus (McCormick et al., 2010), and there is evidence that binge-pattern alcohol exposure during puberty induces long-term changes in HPA axis reactivity (Przybycien-Szymanska et al., 2011). In light of this aforementioned

evidence, one might speculate that repeated administration of highly concentrated doses of ethanol during adolescence increases neural activity in the brain's stress system, which leads to long-term reductions in basal MC activity. However, in opposition to this idea, pharmacological and molecular evidence has shown that stress, anxiety-like responses and depression (Chaki and Okubo, 2007; Chaki and Okuyama, 2005; Kokare et al., 2010; Lim et al., 2012; Liu et al., 2007) all enhance, rather than blunt, MC activity. First, i.c.v. infusion of α-MSH triggers an anxiogenic-like effect (Kokare et al., 2008). Second, the anxiolytic-like effect of ethanol is suppressed by central administration of α-MSH, while pretreatment with the selective MC4-R antagonist HS014 enhances the anxiolytic action of ethanol and markedly blocks ethanol withdrawal anxiety (Kokare et al., 2008). Third, acute emotional stressors (e.g., restraint and forced swimming) evoke mRNA expression of c-fos in a high percentage of pro-opiomelanocortin neurons (Liu et al., 2007). In light of these observations that indicate the existence of a direct positive relation between stress and MC activity, the reduced regional basal α-MSH IR observed in the present study seems unlikely to be related to early stress derived from a binge-pattern alcohol exposure during adolescence.

Given the literature surrounding the role of α-MSH in stress responses (Chaki and Okuyama, 2005; Chaki et al., 2003, 2005; Kokare et al., 2005; Nozawa et al., 2007; Shimazaki and Chaki, 2005), it might seem surprising that an acute ethanol challenge during the test day, which has been shown to be a stressor that activates the HPA axis in ethanol-naïve rats (Ogilvie et al, 1997), caused no significant increases in α-MSH IR in the SP or BEP rats as measured by IHC procedures. Given previous experimental evidence that shows that most protein expression in the hypothalamus peaks 2–3 hr after alcohol injection (Chang et al, 2007), the employment of quantitative approaches (i.e., western blot analyses or quantitative real-time polymerase chain reactions) aimed at quantifying protein levels, together with the inclusion of additional time-windows to assess α-MSH IR and/or the placement of permanent i.p. cannulas to deliver alcohol without handling may help to further clarify whether acute stress due to ethanol injections elicits relevant changes in regional α-MSH expression.

The present data showing site-specific reductions in basal α-MSH IR in adult rats pre-treated with binge-like ethanol exposure during adolescence are consistent with recent pharmacological, behavioral and molecular evidence that suggests a role for endogenous α-MSH in ethanol consumption. Thus, i.c.v. or CeA infusion of the MC3/MC4R agonist MTII has been shown to reduce alcohol consumption in mice (Navarro et al., 2003; 2005), alcohol-preferring AA rats (Ploj et al., 2002) and P rats (York et al., 2011). Additionally, administration of a selective MC4R antagonist into the nucleus accumbens reduces voluntary ethanol consumption (Lerma-Cabrera et al, 2012) and ethanol palatability (Lerma-Cabera et al, 2013). Similarly, in situ hybridization studies have shown increased ratios of POMC/AgRP mRNA in the Arc of alcohol-preferring AA rats (Lindblom et al., 2002). Given the well-known roles of the hypothalamus and the amygdala in ethanol intake (Allen et al., 2011; Gilpin and Roberto, 2012; McBride, 2002; Schepis et al., 2011), the available experimental evidence showing a role for MC signaling in ethanol consumption, and the fact that binge-like ethanol exposure during adolescence increases the probability of ethanol consumption during adulthood (Pascual et al., 2009; Maldonado-Devincci et al., 2010b), it is tempting to propose that long-lasting reduction of α-MSH IR in the Arc, PVN and CeA by binge-like ethanol pretreatment during adolescence might contribute to increased vulnerability to ethanol consumption during adulthood.

As revealed by IHC procedures, the reduction in α-MSH IR could indicate that ethanol inhibits normal endogenous α-MSH signaling via reduced production of α-MSH. The observations that exposure to ethanol inhibits α-MSH IR in the Arc, (a MC-producing brain region), the PVN and the CeA (MC-projecting brain regions), together with the fact that

ethanol administration blunts POMC mRNA in the Arc (Rasmussen et al., 2002; Scanlon et al., 1992; Zhou et al., 2000), leads us to speculate that binge-like ethanol treatment during adolescence disrupts -MSH synthesis during adulthood. Given that, first, MCs are POMC-derived peptides that are generated by extensive post-translational processing involving several enzymes including pro-hormone convertase 1/3 and 2 (PC1/3 and PC2), and second, ethanol exposure reduces POMC IR (Navarro et al, 2012) and the enzyme prohormone convertase PC1/3 IR in the arcuate nucleus of rats (Navarro et al., 2012), we speculate that reductions of basal -MSH IR in the hypothalamus and amygdala in BEP rats stem from the low availability of POMC in -MSH producing cells in the Arc, which, in turn, results from blunted processing of POMC and/or the enzyme pro-hormone convertase PC1/3 following binge-ethanol pretreatment.

The second relevant observation of this study is that acute administration of ethanol during adulthood increased AgRP IR in the Arc in both BEP and SP rats. Given the role played by AgRP in energy balance and caloric intake (de Backer et al., 2011; Ilnytska and Argyropoulus, 2008) and that ethanol has calories, it is possible that ethanol-stimulated AgRP IR resulted from the extra calories that are inherent to ethanol. However, caloric loads (Chang et al., 2005; Ziotopoulou et al., 2000) are associated with decreased AgRP expression, which makes it unlikely that the observed increase in AgRP IR were caused by ethanol's calories. The differences in ethanol-stimulated AgRP IR observed in the pre-treatment groups during adulthood were likely not secondary to group differences in ethanol metabolism because no significant group differences were detected in the BEC reached following any of the two doses of ethanol employed during the test day. Finally, because no group differences in BW were found during adulthood, absolute dosing volume is also not an explanation for the alteration of the dose-response curve for ethanol-stimulated AgRP IR.

The present findings are consistent with previous molecular studies conducted in our laboratory in C57BL/6J and 129/SvJ inbred mice (Cubero et al., 2010), which in tandem with pharmacological evidence support the stimulatory effect of acute ethanol exposure on AgRP signaling and the role of AgRP in ethanol drinking. Thus, C57BL/6J mice, which show twice the level of voluntary ethanol drinking displayed by 129/SvJ mice when offered a 10% ethanol solution (Belknap et al., 1993), show an increase of AgRP IR in the Arc in response to acute ethanol, while AgRP IR in 129/SvJ mice remains unaffected (Cubero et al., 2010). Similarly, central infusion of the AgRP-(83 to 132) fragment and nucleus accumbens administration of the selective MC4R antagonist HS014 both increase ethanol drinking in C57BL/6J mice (Navarro et al., 2005) and rats (Lerma-Cabrera et al., 2012), while genetic deletion of AgRP reduces ethanol self-administration in operant tasks (Navarro et al., 2009).

An interesting hypothesis that has previously been proposed for some peptides that contribute to ethanol intake, such as galanin and orexin (Barson et al., 2011; Schneider et al., 2007) and AgRP (Cubero et al., 2010) states that ethanol-induced increases in peptide signaling are part of a mechanism that involves a positive feedback loop in which ethanol intake rapidly stimulates peptide synthesis, which in turn promotes further excessive binge-like drinking. The present observation that rats showed a significant increase in AgRP IR following administration of ethanol agrees with this loop idea. Additionally, our findings extend the loop hypothesis by suggesting an inverted U pattern that might vary across individuals and strains. A weakness in the present interpretation of the AgRP IR data from BEP animals is that, according to the loop hypothesis, they should drink more ethanol and trigger AgRP synthesis, which contrasts the available evidence that suggests that binge-ethanol exposure during adolescence promotes ethanol consumption during adulthood (Pascual et al., 2009). Nonetheless, given the positive trend (although this trend was non-significant) indicating that BEP animals exhibited increased AgRP signaling in response to

low doses of acute ethanol, it is premature to rule out the loop hypothesis. Additional studies testing higher doses of ethanol will help to better characterize the ethanol dose-AgRP IR response curve in BEP animals and to understand the impact of early binge-ethanol exposure on AgRP stimulation in response to acute ethanol administration and ethanol consumption during adulthood.

5. Conclusion

Here, we show for the first time that binge-like ethanol treatment during adolescence, whether through direct or indirect effects on BW, reduces basal -MSH IR in the hypothalamus and the CeA and changes ethanol-stimulated AgRP IR stimulation during adulthood. Given the role of -MSH and AgRP in energy homeostasis and ethanol drinking, it is possible that reduced -MSH signaling stemming from adolescent binge-like ethanol exposure may contribute to feeding disorders and/or increased vulnerability to initiate ethanol consumption in adulthood (Maldonado-Devincci et al., 2010b; Pascual et al., 2009), while neuroadaptive changes in AgRP signaling may contribute to binge-like ethanol and/or excessive food consumption. Nonetheless, future studies employing techniques aimed at quantifying protein levels (i.e., western blot analyses) will provide a better understanding of ethanol-elicited changes in AgRP and -MSH protein levels.

Acknowledgments

Abbreviations

AA Alko	alcohol rats
AgRP	agouti-related peptide
ANA Alko	non alcohol rats
Arc	hypothalamic arcuate nucleus
BEC	blood ethanol concentration
BEP	binge-like ethanol pre-treatment group
BNST	bed nucleus of the stria terminalis
BW	body weight
CeA	central nucleus of amygdala
CRF	corticotropin releasing factor
DMH	dorsomedial nucleus of the hypothalamus
HPA	hypothalamic-pituitary-adrenal axis
IHC	immunohistochemistry
IR	immunoreactivity
LH	lateral hypothalamus
MC	melanocortin
MC-R	MC receptors
MTII	Melanotan-II

NAc	nucleus accumbens
P	alcohol-preferring rats
PND	postnatal day
POMC	pro-opiomelanocortin
PC1/3	pro-hormone convertase 1/3
PVN	paraventricular nucleus of the hypothalamus
SP	saline pre-treatment group
VMH	ventromedial nucleus of the hypothalamus
VTA	ventral tegmental area
-MSH	-melanocyte stimulating hormone
-MSH	-melanocyte stimulating hormone
-MSH	-melanocyte stimulating hormone

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Highlights

1. We test if binge ethanol exposure to adolescents alters melanocortin IR in adults

2. Ethanol pre-treatment during adolescence reduced basal -MSH IR during adulthood

3. Ethanol pre-treated rats required a higher dose of ethanol to show AgRP IR

4. MC dysfunction may increase vulnerability to ethanol consumption during adulthood
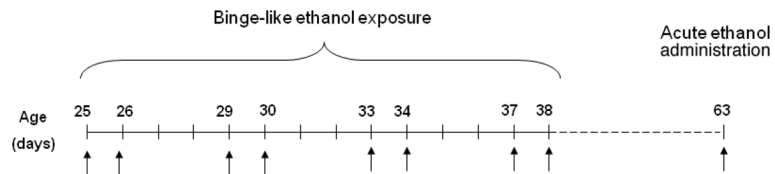
Figure 1.
Schematic temporal representation of ethanol injections during binge-like ethanol pre-treatment during adolescence beginning on PND25. Twenty-five days after the last injection was administered, adult rats were tested on PND63 with one of two acute doses of ethanol (1.5 g/kg or 3 g/kg) or saline to evaluate basal and ethanol-stimulated regional -MSH and AgRP immunoreactivity.
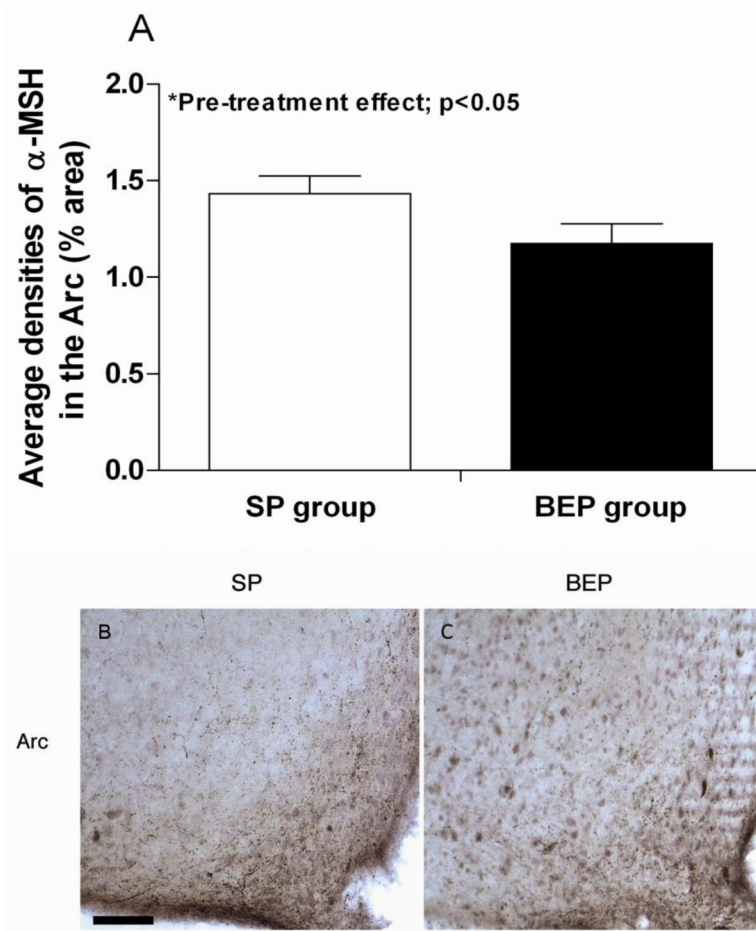
Figure 2.
(A) The graph represents the quantification of -MSH immunoreactivity (IR) (% area) in the arcuate nucleus of the hypothalamus (Arc). As neither the main effect of dose nor the dose x pre-treatment interaction were significant, the -MSH IR data following administration of saline or one of the two possible doses of ethanol were collapsed, and only the statistically significant pre-treatment main effect ($p < 0.05$) is graphically represented. Representative photomicrographs of 50 μm coronal sections showing -MSH immunoreactivity (IR) in SP (B) and BEP (C) animals receiving saline during the test day are depicted in the figure. Images were photographed and quantified at a magnification of 10×. Scale bar = 150 μm. Values are represented as the mean ± SEM.
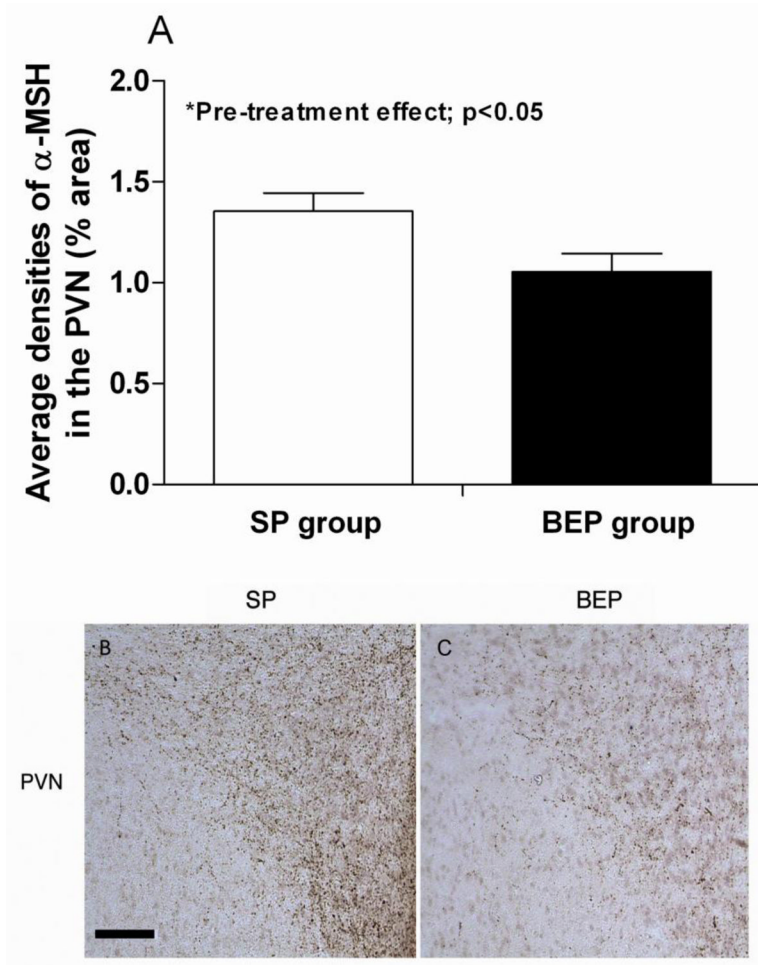
Figure 3.
(A) The graph represents the quantification of -MSH immunoreactivity (IR) (% area) in the paraventricular nucleus of the hypothalamus (PVN). As the main effect of dose and the dose x pre-treatment interaction were not significant, -MSH IR data following administration of saline or one of two possible doses of ethanol are collapsed, and only the statistically significant pre-treatment main effect (p < 0.05) is graphically represented. Representative photomicrographs of 50 μm coronal sections showing -MSH immunoreactivity (IR) in SP (B) and BEP (C) animals receiving saline during the test day are depicted in the figure. Images were photographed and quantified at a magnification of 10×. Scale bar = 150 μm. Values are represented as the mean ± SEM.
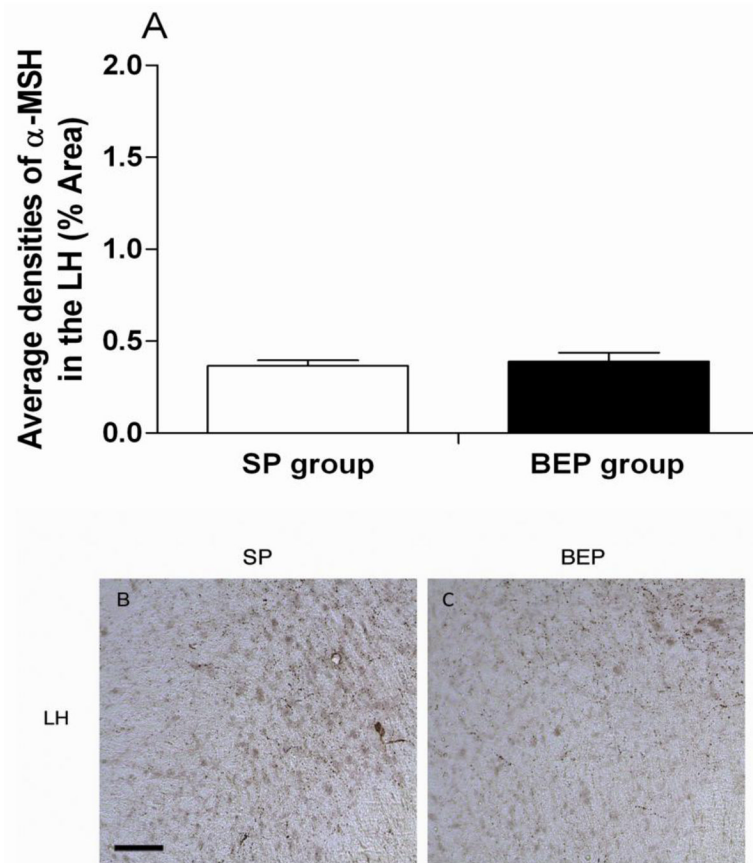
Figure 4.
(A) The graph represents the quantification of -MSH immunoreactivity (IR) (% area) in the lateral hypothalamus (Arc). As neither the main factor nor the interaction attained statistical significance, -MSH IR data following administration of saline or one of two possible doses of ethanol are collapsed, and only the pre-treatment main effect (p > 0.05) is represented. Representative photomicrographs of 50 μm coronal sections showing -MSH immunoreactivity (IR) in SP (B) and BEP (C) animals receiving saline during the test day are depicted in the figure. Images were photographed and quantified at a magnification of 10×. Scale bar = 150 μm. Values are represented as the mean ± SEM.
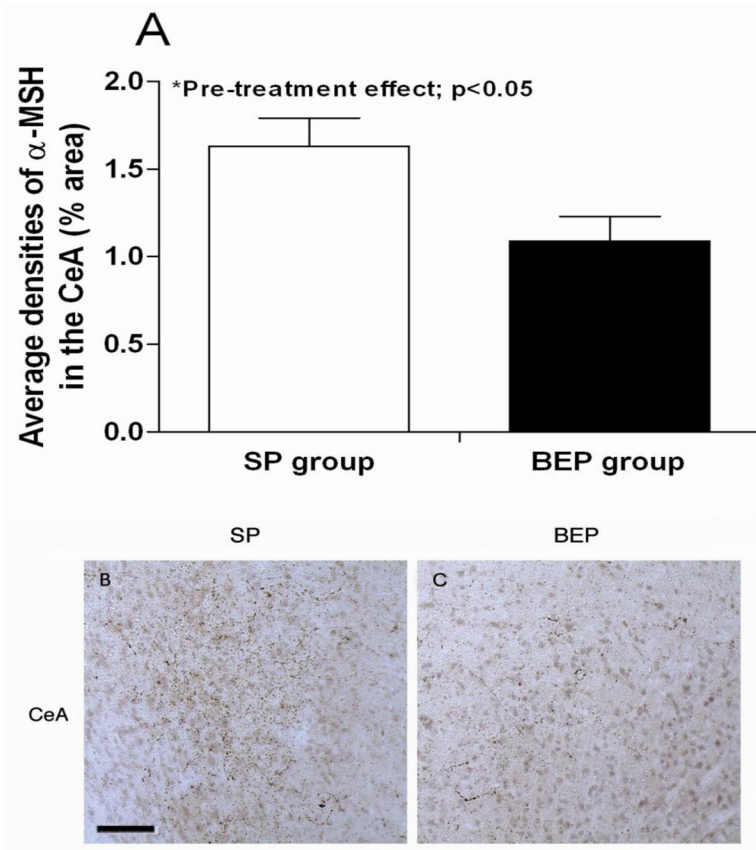
Figure 5.
(A) The graph represents the quantification of -MSH immunoreactivity (IR) (% area) in the central nucleus of the amygdale (CeA). As neither the main effect of dose nor the dose x pre-treatment interaction were significant, -MSH IR data following administration of saline or one of two possible doses of ethanol are collapsed, and only the statistically significant pre-treatment main effect ($p < 0.05$) is graphically represented. Representative photomicrographs of 50 μm coronal sections showing -MSH immunoreactivity (IR) in SP (B) and BEP (C) animals receiving saline during the test day are depicted in the figure. Images were photographed and quantified at a magnification of 10×. Scale bar = 150 μm. Values are represented as the mean ± SEM.
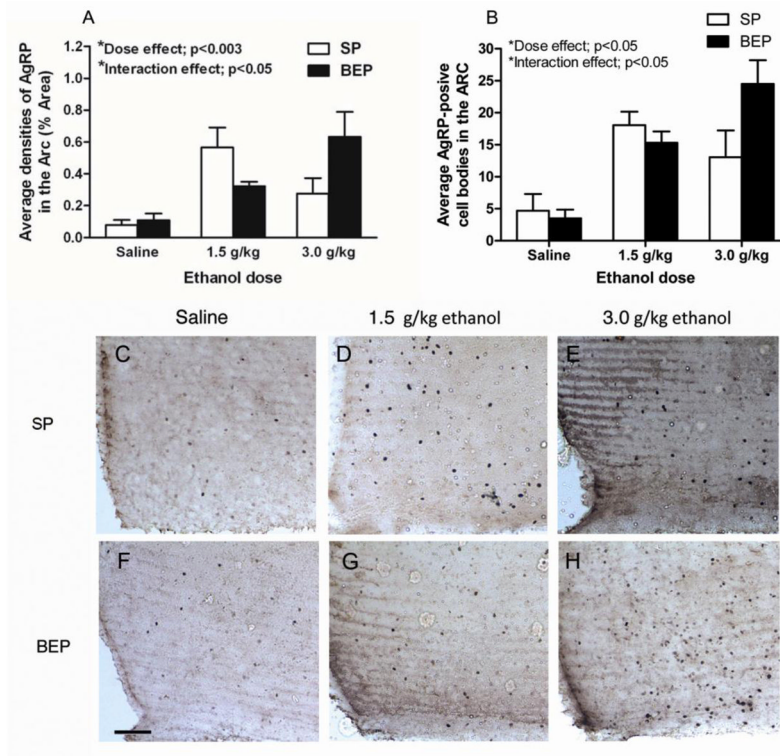
Figure 6.
The graphs represent the quantification of AgRP immunoreactivity (IR) in the arcuate nucleus of the hypothalamus (Arc). Quantification was performed by counting -MSH-positive cell bodies (A) or by measuring the density of -MSH staining (B) using Image J software, which calculated the percent of the total area examined (% area) that showed signal relative to a subthreshold background. The graphs show that administration of 3.0 g/kg doses of ethanol triggered AgRP IR in BEP rats, while 1.5 g/kg doses of ethanol triggered the greatest increase of AgRP IR compared to saline in SP rats. Representative photomicrographs of 50 μm coronal sections showing -MSH immunoreactivity (IR) in SP (C, D, E) and BEP (F, G, H) animals receiving saline during the test day are depicted in the figure. Images were photographed and quantified at a magnification of 10×. Scale bar = 150 μm. Values are represented as the mean ± SEM.