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Proopiomelanocortin peptides are not essential for development of ethanol-induced behavioral sensitization

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Abstract

Background—Behavioral sensitization is a result of neuroadaptation to repeated drug administration and is hypothesized to reflect an increased susceptibility to drug abuse. Proopiomelanocortin (POMC) derived peptides including β -endorphin and α -melanocyte stimulating hormone have been implicated in development of behavioral sensitization and the reinforcing effects of alcohol and other drugs of abuse. This study used a genetically engineered mouse strain that is deficient for neural POMC to directly determine if any POMC peptides are necessary for the development of ethanol-induced locomotor sensitization.

Methods—Adult, female mice deficient for POMC in neurons only (*Pomc*^{-/-} *Tg/Tg*, KO) and wildtype (*Pomc*^{+/+} *Tg/Tg*, WT) littermates were injected once daily with either saline or ethanol (i.p.) for 12–13 days. On ethanol test day (day 13 or 14) all mice from both treatment groups received an i.p. injection of ethanol immediately before a 15-minute analysis of locomotor activity. Blood ethanol concentration (BEC) was measured on ethanol test day immediately following the test session. Baseline locomotor activity was measured for 15 minutes after a saline injection two days later in both groups.

Results—There was no significant difference in BEC between genotypes (WT=2.11±0.06; KO=2.03±0.08 mg/ml). Both WT and nPOMC-deficient mice treated repeatedly with ethanol demonstrated a significant increase in locomotor activity on test day when compared to repeated saline-treated counterparts. In addition, mice of both genotypes in the repeated saline groups showed a significant locomotor stimulant response to acute ethanol injection.

Conclusions—Central POMC peptides are not required for either the acute locomotor stimulatory effect of ethanol or the development of ethanol-induced locomotor sensitization. While these peptides may modulate other ethanol-associated behaviors, they are not essential for development of behavioral sensitization.

Keywords

locomotor activity; mouse; obese; endorphin; melanocyte stimulating hormone

INTRODUCTION

Behavioral adaptations that occur following repeated drug use include tolerance and sensitization. While tolerance is noted by a decrease in a behavioral response with repeated drug administration, sensitization is characterized by an increased behavioral response to administration of the same dose of drug when compared to the initial drug exposure. Drugs

of abuse such as cocaine, amphetamine, ethanol, and nicotine can all produce behavioral sensitization (Babbini and Davis, 1972; Masur et al., 1986; Post and Rose, 1976; Segal and Mandell, 1974). Acute stimulation and development of sensitization are hypothesized to be positively linked to the susceptibility for increased drug self-administration (Robinson & Berridge, 2001).

Locomotor activity is one measure of behavioral sensitization that develops after repeated administration of many drugs, including ethanol (Cunningham & De Carlo, 1992; Masur et al., 1986). Locomotor sensitization occurs when the stimulant effect of the same dose of ethanol is significantly more pronounced following repeated ethanol administration than it is after acute administration. Alternatively, sensitization can also be demonstrated by a leftward shift in the locomotor stimulant dose response curve following repeated administration. Activity at the mu-opioid receptor has been implicated in ethanol-induced locomotor sensitization via pharmacological studies with selective antagonists, suggesting a role for endogenous β -endorphin (β -END) or enkephalins in ethanol sensitization (Pastor & Aragon, 2006).

Proopiomelanocortin (POMC) is a prohormone that is cleaved to form β -END, adrenocorticotropin hormone (ACTH), and alpha-melanocyte stimulating hormone (α -MSH) among other peptides. Centrally, the majority of POMC containing neurons are located in the arcuate nucleus of the hypothalamus with a second, smaller population in the nucleus tractus solitarius. Lesion studies of the arcuate nucleus of the hypothalamus implicate this region in ethanol behaviors such as locomotor activity, behavioral sensitization, sedation, and hypothermia (Crabbe and Dorsa, 1986; Miquel et al., 2003; Sanchis-Segura and Aragon, 2002). In addition, POMC peptides β -END (Hayward et al., 2004; Marinelli et al., 2003; Olive et al., 2001; Zhang & Kelley, 2002) and α -MSH (Navarro et al., 2005; Ploj et al., 2002) have a role in regulation of alcohol self-administration.

While previous studies have examined the involvement of specific POMC peptides or the role of the arcuate in ethanol sensitization, the goal of the present study was to use a genetic model of POMC neural-specific deficiency to determine the contribution of all POMC peptides in the brain on the development of ethanol-induced locomotor sensitization.

MATERIALS AND METHODS

Animals

Mice deficient for neuronal POMC (KO) and wildtype (WT) littermates (*Pomc*^{-/-} *Tg/Tg* and *Pomc*^{+/+} *Tg/Tg*, respectively) were used for these studies (Smart et al., 2006, 2007). Briefly, these mice were made by crossing a global POMC-deficient strain (*Pomc*^{-/-}) with a pHalEx2* transgenic mouse line that expresses a POMC rescue transgene (*Tg*) only in the pituitary. This cross results in compound mutant mice that are deficient for POMC only in neurons, not in the pituitary (*Pomc*^{-/-} *Tg/Tg*), and the wildtype littermates (*Pomc*^{+/+} *Tg/Tg*). These mice were originally maintained on a hybrid B6;D2;129X1;129S6 genetic background and have been backcrossed to C57BL/6J for at least 3 generations. The resulting phenotype for *Pomc*^{-/-} *Tg/Tg* mice is extreme obesity (due to hyperphagia and decreased metabolic rate) with a relatively normal corticosterone diurnal pattern in the females (Smart et al., 2006, 2007).

Adult mice were housed two per cage in clear cages with Purina Rodent Chow (#5001) and water available *ad libitum* throughout the experiment. The vivarium was kept on a 12 h light:dark cycle with lights on at 0700 and off at 1900. All protocols were approved by OHSU's Institutional Animal Care and Use Committee and were in accordance with the

NIH guidelines as established in *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research*.

Ethanol metabolism study

In a separate pilot study, alcohol naïve male, adult *Pomc*^{-/-} *Tg/Tg* (n=4, 77.0±5.7 g body weight) and *Pomc*^{+/+} *Tg/Tg* (n=3, 37.7±1.0 g body weight) mice were used to test the effect of significant differences in body weight and composition between these genotypes on ethanol pharmacokinetics when given an equal amount of ethanol (g) per kg of body weight. Mice were treated with a 2 g/kg dose of ethanol (i.p.; 20% v/v in saline), regardless of genotype, and 20-μl blood samples were taken for blood ethanol concentration (BEC) analysis of ethanol metabolism kinetics at 30, 60 and 120 minutes after injection.

Locomotor activity procedure

Locomotor activity chambers (Med-Associates, St. Albans, VT), each enclosed in a sound attenuating melamine chamber with a fan to mask external noise, were used for measurement of locomotor activity. Test sessions were 15 minutes in length and data were collected in 5 minute bins by a personal computer equipped with Activity Monitor v3.26 (Med-Associates). Chambers were thoroughly wiped down with 10% isopropyl alcohol between subjects. On test days mice were moved to the procedure room for acclimatization at least 45 minutes before the test began.

Procedure

Mice from the C57BL/6J background do not express an ethanol-induced locomotor sensitization as readily as mice of other strains such as the DBA/2J. Since the mice used were backcrossed on the C57BL/6J background, the procedure used was based on a procedure (Lessov et al., 2001) that successfully results in development of an ethanol-induced locomotor sensitization in C57BL/6J mice. The female mice were divided into two groups: repeated saline (RS) and repeated ethanol (RE) (see Table 1 for age, body weight, and number of subjects per group). For 12 or 13 consecutive days, mice in both groups received a daily injection in their home cage with no exposure to the locomotor activity chambers. To minimize the difference in both body weight and composition between the KO and WT mice, younger KO mice were used in this study. However, significant differences in body weight and composition were unavoidable between the genotypes (Table 1) because mice of this strain begin diverging in body weight before adult age is reached (Smart et al., 2006). Due to the differences in body composition and blood ethanol concentrations when mice were administered a fixed dose of ethanol (g) based on kg body weight (see ethanol metabolism study above), the dose of ethanol administered for this study was adjusted by genotype to approximate a similar blood ethanol concentration between WT and KO mice. The doses administered to the WT mice were based on Lessov et al. (2001), and the KO dosage was adjusted (decreased) based on BEC from the ethanol metabolism kinetics study and a small pilot study that also resulted in higher BEC for KO compared to WT mice (unpublished data). The dosage between genotypes was adjusted to match BEC at a given time point, and also resulted in no significant difference in acute ethanol stimulation between genotypes (see results). Wildtype mice received a daily injection of 2.5 g/kg ethanol or equivolume injection of saline, and KO (neural POMC-deficient) mice received 2.0 g/kg ethanol or equivolume saline. On day 13 or 14 all mice from both the RS and the RE groups were injected with ethanol (WT: 2.0 g/kg, KO: 1.2 g/kg) immediately before being placed into a locomotor activity chamber for a 15-minute test session. After the locomotor activity test, the mice were removed from the chamber, and a 20-μl blood sample was taken from the tail for BEC analysis. On day 15 or 16 all mice were injected with saline immediately before being placed in the locomotor activity chamber for a 15-minute test session.

Blood Ethanol Analysis

The 20- μ l blood samples were collected from the tail using calibrated microcapillary glass tubes. The blood sample was then quantitatively transferred into a 2 ml vial containing 500 μ l of a 4mM n-propanol matrix in deionized water solution. The vial was crimped and agitated to mix the matrix and the blood. Similar to the procedure used by Finn et al. (2007), analysis for blood ethanol concentration (BEC) was conducted by ambient headspace gas chromatography (Agilent 6890N GC with a DB-ALC1 column), sampling 30 μ l from each vial. An ethanol standard-curve with at least 6 standards (0.1–3.0 mg/ml) containing an n-propanol internal standard was established for BEC determination.

Data Analysis

To assess any difference in rate of ethanol metabolism between the genotypes, the ethanol metabolism kinetics data were statistically analyzed by a one-way ANOVA comparing the slope of the metabolism kinetics curve as the dependent variable and the genotype as the independent variable. A repeated measures ANOVA was used to compare for differences in BEC over repeated time points between genotypes.

A two-way ANOVA was run on activity data following the saline injection for all groups, with genotype and treatment as the independent variables and horizontal activity counts during the 15-minute session as the dependent variable. As there was no significant main effect of repeated treatment on the saline test day, sensitization to ethanol stimulation for each subject was measured as their difference in horizontal activity counts between the ethanol and saline test days. The locomotor activity data were analyzed by a two-way ANOVA with horizontal locomotor activity counts during the 15 min session as the dependent variable, and genotype and repeated treatment as the independent variables. One-way ANOVAs were run between treatment groups within the genotypes to determine if sensitization developed within each genotype. In addition, a paired t-test was used within each genotype to analyze the acute stimulatory effect of ethanol using only the repeated saline treated mice, comparing 15-minute horizontal activity counts between ethanol- and saline-test days. Blood ethanol concentration measured after the ethanol test session was also analyzed using a two-way ANOVA with treatment and genotype as the independent measures.

RESULTS

Ethanol metabolism kinetics

The POMC neural-deficient mice had significantly higher BECs after receiving the same amount of ethanol (g) per body weight as their WT littermates (Figure 1, main effect of genotype; $F(1, 5)=3.1$, $P=0.001$). However, the rate of ethanol metabolism, as measured by the slope of the line, was not significantly different between genotypes (Figure 1; $F(1,5)=4.3$, $P=0.09$).

Ethanol-induced sensitization study

The body weight of the KO mice was significantly more than the WT mice even though the KO mice were considerably younger than the WT mice on day 1 (Table 1). In order to approximately match the blood ethanol concentration achieved between genotypes, the amount of ethanol (g ethanol/kg body weight) was adjusted per results from our pilot studies (see methods for more details). Thus, despite the difference in ethanol dosing between genotypes, there was no significant main effect of genotype on BEC (Table 1; $P=0.44$) or acute sensitization to ethanol (RS groups only with genotype as independent variable; data not shown, $P=0.26$) following ethanol injection on ethanol test day.

There was a significant difference between genotypes in total distance moved over the 15 minute session following saline injection, with the POMC KO mice showing significantly less activity than the WT controls (WT=1571±107 activity counts, KO= 1019±172 activity counts; $F(1,24)=10.6$, $P=0.003$). Both genotypes from the repeated saline treated groups displayed an acute stimulatory response to ethanol on the ethanol-test day compared to activity on saline-test day (shown as a difference score Figure 2; WT $P=0.03$, KO $P=0.02$, paired t-tests). Significant behavioral sensitization to the locomotor stimulant effects of ethanol was seen in the repeated ethanol injected groups of both genotypes when compared to acute stimulation of the repeated saline injected groups (Figure 2; main effect of treatment, $F(1,24)=14.8$, $P<0.001$; WT, $F(1,15)=8.1$, $P=0.01$; KO, $F(1,9)=12.4$, $P<0.01$).

DISCUSSION

This work shows that POMC peptides in the brain are not essential for development of behavioral sensitization to ethanol. Obese, POMC neural-deficient mice exhibited significantly less activity than the WT mice following saline injection, in agreement with previous observations of these genotypes. However, a similar locomotor stimulatory effect to acute ethanol was seen in both the wildtype and the POMC neural-deficient mice. Following approximately 2 weeks of daily injections with ethanol, significant behavioral sensitization to the locomotor stimulant effects of ethanol was also seen in both genotypes of mice. Although the data presented are in female mice, we have also observed this effect in male mice and have confirmed the effect in mice of both sexes in an independently derived mouse line that also has a neuronal-specific POMC deficiency (Warren et al., 2007).

An attempt was made to minimize the weight difference between genotypes by using older WT and younger KO mice, however, the neuronal POMC KO mice still had a significantly higher body weight when compared to WT mice (Table 1). While the difference in age between the groups is a potential experimental confound, the alternative approach of matching body weights between genotypes via pair-feeding (feeding the KO mice only as much food daily as the WT mice consume) may result in cross-sensitization due to the stress of food-restriction. Neuronal-specific POMC KO mice pair-fed to the same intake as WT mice show significantly increased plasma corticosterone compared to ad libitum fed neuronal-specific POMC KO mice (unpublished data). This pair-feeding approach would introduce the significant chance of cross-sensitization to alcohol into the experimental design, and a possible inability to see further sensitization between the repeated ethanol and repeated saline groups.

Pilot studies in mice suggested that injecting ethanol on a fixed g ethanol per kg body weight would not result in similar blood ethanol concentrations between genotypes given the difference in body weight and composition (see Ethanol Metabolism Kinetics study above). A similar disparity in ethanol pharmacokinetics has been noted between obese and lean humans (Blouin and Warren, 1999; Kalant, 2000; Wang et al., 1992). These findings suggest that dosing based on total body water or percent body weight as water (as opposed to dosing on body weight) is a more accurate regimen for matching blood ethanol concentrations between lean and obese subjects. Ethanol distributes evenly in body water; however, Gundersen and Shen (1966) showed that body water does not increase proportionally to body weight in obese subjects. A study using NMR (Echo MRI 4-in-1, Echo Medical systems, LLC, Houston, Tx) to assess body composition measures including total body water for POMC neural-specific KO and WT mice confirmed that obese KO mice have increased total body water but a much smaller proportion of body weight as water when compared to lean WT mice (total body water-KO=22.2±0.9g, WT=16.5±0.5g; % body weight as water-KO=30.2±0.6%, WT=60.7±1.1%; Sharpe & Low, unpublished data). Thus, obese animals and humans have a smaller proportion of total body weight as water

compared to lean individuals, resulting in a significantly higher BEC when dosed as g ethanol per kg body weight. As there is no literature on how to adjust dosing of ethanol for obese mice, data from our pilot studies were used to guide dosing regimens. The BEC did not significantly differ by genotype or treatment after the mice were removed from the locomotor activity chambers on ethanol-test day. In addition, since both genotypes developed sensitization to the locomotor-stimulant effects of ethanol, it appears that the genotype-specific doses were effective for this study. Together, these results demonstrate the importance in ethanol-administration studies of measuring and accounting for differences in total body water in obese mice to obtain similar BEC between groups with divergent body weights and compositions.

While previous studies suggest an essential role of the POMC peptide β -END in ethanol-induced locomotor stimulation and sensitization, the results from the neural-specific POMC deficient mice used here don't support this hypothesis. Lesion studies using either monosodium glutamate (MSG) or estradiol valerate (EV) to ablate arcuate neurons showed a lack of ethanol-induced locomotor acute stimulation (Sanchis-Segura & Aragon, 2002; Sanchis-Segura et al., 2000; Miquel et al., 2003) and sensitization (Miquel et al., 2003). However, other studies using EV to ablate POMC neurons in the arcuate show a decrease in the number of β -END containing neurons with no significant change in β -END levels (Juárez et al., 2006; Marinelli et al., 2003), suggesting that compensation may occur using this methodology. In addition, the specificity of the MSG and EV for POMC neurons in the arcuate, and the effect on the endogenous levels of other POMC peptides such as α -MSH has not been established. This could be important as α -MSH and β -END are hypothesized to have opposing roles in some behaviors. Furthermore, it is difficult to discern if the effect from destruction of different POMC containing neurons via MSG and EV is specific to the loss of POMC or if it may in part be caused by the loss of other neurotransmitters in those same neurons such as glutamate or GABA, as it does not selectively impair peptide production but rather destroys the POMC-containing neurons.

In contrast to the results of the present studies, pharmacological studies blocking mu-opioid receptors (MOR) suggest that β -END is essential for the development of ethanol-induced locomotor sensitization (Pastor & Aragon, 2006). One possible reason for the difference is that β -END is not the only endogenous agonist at MOR, and thus the effect seen in the pharmacological studies could be due to blockade of both β -END and enkephalin. In support of the results presented here, mutant mice deficient for both β -END and enkephalin acquire ethanol self-administration (Hayward et al., 2004), suggesting that neither of these peptides is essential for the reinforcing effects of ethanol. Also, previous studies have demonstrated an ability of POMC peptides such as β -END and α -MSH to cross the blood-brain barrier (BBB) (Banks and Kastin, 1990). While the possibility that peripheral POMC peptides could have crossed the BBB cannot be excluded by our present data, previously published data show no measurable amounts of the POMC peptide α -MSH in either the global- or the neural-specific POMC deficient mice in the medial-basal hypothalamus despite normal levels of α -MSH in the pituitary of the neural-specific POMC deficient mice (Smart et al., 2006).

Previous studies have shown a decrease in ethanol self-administration due to agonist activity at melanocortin receptors, the cognate receptor for α -MSH (Navarro et al., 2005; Ploj et al., 2002). However, antagonism at melanocortin receptors decreases cocaine self-administration, blocks cocaine conditioned place preference, and attenuates cocaine-induced locomotor sensitization (Hsu et al., 2005). Although other studies have seen that blockade of either the melanocortin or mu-opioid receptors are sufficient to cause disruption of self-administration, our results show that central POMC peptides are not essential for either the acute stimulatory effect of ethanol, or the neuroadaptive measure of ethanol-induced

locomotor sensitization. Since, presumably, POMC peptides are co-released in terminal regions, it is possible that blockade of only one POMC peptide (e.g., α -MSH) could result in an unbalanced situation, with another POMC peptide (β -END) having a more pronounced effect than normal. This problem is avoided by the genetic model used in this study, which deletes all POMC peptides. A possible effect of developmental compensation cannot be ruled out with the current genetic model, however any compensation that might occur is not sufficient to rescue the obesity and hyperphagic phenotypes and it is unknown how it could affect ethanol-related behaviors. Future studies using mutant mice with a conditional ablation or restoration of POMC expression would be required to avoid the issue of any possible developmental compensation.

In summary, neuronal POMC peptides are not essential for either the acute locomotor stimulant effects of ethanol, or the development of ethanol-induced locomotor sensitization. Although POMC peptides such as β -END and α -MSH may regulate ethanol-associated behaviors, they do not appear to be necessary for the development of behavioral sensitization.

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Figure 1.

Ethanol metabolism rate is not different between neural-specific POMC deficient mice (KO, $n=4$) and wildtype (WT, $n=3$) littermates. Blood ethanol concentration (BEC), presented as mean $\pm$ SEM, was measured at 3 time points after injection of a 2 g ethanol/kg body weight dose in a within-subjects design. Using a fixed g ethanol/kg body weight dosing procedure with no regard for body composition or genotype, there is a significant main effect of genotype ($P=0.001$), with KO mice exhibiting a higher BEC at each time point. However, the slopes of the two curves were not different ($P=0.09$), suggesting that the rate of metabolism is not dependent on genotype.

Figure 2.

Wildtype (WT, n=17) and POMC neural-specific deficient mice (KO, n=11) showed both acute stimulation to ethanol (repeated saline-RS groups) and sensitization following repeated ethanol injections (RE vs. RS). The difference in total horizontal activity counts during the 15-minute session between ethanol test day and saline test day is presented for each group (mean±SEM). There was no significant difference in BEC between genotypes or between the treatment groups after the ethanol test session. * denotes statistical significance ($P \leq 0.01$) from RS group of same genotype

Table 1

Demographic information and blood ethanol concentrations for WT and KO mice in sensitization study.

	WT (<i>Pomc</i>^{+/+} <i>Tg/Tg</i>) (n=17)	KO (<i>Pomc</i>^{-/-} <i>Tg/Tg</i>) (n=11)
Age on day 1 (d)	163±4	110±8 *
Body weight day 1 (g)	28.0±0.9	56.1±1.8 *
Subjects per group	RS n=8, RE n=9	RS n=5, RE n=6
Blood ethanol concentration on ethanol-test day (mg/ml), 15–20 min post-injection	2.11±0.06	2.03±0.08

Data are presented as mean±SEM.

* denotes p<0.05 between genotypes