Voluntary Alcohol Drinking Enhances Proopiomelanocortin Gene Expression in Nucleus Accumbens Shell and Hypothalamus of Sardinian Alcohol-Preferring Rats

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Background: Evidence obtained in humans and rodents indicates that beta-endorphin (encoded by the proopiomelanocortin [POMC] gene) is critical in the regulation of alcohol drinking behavior. However, the alcohol effect on POMC gene expression has not been studied in rodent mesolimbic regions, such as the nucleus accumbens (NAc).

Methods: In this study, we first utilized POMC-enhanced green fluorescent protein (EGFP) transgenic mice to visualize POMC neurons and found that POMC-EGFP cells were modestly distributed throughout the NAc shell and core, in addition to the hypothalamic arcuate nucleus. POMC mRNA expression in the NAc of mice and rats was confirmed using reverse transcriptase-polymerase chain reaction and solution hybridization assays. We then investigated whether there are genetically determined differences in basal mRNA levels of POMC and mu opioid receptor (MOP-r) between selectively bred Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats, and whether these mRNA levels are altered in sP rats after alcohol drinking (10%, unlimited access) for 17 days.

Results: Alcohol-naïve sP rats had higher basal POMC mRNA levels than sNP rats only in hypothalamus. Alcohol drinking increased POMC mRNA levels in both the NAc shell (by 100%) and the hypothalamus (by 50%) of sP rats. Although sP rats had lower basal levels of MOP-r mRNA and GTPγS binding in NAc shell than sNP rats, voluntary alcohol consumption had no effect on MOP-r mRNA levels in the NAc shell.

Conclusions: Our results define the distribution of POMC-expressing neurons in the NAc of mice and rats. Higher POMC expression at basal levels in sP rats (genetically determined), along with increases after drinking (alcohol-induced) in the NAc shell and hypothalamus, suggests that the POMC systems play a role in high alcohol preference and consumption.

Key Words: Alcohol Drinking, Hypothalamus, Nucleus Accumbens Shell, POMC, Sardinian Alcohol-Preferring and Nonpreferring Rats.

ALCOHOL HAS BEEN reported to change the activity of the endogenous opioid peptide systems, especially the proopiomelanocortin (POMC) system. POMC is a large peptide precursor that gives rise to several biologically active neuropeptides, including beta-endorphin, adrenocorticotropic hormone (ACTH), beta-lipotropin, and alpha-melanocyte-stimulating hormone. The presence of POMC neurons was originally found to be mainly restricted to rodent hypothalamus (arcuate nucleus), nucleus of the solitary tract, and both anterior and intermediate lobes of pituitary (Mansour et al., 1988; Smith and Funder, 1988). In the rat hypothalamus, alcohol administration was reported to increase POMC mRNA levels after 15 days of alcohol-containing liquid diet (Angelogianni and Gianoulakis, 1993), to decrease it after 7 weeks of alcohol-containing liquid diet (Rasmussen et al., 2002) or cause no change after 14 days of oral gavage (Zhou et al., 2000). In rat anterior pituitary, chronic alcohol administration has been reported to increase POMC mRNA levels after 112 days of voluntary alcohol drinking (Winkler et al., 1995) or decrease after 14 days of continuous alcohol vapor (Dave et al., 1986). Notably, transgenic mice with decreased beta-endorphin exhibit decreased alcohol consumption, indicating that POMC neurons may exert important functional roles in alcohol drinking behavior (Racz et al., 2008). In contrast, another study reported increased alcohol consumption in beta-endorphin-deficient mice (Grisel et al., 1999).

POMC mRNA has been detected in several rat brain regions besides the arcuate nucleus and nucleus of the solitary tract, including the amygdala, cerebral cortex, and hippocampus, although in much lower levels than those in the hypothalamus (Civelli et al., 1982; Zhou et al., 1996).

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Also, using the reverse transcriptase (RT) reaction coupled to the polymerase chain reaction (PCR), POMC mRNA has been found in the nucleus accumbens (NAc), frontal cortex, and ventral tegmental area (VTA; Grauerholz et al., 1998; Leriche et al., 2007). In the NINDS Gene Expression Nervous System Atlas (GENSAT) bacterial artificial chromosome (BAC) transgenic project, using enhanced green fluorescent protein (EGFP) reporter genes incorporated into BAC transgenic mice, it has been shown that POMC-EGFP-positive neurons are found in many dopaminergic mesocorticolimbic regions, including the frontal cortex, ventral striatum, dorsal striatum, amygdala, and hippocampus (www.gensat.org; Gong et al., 2003; Pinto et al., 2004).

Beta-endorphin, the longest endogenous opioid peptide (31 amino acids), primarily acts at mu opioid receptors (MOP-r). Numerous pharmacological studies provide strong evidence that opioid antagonists decrease alcohol consumption, alcohol self-administration, cue-induced reinstatement of alcohol seeking, and relapse-like drinking in rodents and primates (Altschuler et al., 1980; Heyser et al., 1999; Liu and Weiss, 2002; Maccioni et al., 2005; Volpicelli et al., 1986), as well as alcohol drinking, craving for alcohol, and relapse episodes in human alcoholics (Kreek et al., 2002). Alcohol self-administration is reduced in MOP-r knockout mice (Roberts et al., 2000), further suggesting that the MOP-r is involved in the regulation of alcohol drinking. Sardinian alcohol-preferring (sP) rats constitute one of the currently available rat lines selectively bred for high alcohol preference and consumption (Colombo et al., 2006).

Therefore, in the first study herein, related to the neuroanatomical question of whether there are POMC-positive neurons in the NAc, we utilized POMC-EGFP promoter transgenic mice to visualize POMC-EGFP-expressing cells in the NAc core and shell. POMC mRNA expression in NAc of C57B6 mice, Fischer rats, and sNP rats (alcohol nonpreferring counterpart) was further confirmed using RT-PCR assay, and solution hybridization/RNase protection assay, followed by gel electrophoresis. The second study relates to differences in POMC and MOP-r expression in sP and sNP rats before and after alcohol drinking, with 3 aims. The first aim was to investigate whether there are genetically determined differences between sP and sNP rats in POMC and MOP-r gene expression levels, as well as MOP-r [^{35}S]GTPγS binding, in the hypothalamus and mesocorticolimbic regions, including the NAc shell and core. The second aim was to determine whether voluntary alcohol drinking alters the expression levels of these 2 genes in the hypothalamus, and both the NAc shell and the core of sP rats, which was the main goal of this study. To answer this question, we assessed the effect of 17-day alcohol drinking (post the initial phase of acquisition and into the maintenance of alcohol drinking) on these mRNA levels in the sP rats exposed to the standard, home cage 2-bottle "alcohol (10%, v/v) versus water" choice regimen with unlimited access 24 h/d. As expected, sNP rats had extremely low levels of alcohol drinking under this fluid choice condition. However, inclusion of sNP rats was suggested by the need to provide a more complete experimental design as well as to present data on the opposite alcohol drinking behavior and neurochemical alterations between sP and sNP rats. The third and last aim was to examine whether there are genetically determined differences in hypothalamic–pituitary–adrenal (HPA) activity at basal levels between these rat lines and in response to alcohol drinking in sP rats, including POMC mRNA levels in the anterior pituitary, and plasma ACTH and corticosterone (CORT) levels.

MATERIALS AND METHODS

Experiment 1

Distribution of POMC-EGFP-expressing neurons in POMC-EGFP promoter transgenic mice. This study was designed to visualize the POMC-EGFP neurons in the hypothalamus, NAc shell and core, and other mesocorticolimbic regions.

Animals. POMC-EGFP mice (originally generated in CBA/C57Bl6 mice in Dr. Friedman's laboratory at The Rockefeller University) were made by homologous recombination of a POMC gene-containing BAC comprising an EGFP insert, in which the POMC promoter drives EGFP expression (Pinto et al., 2004), and maintained on a C57BL/6J background (see Methods in the Data S1).

Fluorescent Microscopic Observation and Immunohistochemistry for EGFP. For details see Methods in Data S1.

Experiment 2

POMC RT-PCR in the mouse and rat. This study was designed to confirm the POMC mRNA species in the hypothalamus, NAc shell and core, and anterior pituitary.

Animals and Brain Dissections. For details see Methods in Data S1.

Proopiomelanocortin RT-PCR. The specific primer pair used in the PCR was POMC sense (5′-GAG ATT CTG CTA CAG TCG CTC-3′) and POMC antisense (5′-TTG ATG ATG GCG TTC TTG AA-3′) to amplify a region corresponding to a segment of exon 2 and exon 3 [NM 008895]. The mRNAs were reverse-transcribed (RT reaction) and PCR performed as described in Methods in Data S1.

Experiment 3

Genetically determined differences in MOP-r [^{35}S]GTPγS binding in sP and sNP rats. This study was designed to examine striatal levels of MOP-r [^{35}S]GTPγS binding in sP and sNP rats without alcohol exposure (alcohol-naïve rats).

Animals. Male sP and sNP rats from the 69th generation, approximately 75 days old at the start of the study, were housed in the stress-minimized facility at the Neuroscience Institute, National Research Council of Italy, Section of Cagliari, Italy (Methods in Data S1).

DAMGO-Stimulated [^{35}S]GTPγS Binding Autoradiography and Image Analysis Quantification. MOP-r [^{35}S]GTPγS binding was performed as previously described (Sim et al., 1995; see Methods in Data S1).

Experiment 4

Genetically determined differences and effects of alcohol drinking on POMC and MOP-r mRNA levels and HPA hormonal levels in sP and sNP rats. This study was designed to examine both sP and sNP rats with or without exposure to the 2-bottle "alcohol versus water" choice regimen for 17 consecutive days (water/water [alcohol-naïve] and alcohol/water choice).

Animals. Male sP and sNP rats from the 67th generation were used in the stress-minimized facility at the Neuroscience Institute. The housing conditions were identical to those in Experiment 3.

Alcohol Drinking Procedure. At the beginning of the experiment, rats were approximately 75 days old. There were 4 groups of 8 rats: alcohol-naïve and alcohol/water choice sP rats; alcohol-naïve and alcohol/water choice sNP rats. Alcohol-naïve rats had free access to tap water throughout the experimental period. Alcohol/water choice rats consumed alcohol with unlimited access for 24 h/d under the standard, homecage 2-bottle, free choice procedure for 17 consecutive days (see Methods in Data S1).

Preparation of RNA Extracts. For details see Methods in Data S1.

Solution Hybridization Ribonuclease Protection-Trichloroacetic Acid Precipitation Assay. For details see Methods in Data S1.

Radioimmunoassays. For details see Methods in Data S1.

RESULTS

Expression of EGFP from the POMC Promoter in the Hypothalamus, NAc, and Caudate-Putamen of POMC-EGFP Mice

The expression of EGFP immunoreactive protein in the arcuate nucleus of the hypothalamus, NAc, and caudate-putamen was verified by immunohistochemistry using 3 male POMC-EGFP (+) mice, in which the POMC promoter drives EGFP expression. Immunohistochemistry for EGFP immunoreactivity demonstrated the localization of the EGFP-expressing neurons in the NAc (Fig. 1*A*), arcuate nucleus (Fig. 1*B*), and caudate-putamen (Fig. 1*C*). Cells containing POMC-EGFP were scattered throughout the shell and core of the NAc and caudate-putamen.

Detection of POMC mRNA in the Mouse and Rat NAc by Both RT-PCR and Solution Hybridization

To demonstrate the presence of POMC mRNA in the NAc, we used a RT-PCR assay. As expected, we found a 678-bp amplification product in the anterior pituitary, hypothalamus, and NAc in both mice and rats (Fig. 2*A*). As a negative control, the omission of RT resulted in no amplification product (data not shown), indicating that neither genomic DNA nor cDNA contamination accounts for the 678-bp signal. These results indicate that POMC mRNA is present in the NAc of both species.

Selected samples of the hypothalamus, NAc core, and shell were subjected to solution hybridization and RNase treatment followed by gel electrophoresis. Figure 2*B* shows the size distribution of rat POMC antisense probe surviving solution hybridization and RNase treatment. The protected species was approximately 538 bp, corresponding to an

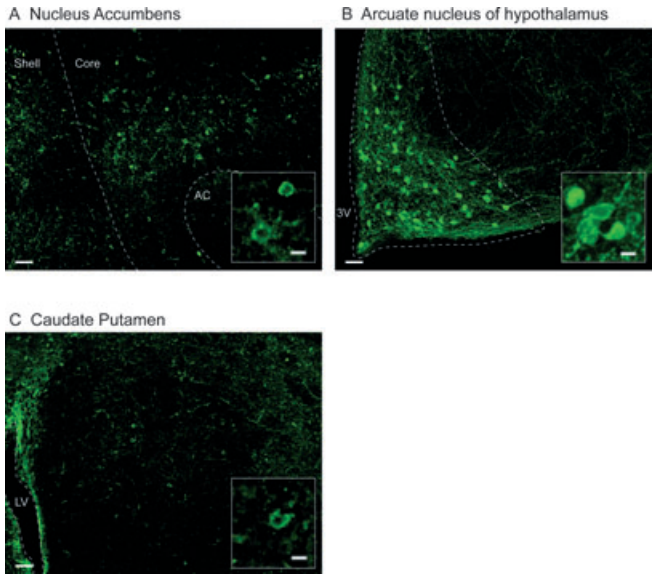

Fig. 1. Localization of proopiomelanocortin-enhanced green fluorescent protein (POMC-EGFP)-expressing neurons in the nucleus accumbens (**A**), arcuate nucleus of the hypothalamus (**B**), and caudate-putamen (**C**) of POMC-EGFP (+) promoter transgenic mice: POMC-EGFP neurons express EGFP immunoreactivity (green). Scale bars, 50 μm in **A**–**C**; 10 μm in the inserts. AC, anterior commissure; 3V, third ventricle; LV, lateral ventricle.

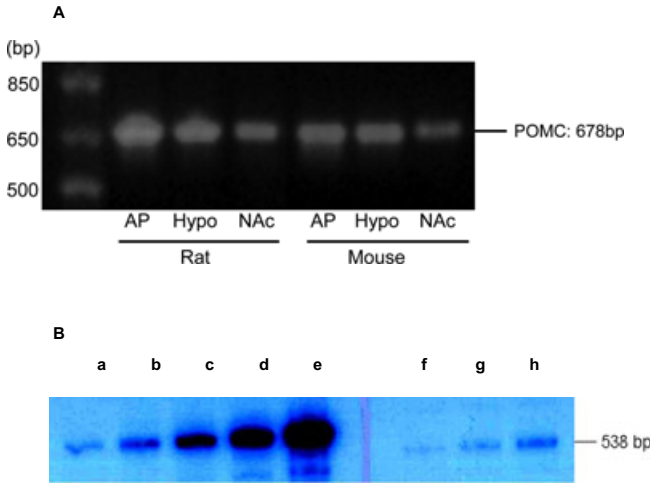

Fig. 2. (**A**) Detection of proopiomelanocortin (POMC) mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) in anterior pituitary (AP), hypothalamus (Hypo), and nucleus accumbens (NAc) of Fischer rats and C57B6 mice. Total RNA was extracted from different regions, cDNA obtained by RT reaction, and PCR performed as described in Materials and Methods section. Gel photograph shows PCR products from the amplification of POMC (678-bp product). (**B**) Representative autoradiograms of the POMC sense transcript standards with total cytoplasmic RNA samples after hybridization with the rat POMC cRNA probe and RNase digestion. The size of the main protected RNA:RNA hybrid is about 538 bp. Lanes a to e: 0.62, 1.25, 2.5, 5, or 10 pg of the POMC sense transcript standards. Lanes f, g, and h: total cytoplasmic RNA samples extracted from the NAc core (1.6 μg), NAc shell (3.1 μg), and hypothalamus (6.2 μg) of alcohol-naive sNP rats, respectively.

RNA:RNA hybrid formed by the hybridization of the full-length cRNA probe (538 bp in length) with total cytoplasmic RNA samples extracted from the hypothalamus, NAc, or anterior pituitary of sNP rats. Consistent with the results from both POMC-EGFP neurons and PCR amplification, the gel electrophoresis further confirmed that POMC mRNA is expressed in the NAc core and shell at relatively low levels in comparison with those found in the hypothalamus.

Genetically Determined Differences Between sP and sNP Rats in DAMGO-Stimulated [^{35}S]GTPγS Binding

Under basal conditions, all the brain regions examined in sP rats presented decreased functional activity of the MOP-r in comparison with those in sNP rats: caudate-putamen ($t = 5.12$, $p < 0.001$), cingulate cortex (Cg; $t = 2.78$, $p < 0.05$), NAc core ($t = 2.98$, $p < 0.01$), and NAc shell ($t = 2.33$, $p < 0.05$; Fig. 3).

Genetically Determined Differences Between sP and sNP Rats in Voluntary Alcohol Drinking

All sP rats' daily alcohol intake rose progressively, averaging approximately 6.5 g/kg/d throughout the 17-day period of exposure. Conversely, daily alcohol intake in alcohol/water choice sNP rats averaged <0.5 g/kg (see details in Results in Data S1).

Genetically Determined Differences Between sP and sNP Rats and Effects of Voluntary Alcohol Drinking on POMC mRNA Levels in the Brain

NAc Shell and Core. In the NAc shell (Fig. 4*A*), 2-way analysis of variance (ANOVA) showed a significant main effect of alcohol consumption, $F(1, 17) = 7.30$, $p < 0.05$, but failed to show a significant alcohol consumption × genotype interaction, $F(1, 17) = 3.82$, $p = 0.06$. POMC mRNA levels in alcohol-naïve sP rats were not different from those of alcohol-naïve sNP rats. However, increased POMC mRNA levels in the NAc shell were observed in the sP rats after voluntary alcohol drinking (alcohol/water choice sP vs. alcohol-naïve sP rats, $p < 0.05$, Newman–Keuls test). Also, the POMC mRNA levels were significantly higher in sP than sNP rats under alcohol/water choice condition ($p < 0.05$).

In the NAc core (Fig. 4*B*), 2-way ANOVA showed a significant main effect of genotype, $F(1, 18) = 5.03$, $p < 0.05$. However, there was no significant main effect of alcohol

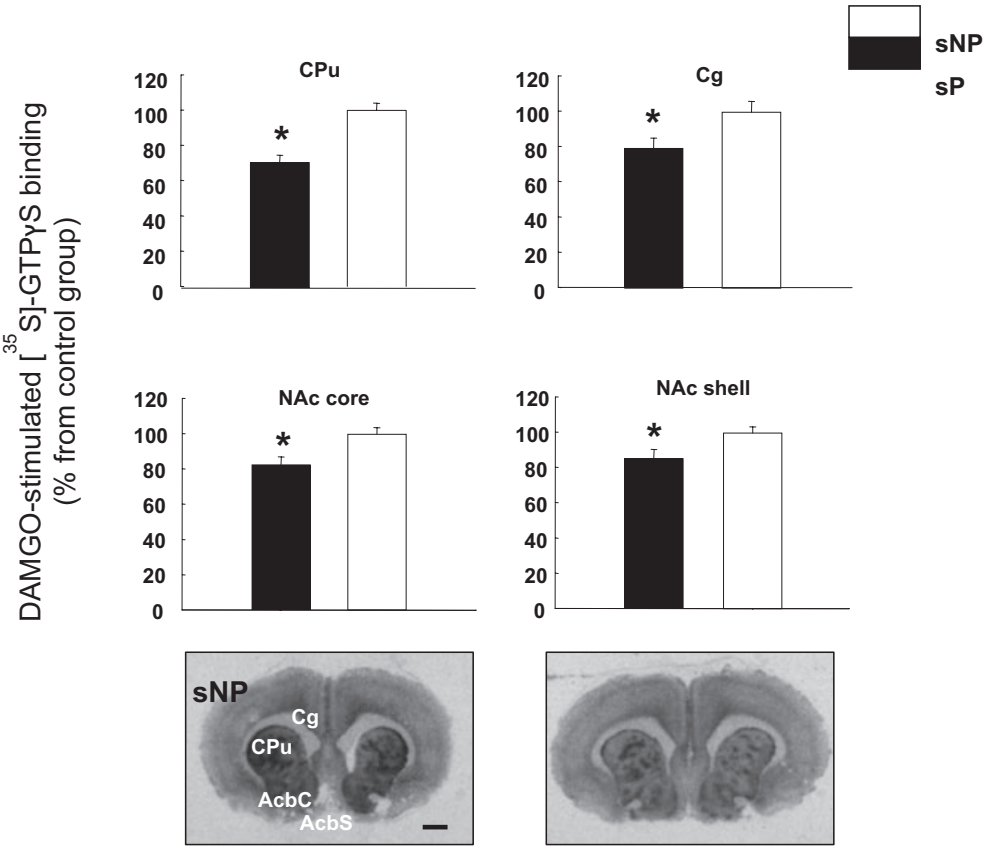

Fig. 3. Genetically determined differences between Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats on DAMGO-stimulated [35S] GTPγS binding in caudate-putamen (CPu), cingulate cortex (Cg), nucleus accumbens shell (NAc shell), and core (NAc core). Columns represent the means + SEM of mu opioid receptor (MOP-r) densities. Values from MOP-r levels in CPu, Cg, NAc shell, and NAc core from sP rats that are significantly different from sNP rats, $*p < 0.05$, $n = 5$ to 6. Bar represents 1 mm. AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell.

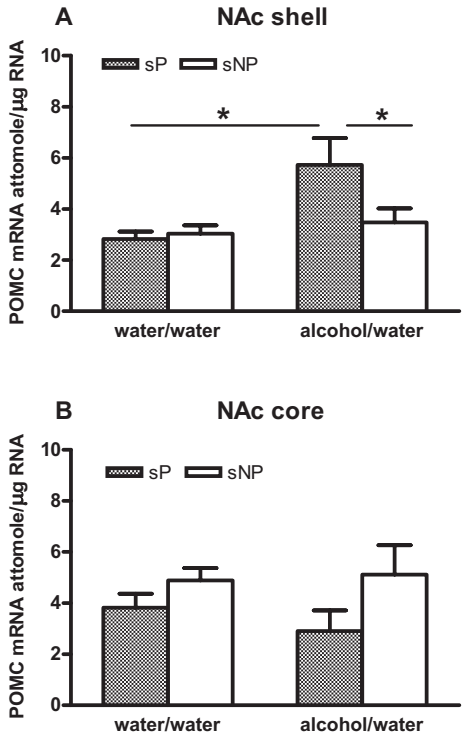

Fig. 4. Genetically determined differences between Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats and effects of voluntary alcohol drinking on proopiomelanocortin (POMC) mRNA levels (attomole/μg total RNA) in the nucleus accumbens (NAc) shell (**A**) and core (**B**). Both sP and sNP rats were offered either water as the sole fluid available (water/water choice) or a free choice between 10% (v/v) alcohol and water (alcohol/water choice) for 17 consecutive days. Each bar is the mean + SEM. Significant differences: *$p < 0.05$, $n = 5$ to 6.

consumption, nor was there a significant alcohol consumption × genotype interaction.

Hypothalamus. Two-way ANOVA revealed a significant main effect of genotype, $F(1, 28) = 18.9$, $p < 0.0005$, and there was a significant alcohol consumption × genotype interaction, $F(1, 28) = 4.77$, $p < 0.05$. Although Newman–Keuls post hoc tests failed to show a significant difference between alcohol-naïve sP and sNP rats ($p = 0.06$), a planned comparison revealed that basal POMC mRNA levels were significantly higher in alcohol-naïve sP rats than alcohol-naïve sNP rats ($p < 0.05$). Increased hypothalamic POMC mRNA levels were observed in sP rats after voluntary alcohol drinking (alcohol/water choice sP vs. alcohol-naïve sP rats, $p < 0.05$, Newman–Keuls test; Fig. 5). Also, POMC mRNA levels were significantly higher in sP than in sNP rats in the alcohol/water choice condition ($p < 0.05$).

Caudate-Putamen, Frontal Cortex, Medial/Basal Amygdala, and Central Nucleus of Amygdala. In the caudate-putamen, frontal cortex, and medial/basolateral amygdala, there was no effect of genotype, alcohol consumption, or

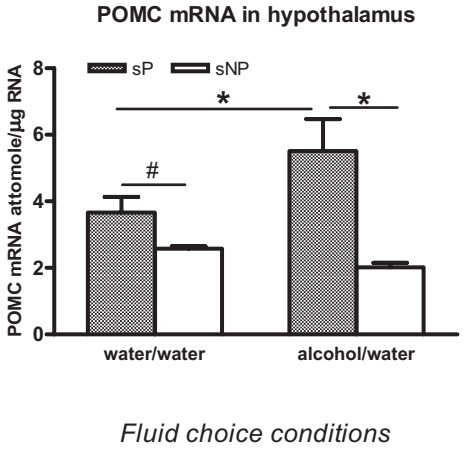

Fig. 5. Genetically determined differences between Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats and effects of voluntary alcohol drinking on proopiomelanocortin (POMC) mRNA levels (attomole/μg total RNA) in the hypothalamus. Both sP and sNP rats were offered either water as the sole fluid available (water/water choice) or a free choice between 10% (v/v) alcohol and water (alcohol/water choice) for 17 consecutive days. Each bar is the mean + SEM. Significant differences: # or *$p < 0.05$, $n = 7$ to 8.

their interaction (Table 1). Because there was a very low expression level of the POMC gene in the central nucleus of amygdala in both sP and sNP rats, we did not try to determine the effects of alcohol drinking in this brain region.

Genetically Determined Differences Between sP and sNP Rats and Effects of Voluntary Alcohol Drinking on MOP-r mRNA Levels in the Brain

NAc Shell and Core. In the NAc shell (Fig. 6*A*), 2-way ANOVA showed a significant main effect of genotype, $F(1, 20) = 13.4$, $p < 0.005$. A planned comparison revealed that basal MOP-r mRNA levels in alcohol-naïve sP rats were significantly lower than those in alcohol-naïve sNP rats ($p < 0.05$). MOP-r mRNA levels in the NAc shell were significantly lower in sP than sNP rats in the alcohol/water choice condition ($p < 0.05$, Newman–Keuls test). In the NAc core (Fig. 6*B*), there was no main effect of genotype, alcohol consumption, or their interaction.

Caudate-Putamen, Frontal Cortex, Medial/Basolateral Amygdala, and VTA. In the caudate-putamen, there was a significant main effect of genotype, $F(1, 28) = 8.0$, $p < 0.05$ (Table 1). Basal MOP-r mRNA levels in alcohol-naïve sP rats were significantly lower than those in alcohol-naïve sNP rats (planned comparisons, $p < 0.05$). The MOP-r mRNA levels were significantly lower in sP than sNP rats in the alcohol/water choice condition ($p < 0.05$, Newman–Keuls test).

In the frontal cortex, there was a significant main effect of genotype, $F(1, 12) = 14.1$, $p < 0.005$ (Table 1). Basal MOP-r mRNA levels in alcohol-naïve sP rats failed to show significant differences from those in alcohol-naïve sNP rats (planned comparisons, $p = 0.06$).

Table 1. Genetically Determined Differences Between Sardinian Alcohol-Preferring (sP) and Nonpreferring (sNP) Rats and Effects of Voluntary Alcohol Drinking on Gene Expression Levels of Proopiomelanocortin (POMC) (A) and Mu Opioid Receptor (MOP-r) (B).

(A) POMC mRNA (Attomole mRNA/μg total RNA)

	sP Rat		sNP Rat	
	Alcohol-naive	Alcohol/water choice	Alcohol-naive	Alcohol/water choice
FCx (n = 8)	1.56 ± 0.26	1.51 ± 0.26	1.72 ± 0.12	1.57 ± 0.36
CPu (n = 6 to 7)	0.79 ± 0.11	0.66 ± 0.04	1.00 ± 0.21	0.91 ± 0.07
MeBLA (n = 5 to 6)	5.20 ± 1.00	3.41 ± 0.58	2.83 ± 0.62	4.26 ± 0.96
Hip (n = 6)	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.03	0.21 ± 0.02

(B) MOP-r mRNA (Attomole mRNA/μg total RNA)

	sP Rat		sNP Rat		
	Alcohol-naive	Alcohol/water choice	Alcohol-naive	Alcohol/water choice	Main effect of genotype by 2-way ANOVA
FCx (n = 4)	0.27 ± 0.01	0.26 ± 0.02	0.36 ± 0.02	0.40 ± 0.06	$F(1, 12) = 14$, $p < 0.005$
CPu (n = 6 to 8)	0.47 ± 0.02*	0.54 ± 0.02	0.61 ± 0.05	0.67 ± 0.05	$F(1, 28) = 8.0$, $p < 0.05$
MeBLA (n = 4)	0.62 ± 0.08	0.49 ± 0.05	0.81 ± 0.12	0.76 ± 0.15	$F(1, 11) = 7.1$, $p < 0.05$
VTA (n = 4)	0.90 ± 0.08	1.08 ± 0.15	0.77 ± 0.11	0.89 ± 0.11	$F(1, 12) = 1.87$, $p = 0.20$

FCx, frontal cortex; CPu, caudate-putamen; MeBLA, medial/basolateral amygdala; Hip, hippocampus; VTA, ventral tegmental area.

*Significant genotype difference between alcohol-naive sP rats and alcohol-naive sNP rats under water/water choice condition: $p < 0.05$.

Note, in B, because there was relatively lower MOP-r mRNA expression level in the FCx, MeBLA, and VTA, the RNA extracts from 2 rats were pooled as 1 sample for the MOP-r assays. Therefore, there was a smaller sample size for each of the 3 regions. Group differences in mRNA levels were analyzed using 2-way (fluid choice condition [water/water or alcohol/water]; rat line [sP or sNP]) ANOVA, followed by the Newman–Keuls post hoc tests.

Although in the medial/basolateral amygdala 2-way ANOVA showed a significant effect of genotype, $F(1, 11) = 7.11$, $p < 0.05$, there was no effect of alcohol consumption, nor was there a significant alcohol consumption × genotype interaction (Table 1). In the VTA, there was no significant effect of genotype, alcohol consumption, or their interaction (Table 1).

Genetically Determined Differences Between sP and sNP Rats and Effects of Voluntary Alcohol Drinking on POMC mRNA Levels in the Anterior Pituitary, and on HPA Hormonal Levels

POMC mRNA Levels in the Anterior Pituitary. Two-way ANOVA showed a significant main effect of genotype, $F(1, 27) = 16.4$, $p < 0.0005$, and a significant alcohol consumption × genotype interaction, $F(1, 27) = 6.00$, $p < 0.05$ (Fig. 7*A*). POMC mRNA levels in alcohol-naïve sP rats were not different from those of alcohol-naïve sNP rats. However, the POMC mRNA levels in the anterior pituitary were significantly lower in sP than sNP rats in the alcohol/water choice condition ($p < 0.01$, Newman–Keuls test).

HPA Hormonal Levels. As shown in Fig. 7*B*, plasma ACTH levels showed a similar pattern to that seen in POMC mRNA in the anterior pituitary. Although 2-way ANOVA showed a significant main effect of genotype, $F(1, 26) = 5.34$, $p < 0.05$, plasma ACTH levels were not significantly lower in sP than sNP rats in the alcohol/water choice condition. In contrast, in plasma CORT levels, there was no significant effect of genotype, alcohol consumption, or their interaction (Fig. 7*C*).

DISCUSSION

POMC-derived peptides, especially beta-endorphin, are distributed in the arcuate nucleus of hypothalamus, and the dopaminergic mesocorticolimbic regions, including the NAc, VTA, and frontal cortex. Increased extracellular level of dopamine in the NAc is a key event in the rewarding and reinforcing actions elicited by alcohol, psychostimulants, opiates, and nicotine (e.g., Di Chiara et al., 1996). Because activation of MOP-r by beta-endorphin is rewarding and modulates dopamine release in the NAc (Spanagel et al., 1991), beta-endorphin may be involved in reinforcing and motivational properties (Amalric et al., 1987; Herz, 1997; Roth-Deri et al., 2008). In addition to the arcuate nucleus, POMC mRNA has also been detected in the NAc at very low levels (Leriche et al., 2007). Therefore, the demonstration of POMC neuron distribution in this region is an essential issue concerning the neural networks containing POMC mRNA and derived peptides in the NAc.

The first aim of the present study was to specifically visualize the NAc POMC neurons, including the core and shell divisions. Using POMC-EGFP transgenic mice in which POMC-expressing neurons were labeled with EGFP and enhanced by immunohistochemistry procedures, we found POMC-EGFP-expressing neurons to be present in modest amounts in both the NAc core and shell of POMC-EGFP mice. Our findings are in agreement with and extend the results of the GENSAT project, showing the expression of POMC-EGFP in the ventral striatum of the POMC-EGFP mice.

The presence of POMC mRNA in the NAc had been reported, using a sensitive RT-PCR technique (Leriche et al.,

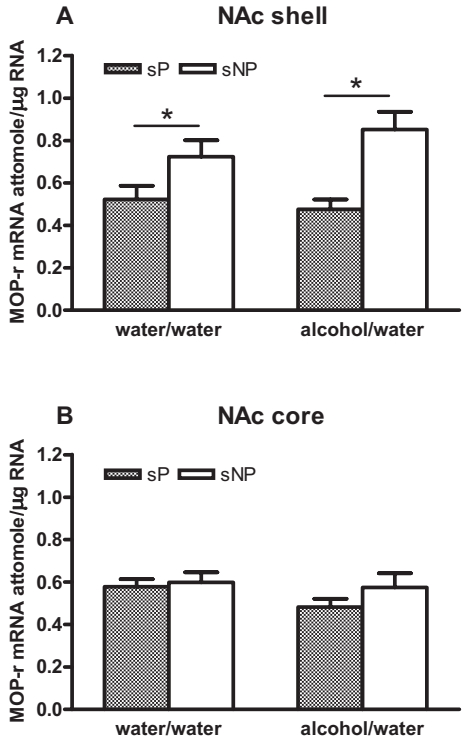

Fig. 6. Genetically determined differences between Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats and effects of voluntary alcohol drinking on mu opioid receptor (MOP-r) mRNA levels (attomole/μg total RNA) in the nucleus accumbens (NAc) shell (**A**) and core (**B**). Both sP and sNP rats were offered either water as the sole fluid available (water/water choice) or a free choice between 10% (v/v) alcohol and water (alcohol/water choice) for 17 consecutive days. Each bar is the mean + SEM. Significant differences: *$p < 0.05$, $n = 7$ to 8.

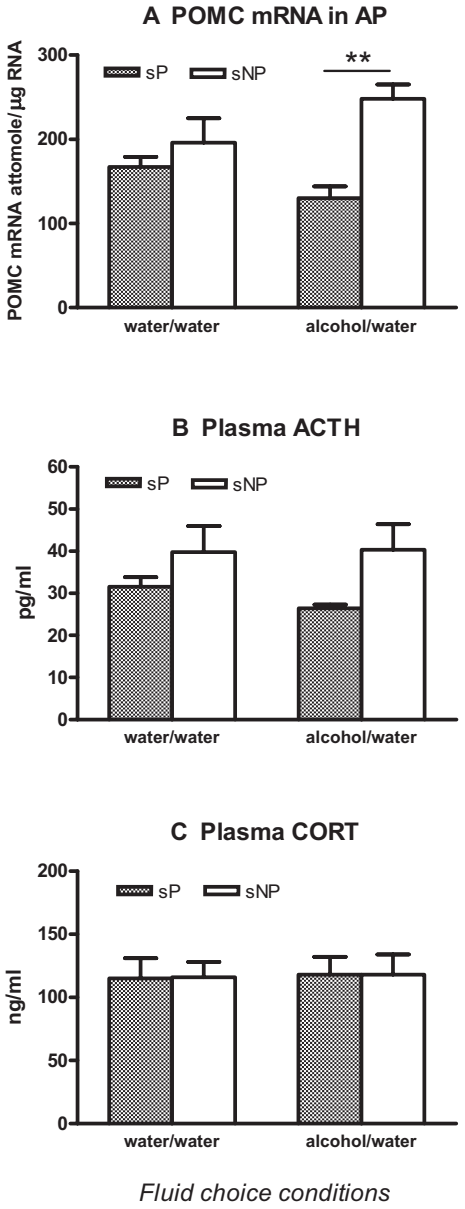

Fig. 7. Genetically determined differences between Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats and effects of voluntary alcohol drinking on proopiomelanocortin (POMC) mRNA levels (attomole/μg total RNA) in the anterior pituitary (AP) (**A**), plasma ACTH (**B**), or plasma corticosterone (CORT) (**C**) levels. Both sP and sNP rats were offered either water as the sole fluid available (water/water choice) or a free choice between 10% (v/v) alcohol and water (alcohol/water choice) for 17 consecutive days. Each bar is the mean + SEM. Significant differences: **$p < 0.01$, $n = 7$ to 8.

2007). We show here that RT-PCR amplification, with the POMC primer specific for rat and mouse POMC mRNA, results in 678-bp PCR products in the NAc of both species, the same size found in the hypothalamus, suggesting that the NAc contains similar POMC mRNA species to that of the hypothalamus. The PCR products obtained were not the result of amplification of genomic DNA or cDNA contamination, because the omission of RT from the RT reaction showed no amplifications. We further used quantitative and specific solution hybridization assays to confirm that POMC mRNA was expressed in the NAc. The relative amount of POMC mRNA in the NAc was about 10% of that found in the hypothalamus. The low POMC mRNA signal observed in the NAc in these mice seems likely due to a small number of POMC-EGFP-containing neurons, as was found in POMC-EGFP mice.

The next 3 aims focused on whether POMC gene expression in several brain regions (particularly the NAc) would differentially respond to alcohol consumption in selectively bred sP rats. Using quantitative solution hybridization assays, we first examined genetically determined differences in POMC levels and found that sP rats displayed markedly higher (about 40%) basal POMC mRNA levels than sNP rats in the hypothalamus only. This suggests a contribution of relatively higher basal POMC gene expression to the genetically determined tendency of sP rats toward enhanced voluntary alcohol consumption. It would be of interest to investigate whether these line differences between sP and sNP rats are also found in other lines of selectively bred P and NP rats. It is noteworthy that our result is consistent

with earlier studies, showing higher basal POMC mRNA levels in the hypothalamus in the AA (Alko, Alcohol) rats or C57BL/6 mice with high alcohol consumption or preference than ANA (Alko, Non-Alcohol) rats or DBA/2 mice with low alcohol consumption or preference (Jamensky and Gianoulakis,1999; Marinelli et al., 2000). We also measured POMC mRNA levels in mesocorticolimbic regions (specifically, the 2 subdivisions of NAc) and hypothalamus of sP rats exposed to 17-day alcohol drinking and found that this voluntary consumption of high amounts of alcohol by sP rats was associated with increases in POMC mRNA levels in the NAc shell (about 100%) and hypothalamus (about 50%), but not NAc core. This result suggests that voluntarily consumed alcohol modulates POMC mRNA expression in the POMC neuron populations in the NAc shell and hypothalamus to a different degree, because the POMC mRNA levels were increased by different magnitudes in these 2 regions. This stimulatory effect on POMC expression in the NAc shell seems to be specific for the POMC gene, as no effect of alcohol drinking was found on MOP-r mRNA levels in this region.

Our finding with respect to POMC mRNA increases in the NAc shell is consistent with an early report showing that alcohol stimulates beta-endorphin release in the rat NAc (Marinelli et al., 2003). Although the stimulatory factors that may influence elevation of POMC mRNA level are not yet fully elucidated in this study, it is possible that an increased beta-endorphin release in the NAc is responsible for the increase in POMC mRNA and biosynthesis to compensate for alcohol-induced peptide depletion. Our results in the hypothalamus of sP rats after 17-day voluntary drinking are in agreement with an earlier study showing that after 15-day alcohol exposure, there is an increase in POMC mRNA levels in the hypothalamus (Angelogianni and Gianoulakis, 1993), while another study reported a decrease after long-term (7-week) alcohol exposure (Rasmussen et al., 2002). Together, our data suggest that enhanced POMC gene expression in the NAc shell and hypothalamus was likely a consequence of high alcohol consumption in sP rats. No change in POMC mRNA levels was observed in sNP rats given alcohol/water choice, likely because of their extremely low levels of alcohol drinking (<0.5 g/kg/d on most days).

Although both the shell and the core express POMC, we observed that the NAc shell appears to be the critical site in response to alcohol drinking. Our findings suggest that POMC neurons in the shell (a region long considered to mediate processes of reward and reinforcement, e.g., Di Chiara, 2002; Koob et al., 1998) contribute to alcohol intake. Most relevant to the present study, a recent report demonstrated that alcohol is self-administered directly into the medial shell, but not the core (Engleman et al., 2009). It has also been reported that cocaine (Rodd-Henricks et al., 2002) or cannabinoids (Zangen et al., 2006) are self-administered directly into the NAc shell. Therefore, the shell (not the core) may be a region in which alcohol and other drugs of abuse contribute to reinforcing effects. Several studies have examined neurotransmitter systems in the NAc shell in the control of alcohol drinking or self-administration, implicating the involvement of glutamate (Goulding et al., 2011), GABA receptors (Nie et al., 2011), neuropeptide Y (Cippitelli et al., 2010), and kappa opioid receptors (Nealey et al., 2011) in modulating alcohol intake. It is tempting to speculate that pharmacological interactions with POMC neurons through these neurotransmitters or neuromodulators may be the initial and specific means by which alcohol exerts its effects in this shell region, although this remains to be determined.

Although interesting and novel, the results of the current study should be interpreted with caution for at least 2 reasons. First, we did not measure levels of POMC-derived peptides; our findings are limited to gene expression. To confirm that the effects observed on the POMC mRNA level were translated to the protein level, we have conducted several experiments using immunohistochemistry for POMC-derived peptides in POMC-EGFP mice. However, we had difficulty in visualizing the immunoreactivity-positive neurons in the mouse NAc, using 3 commercial antibodies against beta-endorphin, ACTH, or POMC with fluorescent microscopy (YZ and KN, unpublished data). It is possible that very low levels of POMC peptides are constitutively expressed or that the POMC mRNA is not translated in this region. Second, it is possible that the current findings may result from changes in other systems known to modulate consummatory behavior. However, it is unlikely that alpha-melanocyte-stimulating hormone (which has an inhibitory effect on food intake) played a role, because high alcohol intake did not result in a general suppression of appetitive or consummatory behaviors in sP rats (Colombo, 1997). Also, in the present study, there was no change in orexin mRNA levels in the lateral hypothalamus after high alcohol drinking in sP rats. Using the opioid receptor antagonist naloxone, it has been demonstrated that the opioid antagonist decreases alcohol self-administration in sP rats (Maccioni et al., 2005). Our data suggest that a genetically determined difference and the effects of alcohol drinking are mediated by the beta-endorphin system.

The activation of corticotropin-releasing factor (CRF) in the hypothalamus contributes to the stimulatory effects of acute alcohol on the HPA axis in rats (Rivier et al., 1990). Of interest, beta-endorphin acting on the MOP-r exerts tonic inhibition of CRF, and then of the HPA axis in both humans and rodents (Kreek and Koob, 1998). Using "binge" alcohol administration by the oral route, we have found that acute "binge" alcohol dramatically activates HPA activity, which is associated with decreased POMC mRNA levels in the hypothalamus (Zhou et al., 2000). In the present study, sP rats displayed increased hypothalamic POMC mRNA levels after alcohol drinking, coupled with reduced levels of plasma ACTH levels, further supporting the concept that there is an inverse association between hypothalamic POMC activity and the HPA axis.

A successful use of a medication for a target indication is the use of naltrexone in the treatment of alcoholism (O'Malley et al., 1992; Volpicelli et al., 1992). Chronic naltrexone

treatment decreases craving and alcohol consumption in alcohol-dependent subjects and results in persistent elevations of HPA activity in alcohol-dependent subjects (Farren et al., 1999; O'Malley et al., 2002). It is hypothesized that the reduction in alcohol drinking by naltrexone may be due to its activation of the HPA axis. Support for this hypothesis can be found in many studies (Schuckit, 1994).

The MOP-r has long been considered a key element related to increased vulnerability to develop alcohol dependence and relapse, and therefore is one of the most important therapeutic targets to treat alcohol dependence and craving (Manzanares et al., 2005). The present study examined MOP-r mRNA levels in sP and sNP rats given alcohol/water choice. As reported earlier (Fadda et al., 1999), in alcohol-naïve sP rats, quantitative autoradiography revealed that MOP-r-binding density is significantly reduced in the NAc shell and caudate-putamen, compared to alcohol-naïve sNP rats. Consistent with MOP-r protein levels, we found that the MOP-r mRNA levels were lower in the same mesolimbic regions of alcohol-naïve sP rats, including the NAc shell, caudate-putamen, frontal cortex, and basolateral amygdala, in comparison with alcohol-naïve sNP rats. Differences in MOP-r densities were reported in rats presenting high and low preference for alcohol consumption (Cowen et al., 1999; Parkes and Sinclair, 2000). Our results are also consistent with lower basal MOP-r mRNA levels in P than NP rats (June et al., 2004); notably, P/NP and sP/sNP rats have been selectively bred using similar procedures and criteria (Bell et al., 2006; Colombo et al., 2006). Indeed, sP rats presented lower basal levels of MOP-r binding in the caudate-putamen, Cg, NAc shell, and core compared to those of sNP rats. These results suggest that lower MOP-r functional activity is associated with increased vulnerability for alcohol consumption.

In summary, our results demonstrate the existence of genetically determined higher basal levels of POMC gene expression in the hypothalamus of selectively bred sP rats, in comparison with sNP rats. Our main finding shows a significant increase in POMC gene expression levels in both the NAc shell and the hypothalamus of sP rats during the initial phases of acquisition and maintenance of alcohol drinking. POMC-EGFP neurons were visualized in the NAc shell and core. Because increases in POMC neuronal activity are thought to be involved in several reward-related behaviors, we suggest that the observed alterations in the POMC systems in the NAc shell and hypothalamic arcuate nucleus contribute, at least in part, to the high alcohol drinking behavior of sP rats.

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REFERENCES

Altschuler HL, Phillips PE, Feinhandler DA (1980) Alteration of ethanol self-administration by naltrexone. Life Sci 26:679–688.

Amalric M, Cline EJ, Martinez JL Jr, Bloom FE, Koob GF (1987) Rewarding properties of beta-endorphin as measured by conditioned place preference. Psychopharmacology 91:14–19.

Angelogianni P, Gianoulakis C (1993) Chronic ethanol increases pro-opiomelanocortin gene expression in the rat hypothalamus. Neuroendocrinology 57:106–114.

Bell RL, Rodd ZA, Lumeng L, Murphy JM, McBride WJ (2006) The alcohol-preferring P rat and animal models of excessive alcohol drinking. Addict Biol 11:270–288.

Cippitelli A, Damadzic R, Hansson AC, Singley E, Sommer WH, Eskay R, Thorsell A, Heilig M (2010) Neuropeptide Y (NPY) suppresses yohimbine-induced reinstatement of alcohol seeking. Psychopharmacology 208:417–426.

Civelli O, Birnberg N, Herbert E (1982) Detection and quantitation of pro-opiomelanocortin mRNA in pituitary and brain tissues from different species. J Biol Chem 257:6783–6787.

Colombo G (1997) Ethanol drinking behaviour in Sardinian alcohol-preferring rats. Alcohol Alcohol 32:443–453.

Colombo G, Lobina C, Carai M, Gessa G (2006) Phenotypic characterization of genetically selected Sardinian alcohol-preferring and -non preferring rats. Addict Biol 11:324–338.

Cowen MS, Rezvani AH, Jarrott B, Lawrence AJ (1999) Ethanol consumption by Fawn-Hooded rats following abstinence: effect of naltrexone and changes in mu-opioid receptor density. Alcohol Clin Exp Res 23:1008–1014.

Dave JR, Eiden LE, Karanian JW, Eskay RL (1986) Ethanol exposure decreases pituitary corticotropin-releasing factor binding, adenylate cyclase activity, proopiomelanocortin biosynthesis and plasma beta-endorphin levels in the rat. Endocrinology 118:280–286.

Di Chiara G (2002) Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. Behav Brain Res 137:75–114.

Di Chiara G, Acquas E, Tanda G (1996) Ethanol as a neurochemical surrogate of conventional reinforcers: the dopamine-opioid link. Alcohol 13:13–17.

Engleman EA, Ding ZM, Oster SM, Toalston JE, Bell RL, Murphy JM, McBride WJ, Rodd ZA (2009) Ethanol is self-administered into the nucleus accumbens shell, but not the core: evidence of genetic sensitivity. Alcohol Clin Exp Res 33:2162–2171.

Fadda P, Tronci S, Colombo G, Fratta W (1999) Differences in the opioid system in selected brain regions of alcohol preferring (sP) and alcohol non preferring (sNP) rats. Alcohol Clin Exp Res 23:1296–1305.

Farren CK, O'Malley S, Grebski G, Maniar S, Porter M, Kreek MJ (1999) Variable dose naltrexone-induced hypothalamic-pituitary-adrenal stimulation in abstinent alcoholics: a preliminary study. Alcohol Clin Exp Res 23:502–508.

Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N (2003) A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature 425:917–925.

Goulding SP, Obara I, Lominac KD, Gould AT, Miller BW, Klugmann M, Szumlinski KK (2011) Accumbens Homer2-mediated signaling: a factor contributing to mouse strain differences in alcohol drinking? Genes Brain Behav 10:111–126.

Grauerholz BL, Jacobson JD, Handler MS, Millington WR (1998) Detection of pro-opiomelanocortin mRNA in human and rat caudal medulla by RT-PCR. Peptides 19:939–948.

Grisel J, Mogil J, Grahame N, Rubinstein M, Belknap J, Crabbe J, Low M (1999) Ethanol oral self-administration is increased in mutant mice with decreased beta-endorphin. Brain Res 835:62–67.

Herz A (1997) Endogenous opioid systems and alcohol addiction. Psychopharmacology 129:99–111.

Heyser C, Roberts A, Schulteis G, Koob G (1999) Central administration of an opiate antagonist decreases oral ethanol self-administration in rats. Alcohol Clin Exp Res 23:1468–1476.

Jamensky NT, Gianoulakis C (1999) Comparison of the proopiomelanocortin and proenkephalin opioid peptide systems in brain regions of the alcohol-preferring C57BL/6 and alcohol-avoiding DBA/2 mice. Alcohol 18:177–187.

June HL, Cummings R, Eiler WJ 2nd, Foster KL, McKay PF, Seyoum R, Garcia M, McCane S, Grey C, Hawkins SE, Mason D (2004) Central opioid receptors differentially regulate the nalmefene-induced suppression of ethanol- and saccharin-reinforced behaviors in alcohol-preferring(P) rats. Neuropsychopharmacology 29:285–299.

Koob GF, Sanna PP, Bloom FE (1998) Neuroscience of addiction. Neuron 21:467–476.

Kreek MJ, Koob GF (1998) Drug dependence: stress and dysregulation of brain reward pathways. Drug Alcohol Depend 51:23–47.

Kreek MJ, LaForge KS, Butelman E (2002) Pharmacotherapy of addictions. Nat Rev Drug Discov 1:710–726.

Leriche M, Cote-Vélez A, Méndez M (2007) Presence of pro-opiomelanocortin mRNA in the rat medial prefrontal cortex, nucleus accumbens and ventral tegmental area: studies by RT-PCR and in situ hybridization techniques. Neuropeptides 41:421–431.

Liu X, Weiss F (2002) Additive effect of stress and drug cues on reinstatement of ethanol seeking: exacerbation by history of dependence and role of concurrent activation of corticotropin-releasing factor and opioid mechanisms. J Neurosci 22:7856–7861.

Maccioni P, Serra S, Vacca G, Orrù A, Pes D, Agabio R, Addolorato G, Carai MAM, Gessa GL, Colombo G (2005) Baclofen-induced reduction of alcohol reinforcement in alcohol-preferring rats. Alcohol 36:161–168.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ (1988) Anatomy of CNS opioid receptors. Trends Neurosci 11:308–314.

Manzanares J, Ortiz S, Oliva JM, Pérez-Rial S, Palomo T (2005) Interactions between the cannabinoid and opioid receptor systems in the mediation of alcohol effects. Alcohol Alcohol 40:25–34.

Marinelli PW, Kiianmaa K, Gianoulakis C (2000) Opioid propeptide mRNA content and receptor density in the brains of AA and ANA rats. Life Sci 66:1915–1927.

Marinelli PW, Quirion R, Gianoulakis C (2003) A microdialysis profile of beta-endorphin and catecholamines in the rat nucleus accumbens following alcohol administration. Psychopharmacology 169:60–67.

Nealey KA, Smith AW, Davis SM, Smith DG, Walker BM (2011) κ-opioid receptors are implicated in the increased potency of intra-accumbens nalmefene in ethanol-dependent rats. Neuropharmacology 61:35–42.

Nie H, Rewal M, Gill TM, Ron D, Janak PH (2011) Extrasynaptic delta-containing GABA(A) receptors in the nucleus accumbens dorsomedial shell contribute to alcohol intake. Proc Natl Acad Sci USA 108:4459–4464.

O'Malley S, Krishnan-Sarin S, Farren C, Sinha R, Kreek MJ (2002) Naltrexone decreases craving and alcohol self-administration in alcohol-dependent subjects and activates the hypothalamic-pituitary-adrenocortical axis. Psychopharmacology 160:19–29.

O'Malley SS, Jaffe A, Chang G, Schottenfeld RS, Meyer RE, Rounsaville BJ (1992) Naltrexone and coping skills therapy for alcohol dependence: a controlled study. Arch Gen Psychiatry 49:881–887.

Parkes H, Sinclair JD (2000) Reduction of alcohol drinking and upregulation of opioid receptors by oral naltrexone in AA rats. Alcohol 21:215–221.

Pinto S, Roseberry AG, Liu H, Diano S, Shanabrough M, Cai X, Friedman JM, Horvath TL (2004) Rapid rewiring of arcuate nucleus feeding circuits by leptin. Science 304:110–115.

Racz I, Schürmann B, Karpushova A, Reuter M, Cichon S, Montag C, Fürst R, Schütz C, Franke PE, Strohmaier J, Wienker TF, Terenius L, Osby U, Gunnar A, Maier W, Bilkei-Gorzó A, Nöthen M, Zimmer A (2008) The opioid peptides enkephalin and beta-endorphin in alcohol dependence. Biol Psychiatry 64:989–997.

Rasmussen DD, Boldt BM, Wilkinson CW, Mitton DR (2002) Chronic daily ethanol and withdrawal: 3. Forebrain pro-opiomelanocortin gene expression and implications for dependence, relapse, and deprivation effect. Alcohol Clin Exp Res 26:535–546.

Rivier C, Imaki T, Vale W (1990) Prolonged exposure to alcohol: effect on CRF mRNA levels, and CRF- and stress-induced ACTH secretion in the rat. Brain Res 520:1–5.

Roberts A, McDonald J, Heyser C, Kieffer B, Matthes H, Koob G, Gold L (2000) μ-Opioid receptor knockout mice do not self-administer alcohol. J Pharmacol Exp Ther 293:1002–1008.

Rodd-Henricks ZA, McKinzie DL, Li TK, Murphy JM, McBride WJ (2002) Cocaine is self-administered into the shell but not the core of the nucleus accumbens of Wistar rats. J Pharmacol Exp Ther 303:1216–1226.

Roth-Deri I, Green-Sadan T, Yadid G (2008) Beta-endorphin and drug-induced reward and reinforcement. Prog Neurobiol 86:1–21.

Schuckit M (1994) Low level of response to alcohol as a predictor of future alcoholism. Am J Psychiatry 151:184–189.

Sim LJ, Selley DE, Childers SR (1995) In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[gamma-[35S]thio]-triphosphate binding. Proc Natl Acad Sci USA 92: 7242–7246.

Smith AI, Funder JW (1988) Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. Endocr Rev 9:159–179.

Spanagel R, Herz A, Bals-Kubik R, Shippenberg TS (1991) Beta-endorphin-induced locomotor stimulation and reinforcement are associated with an increase in dopamine release in the nucleus accumbens. Psychopharmacology 104:51–56.

Volpicelli JR, Alterman AI, Hayashida M, O'Brien CP (1992) Naltrexone in the treatment of alcohol dependence. Arch Gen Psychiatry 49:876–880.

Volpicelli JR, Davis MA, Olgin JE (1986) Naltrexone blocks the post-shock increase of ethanol consumption. Life Sci 38:841–847.

Winkler A, Rosker I, Furkert J, Fickel J, Melzig MF (1995) Effects of voluntary ethanol ingestion on the POMC gene expression in the rat pituitary and on the plasma beta-endorphin content. Alcohol Alcohol 30:231–238.

Zangen A, Solinas M, Ikemoto S, Goldberg SR, Wise RA (2006) Two brain sites for cannabinoid reward. J Neurosci 26:4901–4907.

Zhou Y, Franck J, Spangler R, Maggos C, Ho A, Kreek MJ (2000) Reduced hypothalamic POMC and anterior pituitary CRF_1 receptor mRNAs after acute, but not chronic, daily "binge" intragastric alcohol administration. Alcohol Clin Exp Res 24:1575–1582.

Zhou Y, Spangler R, LaForge KS, Maggos CE, Ho A, Kreek MJ (1996) Modulation of CRF-R1 mRNA in rat anterior pituitary by dexamethasone: correlation with POMC mRNA. Peptides 17:435–441.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Genetically determined differences between sP and sNP rats in voluntary alcohol drinking, and the effects on orexin and ppDyn in RNA levels in the lateral hypothalamus, and plasma prolactin levels, with methods.

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