Period 2 gene deletion abolishes β-endorphin neuronal response to ethanol

Maria Agapito, **Nadia Mian**, **Nadka I. Boyadjieva**, and **Dipak K. Sarkar**[*]
Endocrine Program, Department of Animal Sciences, Graduate Program of Neuroscience, Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA

Abstract

Background—Ethanol exposure during early life has been shown to permanently alter the circadian expression of clock regulatory genes and the β-endorphin precursor proopiomelanocortin (POMC) gene in the hypothalamus. Ethanol also alters the stress- and immune-regulatory functions of β-endorphin neurons in laboratory rodents. Our aim was to determine whether the circadian clock regulatory *Per2* gene modulates the action of ethanol on β-endorphin neurons in mice.

Methods—*Per2* mutant (*mPer2^Brdml*) and wild type (C57BL/6J) mice were used to determine the effect of *Per2* mutation on ethanol-regulated β-endorphin neuronal activity during neonatal period using an *in vitro* mediobasal hypothalamic (MBH) cell culture model and an *in vivo* milk formula feeding animal model. The β-endorphin neuronal activity following acute and chronic ethanol treatments, was evaluated by measuring the peptide released from cultured cells or peptide levels in the MBH tissues, using enzyme-linked immunosorbent assay (ELISA).

Results—*Per2* mutant mice showed a higher basal level of β-endorphin release from cultured MBH cells and a moderate increase in the peptide content in the MBH in comparison to control mice. However, unlike wild type mice, *Per2* mutant mice showed no stimulatory or inhibitory β-endorphin secretory responses to acute and chronic ethanol challenges *in vitro*. Furthermore, *Per2* mutant mice, but not wild type mice, failed to show the stimulatory and inhibitory responses of MBH β-endorphin levels to acute and chronic ethanol challenges *in vivo*.

Conclusions—These results suggest for the first time that the *Per2* gene may be critically involved in regulating β-endorphin neuronal function. Furthermore, the data revealed an involvement of the *Per2* gene in regulating β-endorphin neuronal responses to ethanol.

There are clinical reports uncovering a circadian rhythmicity of alcohol intoxication and overdose (Raymond et al., 1992). There also appears to be a relationship between alcohol drinking behavior and altered body circadian functions. In line with these views, alcohol intake has been shown to alter circadian blood pressure (Nakashita et al., 2009; Ohkubo et al., 2009), core body temperature (Devaney et al., 2003), hormone release in humans (Röjdmark et al., 2001), circadian phase shifting and free running period in mice (Seggio et al., 2009), circadian phase resetting and nocturnal activity patters in the hamster (Ruby, 2009), and rhythmicity in hypothalamic proopiomelanocortin (POMC) neurons in rats (Chen et al., 2006). Maternal alcohol drinking profoundly and permanently affects circadian function in the offspring. It is known that prenatal ethanol exposure alters core body temperature, corticosterone rhythms (Handa et al., 2007), NK cell function rhythms (Arjona et al., 2006a), phase shifting ability (Sakata-Haga et. al, 2006), and rhythmic pituitary-

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[*]Corresponding author address: Dr. Dipak K. Sarkar, Professor II and Director, Program of Endocrinology, The State University of New Jersey, Endocrine Research Facility, 67 Poultry Farm Lane, New Brunswick, NJ 08901, USA. Phone: 732-932-1529; Fax: 732-932-4134; sarkar@aesop.rutgers.edu.

adrenal function in rats (Taylor et al., 1982), as well as circadian blood pressure and the heart rate of adolescent humans (Alvarez et al., 1995).

Why is the body's circadian system so vulnerable to alcohol toxicity? One logical explanation for the circadian system vulnerability, may point to a possible alcohol specific target for the gene(s) that regulate circadian functions. The circadian clock-work involves the interaction of the so-called clock genes such as the *Period* (*Per*1, *Per*2, *Per*3), *Clock*, *Bmal*1 and *Cryptochrome* (*Cry*1, *Cry*2) genes into two tightly coupled transcriptional and translational feedback loops, thereby self-sustaining a circadian period of activity of the cell and modulating the expression of clock-controlled genes (Ko and Takahashi, 2006). Of these genes, the *Per*2 gene appears to be a putative target for alcohol. Support for this view comes from evidence showing that chronic alcohol exposure alters the circadian expression of Period genes in the hypothalamus of adult rats (Chen et al., 2004). Similarly, prenatal alcohol feeding alters circadian expression of Period genes in the hypothalamus and peripheral tissues (Chen et al., 2006; Arjona et al, 2006b; Farnell et al, 2008). Additionally, *Per*2 gene mutant (*Per*2Brdm1) mice display enhanced alcohol consumption and preference (Spanagel et al., 2005a), whereas *Per*1Brdm1 mutant mice do not show such an enhancement in alcohol drinking behavior (Zghoul et al., 2007). Evidence has shown, alcoholics with a specific set of polymorphisms in the *Per2* gene consume less alcohol than alcoholics without the polymorphisms (Spanagel et al., 2005a, b). Hence, *Per2* appears to be a targeted gene where alcohol may act on to alter circadian functions. In order to more closely elucidate the role of the circadian clock in ethanol's effects on the brain, we examined the effect of the *Per*2 mutation on hypothalamic β-endorphin neuronal sensitivity to ethanol.

MATERIALS AND METHODS

Animal use

Per2 mutant (*mPer2Brdml*) and wild type male and female mice of the same genetic background (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME). *mPer2Brdml* mice carry a mutant *mPer2* gene with a deletion in the PAS dimerization domain, which is critical for the interaction with other clock proteins (Zheng et al., 1999), thus rendering a non-functional PER2 protein. Although the *Per*2Brdml mutant mice have an albino phenotype, because it was engineered with a tyrosinase gene deficiency that affects the ability to make melanin, the *Per*2Brdml mutant mice has the same C57BL/6 genetic background with only a difference of the aforementioned gene deficiency. It has been shown that the albino mice strains 129/J, RF/J, SWR/J, AKR/J, A/J, and BALB/cByJ have a tyrosinase gene deficiency with no significant relationship between albinism and mean τ_{DD}, the endogenous (free-running) period of the circadian pacemaker measured in constant environmental darkness (Schwartz and Zimmerman, 1990). This suggests that the albino phenotype trait will have no implications on any circadian related studies. Similarly, gradual ethanol exposure has been shown to produce an increase in alcohol preference in both C57BL/6 mice with normal tyrosinase activity and in BALB/cByJ mice with tyrosinase deficiency (Blizard et al., 2004; Middaugh et al., 1999). Furthermore, these two mouse strains (C57BL/6By and BALB/cByJ) showed no differences in their hypothermic response or the brain level of cGMP to the same ethanol dose (Church and Feller, 1979). These data support that the tyrosinase deficiency will have very little consequences in alcohol-response studies. Mice were maintained under constant environmental conditions on a 12 h light/12 h dark cycle (lighting period from 7:00 a.m. to 7:00 p.m. with ad libitum food and water. The *mPer2 Brdml* mutant mice were routinely genotyped to verify the *Per*2 gene mutation. The primers used for detecting the Per2 gene were the following: forward1-cttgggtggagaggctattc, forward2-cattggggaggcacaagtcag, reverse1-aggtgagatgacaggagatc; reverse2-gagctgcgaacacatcctca. Male and female *mPer2Brdml* mice or male and female C57BL/6J mice were bred to produce neonates for MBH cell cultures or in vivo studies.

In vivo studies

In the in vivo study, postnatal day-2 (PD2) old C57BL/6 pups and Per2 [Brdml] mutant pups (both sexes) were fed by intubation with milk formula containing either alcohol (alcohol-fed) or an isocaloric volume of maltose dextrin (pair-fed) as originally described by Goodlet et al. (1998) and modified by Sarkar et al. (2007), or pups were undisturbed (*ad libitum*-fed). The alcohol–fed groups were given a milk formula containing ethanol (11.34%; vol/vol; 0.1–0.2 ml/animal; during a period of 1 minute), yielding a total daily ethanol dose of 2.5g/kg. The feeding was conducted at 1000 and 1200 h at PD7 and animals were sacrificed at 1300 h (3 h after the initiation of alcohol feeding) for acute ethanol treatment or conducted daily for 5 days between PD\ for chronic ethanol administration. Tissue samples were collected from all groups at 1300 h on PD7. The amount fed to the animals equaled 33% of their mean body weight (milliliters per gram). After feeding, the pups were immediately returned to the litter. One hour after the last feeding, six of each *ad libitum*-fed (AD), pair-fed (PF) or alcohol-fed (AF) neonates from six separate liters were sacrificed, their brains dissected, mediobasal hypothalami (MBH) obtained and immediately frozen for β-endorphin measurements. We have previously shown that feeding of ethanol-containing milk formula in laboratory rats increases blood level of alcohol (alcohol content was 205.15 ± 22.5 and 242.75 ± 23.75 mg/dl; N = 5) 1 and 2 h after the last feeding and undetectable 4 h after the last feeding. Animal care and treatment were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy. The animal protocol used was approved by the Rutgers Animal Care and Facilities Committee.

Mediobasal hypothalamic cell cultures

The method for mouse MBH cell cultures was adopted from the previously published methods of our laboratory (Boyadjieva et al., 1994) for rat MBH cells. In brief, C57BL/6 and *Per*2 mutant neonates (both sexes) were sacrificed at day 1 of age, and the fetuses were removed by aseptic surgical procedure. Brains from the fetuses were immediately removed and the MBHs were separated and placed in ice-cold Hanks' Balanced Salt Solution (HBSS) containing antibiotic solution (100 U/ml penicillin,100 μg/ml streptomycin, and 250 ng/ml amphotericin B),0.1% bovine serum albumin, and 200 μM ascorbic acid (all from Sigma-Aldrich, St. Louis, MO). The block of the MBH extended approximately 1 mm rostral to the optic chiasma and just caudal to the mammillary bodies, laterally to the hypothalamic sulci, and dorsally to ~2 mm deep. This part of the hypothalamus is known to contain glial cells and neuroendocrine neurons, produces β-endorphin, dopamine, thyrotropin-releasing hormones, and growth hormone-releasing hormone as well as glial cells (Brown, 1998). We have previously characterized the similar MBH culture system in regards to β-endorphin neuronal responses to ethanol. In MBH cultures, ethanol acutely stimulates β-endorphin secretion and POMC mRNA levels while chronically inhibiting β-endorphin secretion and POMC mRNA levels (Boyadjieva et al., 2004; Pastorcic et al., 1994). The β-endorphin neuronal responses to acute and chronic ethanol treatments in MBH cell cultures are similar to those seen in hypothalamic tissues of adult rodent brains (Boyadjieva et al., 2001; Rasmussen e al.,1998, 2000). Also in MBH cell cultures, like in the adult hypothalamus, ethanol action on β-endorphin neurons is mediated by catalase activated production of acetaldehyde (Reddy et al., 1995; Pastor and Aragon, 2008). These data support that the MBH cell culture model is useful in determining cellular mechanisms of ethanol actions on β-endorphin neurons.

MBH cells were then sedimented at 400 g for 10 min. Pellets were resuspended in HEPES-buffered Dulbecco's Modified Eagle's Medium (HDMEM, 4.5 g/l glucose; Sigma, St. Louis, MO). Cells were cultured into 25-cm^2 polyornithine coated tissue culture flasks (2.5 million cells/flask) in HDMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin. On day 3, the culture medium was replaced with HDMEM containing serum

supplement (30 nM selenium, 20 nM progesterone, 1 mM iron-free human transferrin, 5 mM insulin, and 100 mM putrescin) and 1% penicillin/streptomycin. This medium was changed every 3 days until the release experiment.

Release studies in vitro

These studies were conducted after maintaining the MBH neurons in cultures for 8–9 days. The cultures were treated with 2 ml of serum-free chemically defined medium containing various doses of ethanol (25, 50, 100 mM) or no ethanol. In order to evaluate the changes of β-endorphin release (during a 3 h period) in the media following acute and chronic ethanol treatments, cultures were fed with fresh medium containing ethanol at 0, 3, 6, 21, 24 and 45 h. The conditioned media from each culture was collected at 3, 6, 24 and 48 h after the initiation of the treatment. The conditioned medium samples were collected in 12×75 mm glass tubes and stored frozen at $-20°C$ until use for measurements of β-endorphin levels. At the end of the treatment, the cells from each culture were extracted and measured for the total cellular protein level.

Protein measurement

The release of β-endorphin from the cultured MBH neurons was measured by determining the concentration of the peptide in the medium by enzyme immunoassay (EIA) using a kit purchased from Peninsula Laboratories, LLC (Torrance, CA). The assay was conducted according to the manufacturer's protocol. The peptide release rate during a 3 h period of each culture was calculated using the media β-endorphin concentration (pg/ml) per μg of total cell protein, which was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Statistics

The mean ± SEM of the data were determined and presented in the text. Data obtained in the studies dealing with ethanol time effects on β-endorphin release of each strain were compared using one-way ANOVA followed by Newman Keuls post-hoc test. Ethanol time effects on β-endorphin release between *Per2* mutant and wild type mice were statistically evaluated using two-way ANOVA followed by the Bonferroni *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

Comparison of the effects of ethanol on β-endorphin release from MBH cells of control and Per2 mutant mice in cultures

It has been shown previously that β-endorphin release from MBH cells is elevated after acute (1–12 hr) ethanol treatment, but decreased following chronic (24–48 hr) ethanol treatment (Sarkar and Minami, 1990: Boyadjieva et al., 1997; Poplawski, et al., 2005). To evaluate the effect of *Per2* gene deletion on β-endorphin neuronal response to ethanol, the opioid secretory responses to various doses of ethanol at different time points were determined in cultured MBH cells of C57BL/6 and *Per*2 mutant mice.

As anticipated, MBH cells of control mice demonstrated significant β-endorphin-secretory response to acute ethanol treatments for a period of 3 and 6 h in a concentration-dependent-manner (Fig. 1). The effective doses of ethanol that were able to significantly elevate β-endorphin-release were within the range of 50 and 100 mM. However, MBH cells of Per2 mice failed to show the β-endorphin-secretory response to acute treatment with either of the ethanol doses (25–100 mM) studied. Interestingly, the basal release rate of β-endorphin from MBH cells of *Per*2 mice was significantly higher (2–5-fold) than MBH cells of control mice (Figs. 1 and 2).

When the effect of chronic ethanol treatment on β-endorphin-release from MBH cells of control mice was examined, the study revealed a significant inhibition of β-endorphin-release at 24 and 48 h by all doses (25, 50 and 100 mM) of ethanol. Chronic treatment with these ethanol doses failed to change β-endorphin-release from MBH cells of *Per*2 mice. This data suggests that *Per*2 deletion increases the basal release of β-endorphin neurons, but markedly alters the ability of these neurons to respond to ethanol.

Comparison of the effects of ethanol exposure on the MBH level of β-endorphin in control and Per2 mutant mice

The *in vitro* analyses of the effects of ethanol exposure on β-endorphin release from MBH cells of C57BL/6 and *Per*2 mutant mice clearly identified a role for this clock-regulating gene in regards to ethanol action. In the *in vitro* study, isolated MBH cells in culture were devoid of the influence of the rest of the central and peripheral nervous systems. Hence, ethanol effects on β-endorphin neuron were also determined in vivo.

Using milk formula, alcohol was administered acutely (3 h) or chronically (5 days) in neonatal mice. We used a 3 h treatment period for acute alcohol treatment, since this time period produced stimulatory effects on β-endorphin release in culture. We used a 5-day treatment period for chronic ethanol treatment. Although a 48 h ethanol treatment period was able to reduce β-endorphin release from MBH cells in culture, we treated the animal with ethanol for 5 days in order to establish the chronic ethanol effect on β-endorphin neurons during the equivalent of the third trimester of human pregnancy. When the MBH tissue levels of β-endorphin were compared between AD and PF groups after 3 h of feeding, no significant differences in the protein levels were found between these two treatment groups in C57BL/6 or in *Per*2 mutant mice (Fig. 3), suggesting that the animal handling or the formula feeding protocols did not produce any significant effect on this protein levels. However, the β-endorphin level in MBH tissue was higher in acute AF group than in AD and PF groups in C57BL/6 mice but not in *Per*2 mutant mice (Fig. 3). At day 5 of milk formula feeding, the β-endorphin level in MBH of PF group did not significantly differ from AD groups of C57BL/6 mice, however alcohol fed (AF) C57BL/6 mice showed a significant reduction in the peptide level in MBH tissue (Fig. 4). In *Per*2 mutant mice, the β-endorphin levels in MBH tissues were similar in all groups. These results suggest that, like in vitro, acute ethanol treatment increases while chronic ethanol treatment reduces the β-endorphin levels in MBH of C57BL/6 mice but not in *Per*2 mutant mice.

DISCUSSION

In this paper, we report for the first time the involvement of the *Per*2 gene in the β-endorphin neuronal response to ethanol. We showed here that the *Per*2 mutation prevents the β-endorphin stimulatory response to acute ethanol, and the β-endorphin inhibitory response to chronic ethanol treatments. Because the effect was observed not only *in vivo* but also *in vitro* cell culture systems where β-endorphin neurons may lack the influence of the central clock due to lack of the integrated neurocircutry required for SCN control mechanism, it could be suggested that the *Per*2 gene regulates the internal clock to control β-endorphin neuronal response to ethanol. In support of this view is the data that prenatal ethanol alters Per2 and POMC mRNA levels in the arcuate nucleus of the adult rat hypothalamus (Chen et al., 2006). Additionally, Per2 gene is identified in laser captured microdissected β-endorphin neurons (Chen et al., 2006).

We also demonstrated an increase in the basal release rate of β-endorphin in cells of MBH from *Per*2 mutants suggesting that the clock gene might have an inhibitory control over β-endorphin production and release. However, basal β-endorphin levels in the MBH tissue was similar in both C57BL/6 and in *Per*2 mutant mice, suggesting that clock gene inhibitory

influence over β-endorphin production and release may be overcome by the stimulatory inputs from other CNS areas under the *in vivo* condition.

A core clock mechanism in the mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 are positive regulators and three m*Period* (m*Per*) genes are involved in negative feedback. Clock gene products BMAL1 and CLOCK have been shown to regulate circadian expression of several genes through their binding to E-boxes (Jin et al., 1999). Thus, it is possible that the molecular circadian clock directly regulates β-endorphin producing POMC gene expression since there are putative E-box binding sites in the mouse POMC promoter (Therrien, 1993).

Our data indicated a direct action of Per2 gene in regulation of POMC gene response to ethanol. This view is in agreement of the finding by Spanagel et al, who showed increased ethanol preference following Per2 mutation possibly due to altered excitatory neurotransmission and that the increase is not related to circadian timing (Spanagel et al., 2005a). Additional support for the involvement of the *Per*2 gene in regulating ethanol sensitivity comes from the indirect evidence that ethanol-induced suppression of immune function is correlated with the altered expression of the *Per*2 gene in splenocytes (Arjona et al., 2004) and m*Per*2 mutation disrupts circadian expression of cytolytic factors in splenocytes (Arjona and Sarkar, 2006).

Another key question is the pathophysiological relevance of the circadian regulation of β-endorphin neurons, especially in the context of fetal alcohol spectrum disorders. β-Endorphin peptide and its precursor protein POMC, control a wide variety of physiological functions, including stress control, feeding, immune functions, tumor control, positive reinforcement and motivational properties of alcohol (De Wied et al., 1982; Gianoulakis, 1996; Konturek et al., 2005; Sarkar et al., 2008; Boyadjieva et al., 2009). Studies in laboratory rodents indicated that prenatal ethanol exposure produces significant alteration in circadian expression of *Per*2 and POMC genes in the hypothalamus (Chen et al., 2006), reduces immune function (Arjona et al., 2006), increases the incidence of cancers (Hilakivi-Clarke et al., 2004), and increases alcohol-drinking behavior (Chotro et al., 2003). Mutations in *Per* genes in mice also have been shown to produce similar effects on β-endorphin neurons (Figs 1–4), the immune system (Arjona and Sarkar, 2006), cancer development (Lee, 2006) and alcohol drinking behavior (Spanagel et al., 2005). Furthermore, the prenatal ethanol-induced stress axis dysfunction (Weinberg et al., 2008; Sarkar et al., 2007) is associated with abnormal expression of the *Per*2 gene in β-endorphin neurons (Arjona and Sarkar, 2006), which participate in stress control mechanisms (Sarkar et al., 2007; Boyadjieva et al., 2009). Together these data provide support for the notion that the abnormality in *Per*2 gene function may participate in induction of many prenatal ethanol-induced pathophysiologies. Finally, much research is needed in order to further characterize the interaction between ethanol and *Per*2 gene, as well as the implications of this interaction for health.

Acknowledgments

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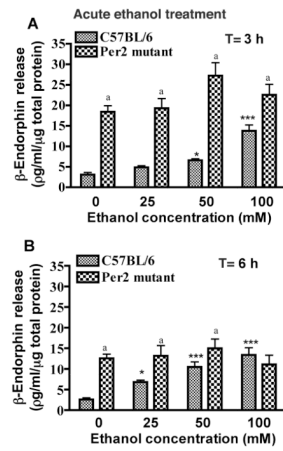
Fig. 1. Effects of acute ethanol exposure on β-endorphin release from mediobasal hypothalamic cells of C57BL/6 mice and *Per*2 mutant mice in primary cultures
MBH cell cultures were treated with (25, 50, 100 mM) or without ethanol for 3 hrs (A) or 6 hrs (B). T = time of treatment. Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. [a] P< 0.05, significantly different between two strains at the same dose.
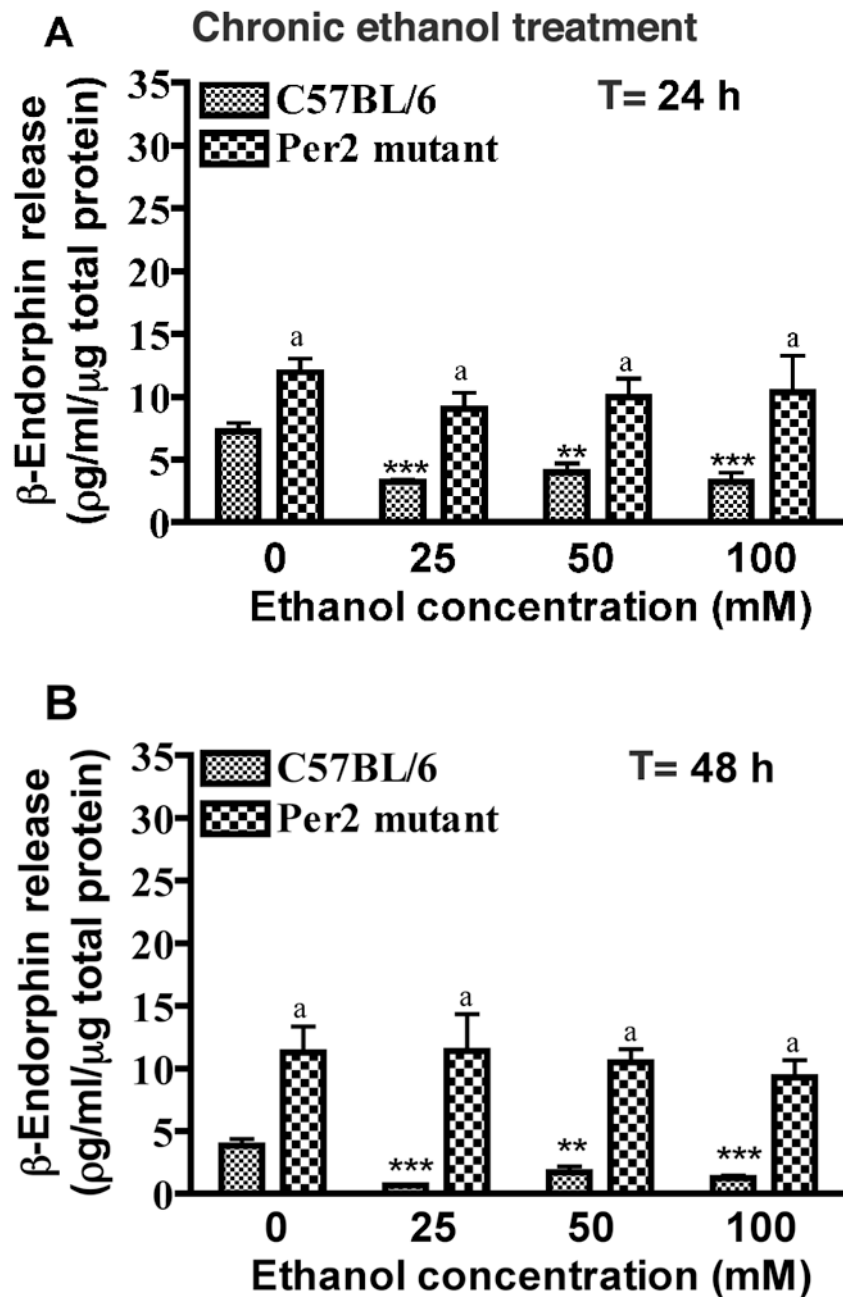
Fig. 2. Effects of chronic ethanol exposure on β-endorphin release in mediobasal hypothalamic cells of C57BL/6 mice and *Per*2 mutant mice

MBH cell cultures were treated with (25, 50, 100 mM) or without ethanol for 24 (A) to 48 hrs (B). T = time of treatment. Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. [a] P< 0.01, significantly different between two strains at the same dose.
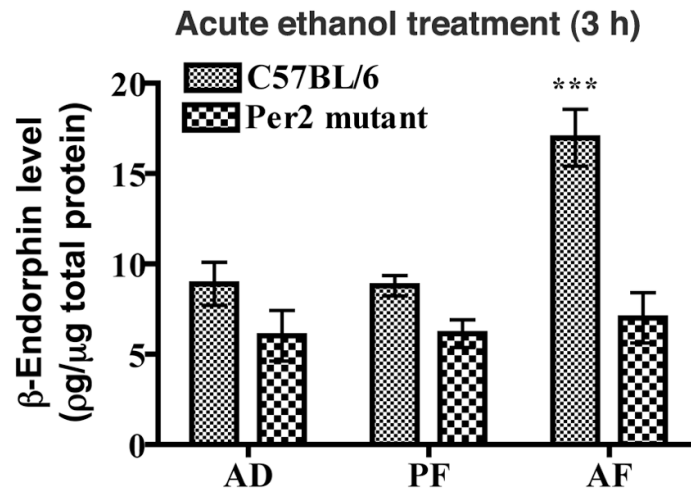
Figure 3. Effects of acute ethanol administration on β-endorphin levels in mediobasal hypothalami of C57BL/6 and *Per2* mutant mice

Postnatal rats were fed milk formula containing ethanol (AF) or no ethanol (PF) or left in the litter (AD) for 1 day. T = time of treatment. Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. [a] P< 0.01, significantly different between two strains at the same dose.
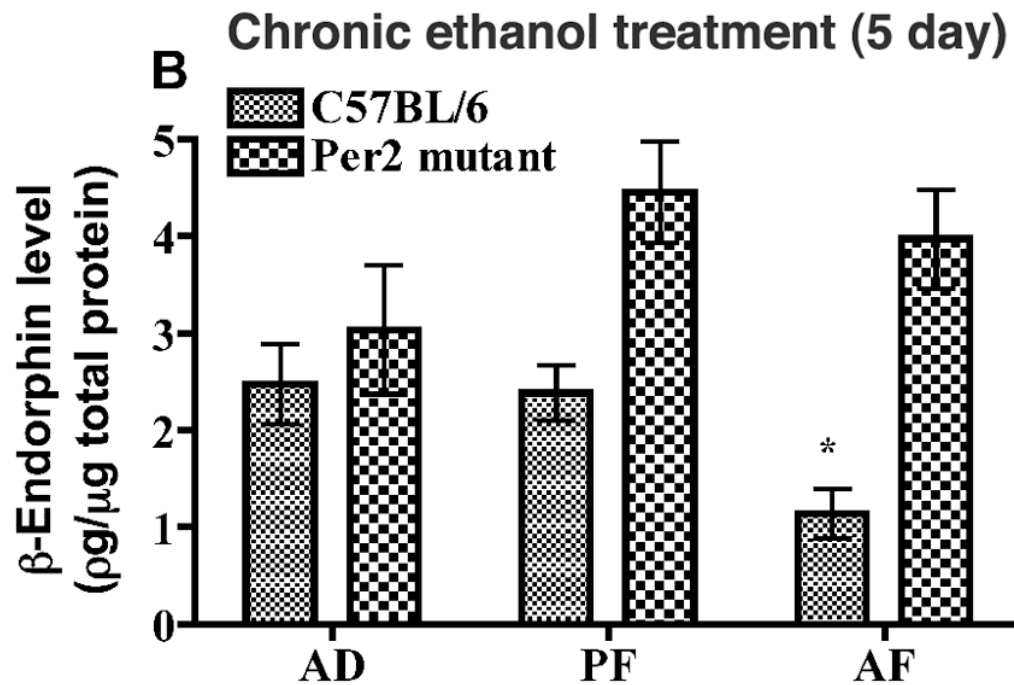
Figure 4. Effects of chronic ethanol administration on β-endorphin levels in mediobasal hypothalami of C57BL/6 and *Per*2 mutant mice
Postnatal rats were fed milk formula containing ethanol (AF) or no ethanol (PF) or left in the litter (AD) for 5 days. T = time of treatment. Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. a P< 0.01, significantly different between two strains at the same dose.