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Long-term ethanol effects on acute stress responses: modulation by dynorphin

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ABSTRACT

The brain stress-response system is critically involved in the addiction process, stimulating drug consumption and the relapse to drug taking in abstinent addicts. At the same time, its functioning is affected by chronic drug exposure. Here, we have investigated the role of the endogenous opioid peptide dynorphin as a modulator of effects of long-term ethanol consumption on the brain stress-response system. Using the two-bottle choice paradigm, we demonstrate an enhanced ethanol preference in male dynorphin knockout mice. Exposure to mild foot shock increased ethanol consumption in wild-type control littermates, but not in dynorphin-deficient animals. Blood adrenocorticotropic hormone levels determined 5 minutes after the shock were not affected by the genotype. We also determined the neuronal reactivity after foot shock exposure using c-Fos immunoreactivity in limbic structures. This was strongly influenced by both genotype and chronic ethanol consumption. Long-term alcohol exposure elevated the foot shock-induced c-Fos expression in the basolateral amygdala in wild-type animals, but had the opposite effect in dynorphin-deficient mice. An altered c-Fos reactivity was also found in the periventricular nucleus, the thalamus and the hippocampus of dynorphin knockouts. Together these data suggest that dynorphin plays an important role in the modulation of the brain stress-response systems after chronic ethanol exposure.

Keywords Alcohol, c-Fos, ethanol preference, stress hormone.

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INTRODUCTION

The brain stress-response system is critically involved in different stages of the addiction process (Koob & Volkow 2010). On the one hand, stress is one of the main factors stimulating drug consumption and the relapse to drug taking in abstinent addicts (Delaney *et al.* 2002). Chronic drug exposure on the other hand, affects the brain stress-response systems. Thus, drug abuse is often accompanied by enhanced brain stress responses, which in turn may contribute to the addiction process (Koob & Volkow 2010).

The endogenous opioid system is a modulator of drug reward and stress responses. It consists of endogenous opioid peptides (enkephalin, β-endorphin, dynorphin, nociceptin) and their G-protein coupled receptors [mu- (MOR), delta- (DOR), kappa-opioid receptors (KOR), opioid receptor-like receptor 1 (ORL1)]. Enkephalins and endorphins activate MOR and DOR, whereas dynorphins bind selectively to KOR and nociceptin to ORL1 (Kieffer & Gaveriaux-Ruff 2002). The MOR has been suggested to constitute a 'gateway to drug addiction' (Contet, Kieffer & Befort 2004) because it is essential for the reinforcing effects of many drugs of abuse. The hedonic control of MOR is opposed by KOR activation which is aversive and may contribute to the negative emotional effects of drug withdrawal (Shippenberg, Zapata & Chefer 2007).

Thus, alcohol consumption was reduced in MOR (Roberts *et al.* 2000) and β-endorphin (Racz *et al.* 2008) knockout animals. However, other studies in knockout mice have delivered contradictory results. Earlier studies by Grisel *et al.* (1999) presented elevated ethanol self-administration in β-endorphin knockout animals. MOR knockout mice and wild-type animals treated with the MOR antagonist, naloxonazine showed a reduction in ethanol-induced dopamine release in the nucleus accumbens (Job *et al.* 2007). Rats and mice selectively bred for higher ethanol preference showed a positive correlation with an elevated MOR function (de Waele, Kiianmaa & Gianoulakis 1995; de Waele & Gianoulakis 1997; Soini, Hyytia & Korpi 2002).

Acute treatment with the KOR agonist U50,488 attenuated ethanol self-administration in two-bottle choice paradigm (Lindholm *et al.* 2001) and decreased ethanol-induced conditional place preference (Logrip, Janak & Ron 2009), while chronic administration of KOR agonist potentiated ethanol-induced place preference. It has been suggested that the repeated exposure to KOR agonists prolongs the dysphoric state and potentiates the rewarding effect of drugs of abuse (Sperling *et al.* 2010; Wee & Koob 2010). Nor-binaltorphimine (nor-BNI), a KOR antagonist, causes a long-lasting increase in ethanol consumption in Lewis rats (Mitchell, Liang & Fields 2005). Other studies found an opposite effect with other rat strains. For example, Wistar rats showed a progressive attenuation in excessive alcohol self-administration after exposure to nor-BNI. This was selective for alcohol dependent rats and not observed in non-dependent animals (Walker, Zorrilla & Koob 2011). When injected directly into the nucleus accumbens, nor-BNI increases, while U50,488 decreases the dopamine level in this brain area (Spanagel, Herz & Shippenberg 1992; Lindholm *et al.* 2007). In addition, mice lacking the KOR have elevated dopamine levels in the nucleus accumbens (Chefer *et al.* 2005).

Chronic ethanol treatment decreases MOR activity via tolerance-like neuroadaptation, while the KOR becomes activated or sensitized, which results in compulsive ethanol consumption (Wee & Koob 2010). The latest studies using ethanol dependent animals support the hypothesis that dynorphin/KOR signalling becomes dysregulated during ethanol dependence and thus produces a negative emotional state that results in exaggerated ethanol intake during acute withdrawal (Walker & Koob 2008; Nealey *et al.* 2011).

Stress engages the endogenous opioid system in limbic structures (Shirayama *et al.* 2004), which contributes to the initiation, modulation and termination of stress responses (Drolet *et al.* 2001; Kieffer & Gaveriaux-Ruff 2002). The role of dynorphin/KOR signalling as a modulator of stress responsivity in particular has recently attracted much interest (McLaughlin, Marton-Popovici & Chavkin 2003; Knoll & Carlezon 2010). Stress exposure triggers the release of dynorphin in limbic structures through increased neuronal activity and/or as a consequence of the cellular effects of stress hormones. The subsequent activation of KORs is thought to contribute to the negative emotional effects of the stress exposure, which motivates the individual to escape from the stressor.

The focus of the present study was to examine the role of dynorphin on the interaction of long-term ethanol exposure and acute stress. We hypothesized that ethanol is more reinforcing in the dynorphin knockout animals and that dynorphin plays an important role in the modulation of stress-induced ethanol drinking. Thus, dynorphin-deficient mice and wild-type controls consumed ethanol for several weeks before being exposed to a mild foot shock stressor. Effects of chronic ethanol consumption on behavioural, hormonal and cellular responses to this stressor were recorded.

METHODS

Animals

Mice with a deletion of the prodynorphin gene ($Pdyn^{-/-}$) and wild-type littermates ($Pdyn^{+/+}$) used in this study were generated in our laboratory as described by Kriegsfeld & Nelson (1998 and Zimmer *et al.* (2001). $Pdyn^{-/-}$ mice were crossed for more than 10 generations to C57BL/6J mice and were therefore congenic for this genetic background. The genotype of the animals was confirmed by polymerase chain reaction using multiple primers. All animals were housed individually under reversed light–dark conditions (lights on at 7:00 PM, lights off at 9:00 AM). Our animal facility chose this set of light–dark cycle to improve the reproductive function of the mice (Kriegsfeld & Nelson 1998). The animals had free access to food and water. Experiments were conducted with 8- to 10-week-old mice. For the two-bottle choice experiments, we used 38 male and 42 female $Pdyn^{-/-}$ animals, and 40 male and 40 female $Pdyn^{+/+}$ mice. Fifteen to 20 animals per genotype and sex served as controls. The following experimental groups were used and each groups consisted of 20 animals per genotype and sex: animals receiving water, without stress exposure, animals receiving two-bottle choice, without stress exposure, animals receiving water and exposed to foot shock stress, and animals receiving two-bottle choice and exposed to foot shock stress. After the foot shock procedure, the groups were further split as follows: 10 animals of the 20 were returned back to the home cage to determine the stress-induced ethanol consumption, 7 animals were used for the plasma adrenocorticotropic hormone (ACTH) measurement and 3 animals were used for the immunohystochemistry. Experimental procedures complied with all regulations for animal experimentation in Germany and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein-Westfalen, Germany (AZ: 8.87-50.10.35.08.324).

Ethanol preference and stress-induced alcohol consumption

Ethanol preference was assessed using the two-bottle choice procedure as previously described by Racz *et al.* (2003). Briefly, two drinking bottles with 8% v/v alcohol (EtOH) or drinking water were available to the animals *ad libitum*. In order to avoid a possible side preference, the

positions of bottles were changed daily. The drinking bottles (g), the body weight (g) and food (g) were weighed twice a week. The ratio of alcohol to total fluid consumption and the amount of consumed ethanol (g/kg) were calculated after every measurement. These data were used to assess the average daily and weekly consumption. In the stable drinking phase (at least 5 weeks of two-bottle choice), blood alcohol levels were determined. For this, we collected blood from the orbital sinus under a short isoflurane anaesthesia. The blood samples were centrifuged (4000 rpm, 20 minutes, 4°C), plasmas were collected and frozen immediately. Plasma alcohol levels were determined using a NAD-ADH Reagent Multiple Test Vial (Sigma N7160; Sigma Aldrich, Saint Louis, MO, USA). To control for the possible leakage of the bottles, we used an empty cage containing one bottle with water and one bottle with 8% ethanol solution, which was handled the same way as the other cages. We subtracted the leakage of the bottles measured in the empty cages when we calculated the water and ethanol consumptions of the animals.

From the fifth week of the two-bottle choice, the animals presented a stable ethanol preference and intake (Racz *et al.* 2003). We let them drink for three additional weeks and exposed them to a mild foot shock. They were placed in an isolated, dark chamber with a continuous background white noise (65 dB) for 5 minutes. Warning signals (sound and light) were presented a few seconds before intermittent electric foot shocks (intensity 0.5 mA, duration 100 ms, interval between shocks 55–60 seconds) delivered five times through a grid floor. We recorded the behavioural responses (jumping reactions) of the animals during the stress procedure and then calculated the mean of the five startle reactions.

The mice were then returned to their home cages, and the alcohol and water consumption was determined 24 and 72 hours after the shock and calculated as the average daily consumption. These values were compared to the average daily ethanol consumption of the last 5 weeks before the stress exposure.

Plasma ACTH measurement

The plasma ACTH levels were determined as described by Bilkei-Gorzo *et al.* (2008). Briefly, seven animals per groups were killed 5 minutes after the foot shock by decapitation and trunk blood was collected. ACTH concentrations were determined from the plasma using a radioimmunoassay (MP Biomedicals, Illkirch, France). Basal stress hormone levels were measured from mice that were individually housed and received either water and alcohol, or just water and were put into the startle box without exposure to foot shocks. Here, blood collection was performed as described for the stressed animals.

Detection of c-Fos like immunoreactivity

Three wild-type and knockout animals were killed 90 minutes after foot shock stress and c-Fos expression was evaluated as described by Bilkei-Gorzo *et al.* (2008). The control groups were handled in the same way, except that they were not exposed to foot shock. Animals were sacrificed by cervical dislocation, their brains were removed, shock frozen in isopentane and kept at −80°C until further use. Brains were sliced in 16 µm slices and mounted on glass slides. The sections were fixed for 30 minutes in 4% formaldehyde and then incubated for 15 minutes in 0.3% H_2O_2, for 60 minutes in 0.5% Triton X-100, and for 2 hours in 3% bovine serum albumin (BSA). After blocking the non-specific binding sites with BSA solution, the slices were kept for 48 hours at 4°C in rabbit anti-c-Fos antibody solution (Calbiochem Ab-5, 1:15,000; Merck KGaA, Darmstadt, Germany). After washing, the slides were soaked in biotinylated donkey anti-rabbit immunoglobulin G solution for 2 hours (1:500 in phosphate-buffered saline containing 0.5% BSA; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and in avidin-biotin complex (ABC) solution for 60 minutes. The presence of antibody–antigen complex was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB) staining. First, we incubated the slides for 5 minutes in the chromogen solution containing 0.5 mg/ml DAB, 0.5 mg/ml NH_4Ni-sulphate in 50 mM (pH 7.3) Tris-buffer. The reaction was started by adding 30% H_2O_2 (0.14 ml/ml reaction mixture) and stopped after 9 minutes washing with 50 mM Tris-buffer (pH 7.3). The signal intensity was quantified using the NIS-Elements software (Nikon, Melville, NY, USA) and expressed as the average number of cells exhibiting c-Fos-like immunoreactivity (c-Fos IR) per mm^2 per brain area. The areas of measurement were selected to best represent the corresponding brain region. These areas were identical for all groups. We evaluated three mice per group with at least four sections per mouse. The following areas were analyzed: basolateral amygdala, paraventricular nucleus (PVN), dentate gyrus of the hippocampus and the periventricular thalamic nucleus.

Statistical analysis

Statistical analysis was performed using a STATISTICA software package (version 7.1 Statsoft, Inc., 2005; StatSoft GmbH, Berikon, Switzerland). We used repeated measurement analysis of variance (ANOVA) for the analysis of ethanol consumption and preference (strain and sex as main factors, and time as within effect). The stress effect on ethanol consumption was analyzed using one-way ANOVA followed by the Fisher least significant difference test. The behavioural reactions were calculated

by two-way ANOVA (strain and ethanol consumption were the main factors). Serum ACTH level and c-Fos IR was analyzed by two-way ANOVA, where ethanol treatment and foot shock were the main factors. All significance tests were two-tailed and considered as significant at $P < 0.05$.

RESULTS

Ethanol consumption

Female mice showed a significantly higher preference for EtOH than male animals ($F_{1, 276} = 7.78$, $P < 0.0001$) and are thus presented separately in Fig. 1. Pdyn$^{-/-}$ females consumed significantly more EtOH than Pdyn$^{+/+}$ females ($F_{1, 122} = 6.66$, $P = 0.01$), but they showed the same ethanol preference ($F_{1, 122} = 0.001$, $P = 0.98$). Thus, female knockout mice drank more ethanol solution and also more water. Female mice from both strains showed a significant increase in EtOH preference during the 7-week drinking period ($F_{6, 732} = 35.69$, $P < 0.0001$). Ethanol consumption of male Pdyn$^{-/-}$ animals was also significantly higher compared to wild-type males ($F_{1, 154} = 13.7$, $P < 0.001$), and they also showed a significantly higher preference for alcohol ($F_{1, 154} = 13.7$, $P = 0.01$). The measurement of serum alcohol level demonstrated the same difference that in ethanol consumption. The serum alcohol levels were higher in Pdyn$^{-/-}$ mice (male 125.7 ± 18.3 mg/dl, female 132.1 ± 17.5 mg/dl) compared with Pdyn$^{+/+}$ animals (male 46.2 ± 18.3 mg/dl, female 59.1 ± 17.5 mg/dl).

Stress reactivity

Mild foot shocks caused a significant, but transient increase in EtOH consumption and preference in male Pdyn$^{+/+}$ mice 24 hours after the stress procedure ($F_{2, 211} = 7.52$, $P = 0.037$). However, after 72 hours this effect was normalized, EtOH preference and consumption returned to the same levels that were measured before the shock (Fig. 2). In contrast, high EtOH drinking was maintained in Pdyn$^{-/-}$ male animals after stress exposure ($F_{2, 76} = 1.67$, P = ns). Female mice showed a high EtOH consumption without stress exposure, which was not changed after stress exposure (Fig. 2). We detected a slight but significant ($F_{2, 61} = 3.36$, $P = 0.013$) elevation

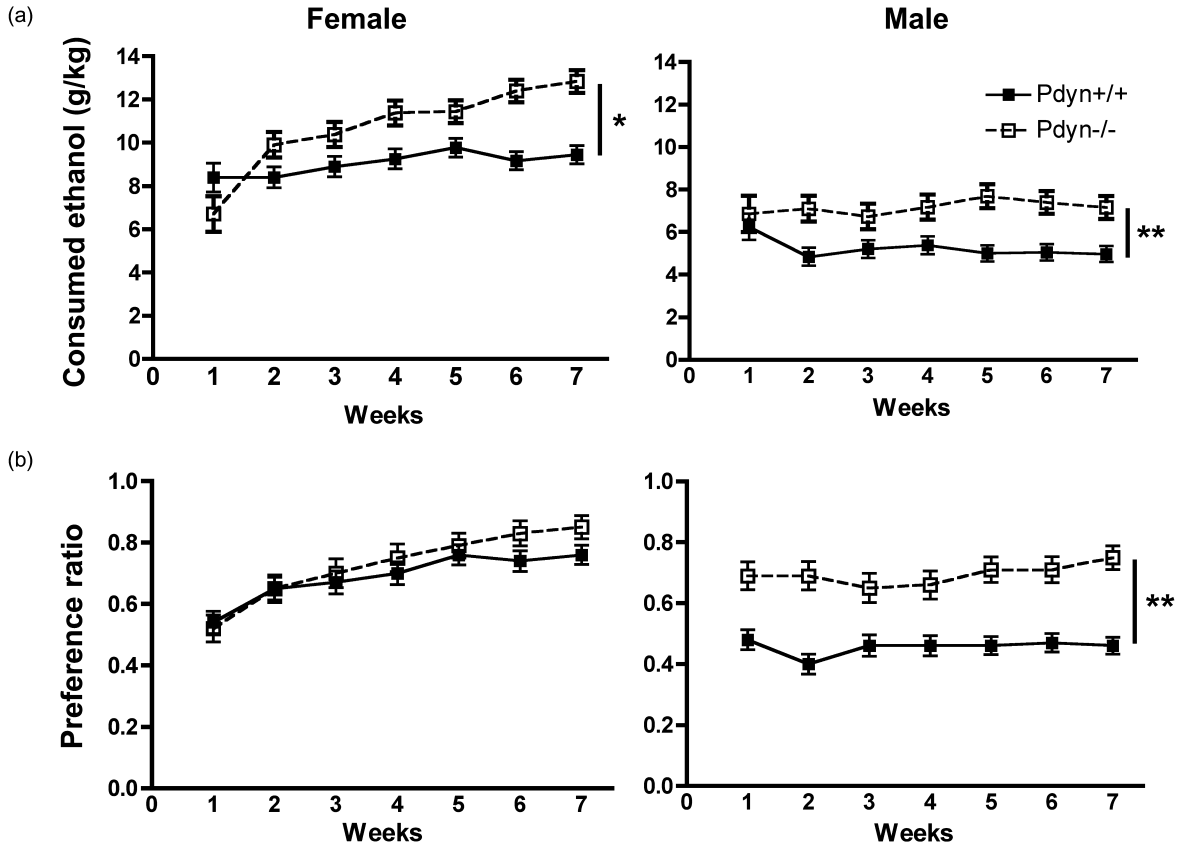

Figure 1 Ethanol consumption and preference of Pdyn$^{-/-}$ and Pdyn$^{+/+}$ animals. (a) Pdyn$^{-/-}$ mice consumed more ethanol than Pdyn$^{+/+}$ animals. In both strains, the alcohol consumption of female mice was higher in comparison to male animals. (b) Female mice of both genotypes showed a significantly increasing preference for alcohol during the 7-week drinking period. The male Pdyn$^{-/-}$ mice also displayed a higher ethanol preference compared with control littermates. $n = 20$/gender/strain; *$P < 0.05$; **$P < 0.005$

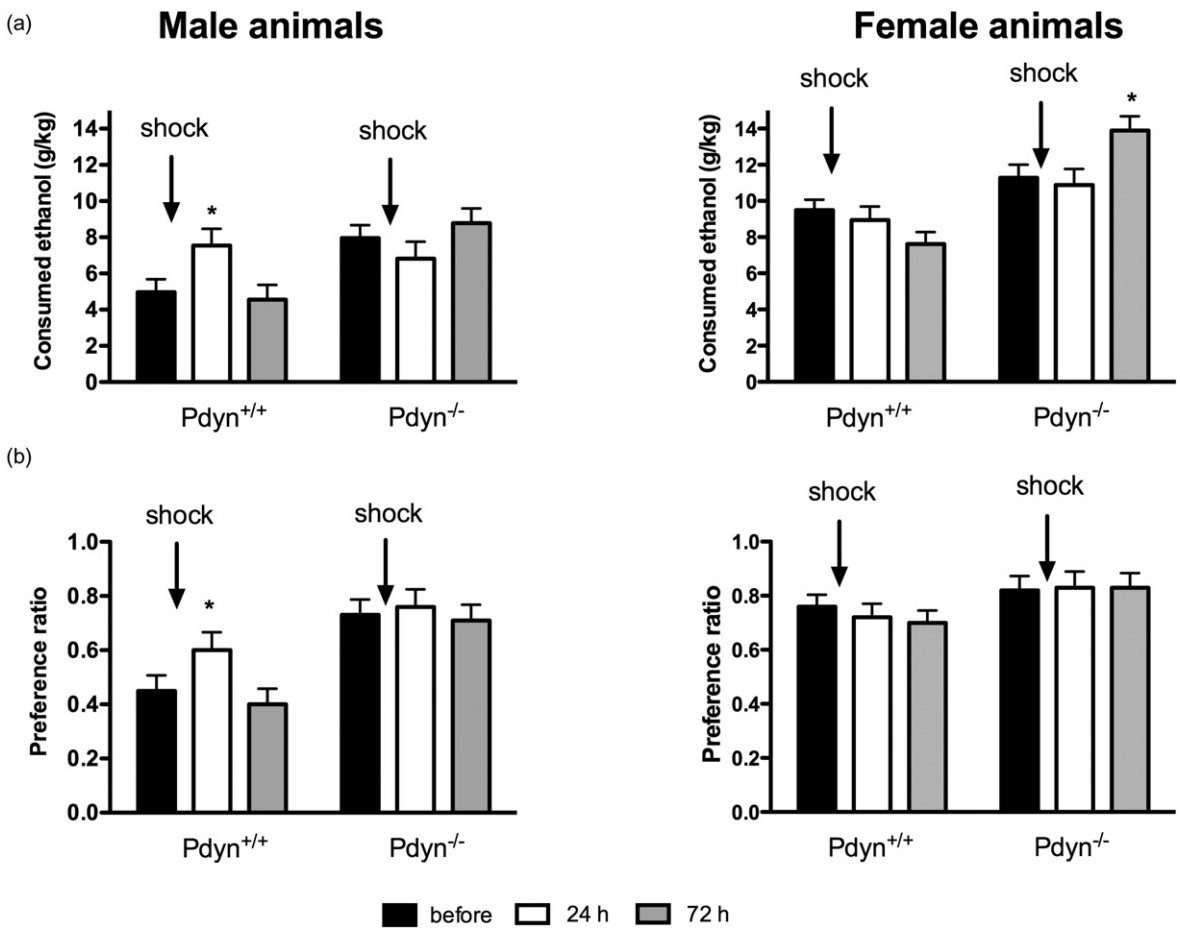

Figure 2 Stress-induced ethanol consumption of Pdyn$^{-/-}$ and Pdyn$^{+/+}$ animals. (a) Pdyn$^{+/+}$ male mice drank significantly more alcohol and showed significantly higher preference (b) for ethanol 24 hours after the mild foot shock exposure. In both strains the female mice did not present any stress-induced changes in ethanol consumption (a) and preference (b) 24 hours after the shock. Seventy-two hours afterwards, the ethanol consumption and preference returned to the same level as before the shock procedure in the wild-type mice. The Pdyn$^{-/-}$ animals consumed significantly more alcohol (a) and showed significantly higher preference (b) for alcohol before the stress and presented no changes after the foot shock. Seventy-two hours after the foot shock, the female knockout mice presented a slight, but significant elevation in ethanol consumption but not in ethanol preference. The term 'before' comprises the mean daily ethanol consumption of the last 5 weeks before the shock procedure. $n = 20$/gender/strain; *$P = 0.05$

in ethanol consumption 72 hours after the shock exposure, although the ethanol preference remained unchanged. It is important to note that the foot shock produced a significantly lower startle reactivity in ethanol-naïve Pdyn$^{-/-}$ mice compared with Pdyn$^{+/+}$ mice ($F_{1, 209} = 9.04$, $P = 0.003$). However, *post hoc* test revealed significant differences only between female Pdyn$^{+/+}$ and Pdyn$^{-/-}$ animals ($P = 0.02$). If the mice had been exposed to ethanol in the two-bottle choice paradigm, the startle responses were significantly lower ($F_{1, 209} = 57.45$, $P < 0.0001$) with no significant difference found between the genotypes (Fig. 3).

The mild foot shock caused a significant elevation in plasma ACTH level in both Pdyn$^{+/+}$ and Pdyn$^{-/-}$ mice ($F_{3, 108} = 6.6$, $P < 0.001$). This effect was independent from the long-term ethanol consumption (Fig. 3). The ethanol-naïve and chronic alcohol-drinking animals showed the same stress-induced increase in ACTH level ($F_{3, 108} = 3.04$, $P =$ ns).

We next investigated the pattern of neuronal activity after foot shock stress with and without chronic EtOH exposure using c-Fos immunostaining (Fig. 4) in several brain areas involved in stress responsivity. Virtually no c-Fos IRs was detected in stress-free, alcohol naïve animals independently from the genotype and the examined brain area. An exception was the thalamus, where we found elevated basal c-Fos IR in Pdyn$^{-/-}$ animals.

In the basolateral amygdala, a brain area involved in emotional memory formation after stress, c-Fos expression was significantly increased in both Pdyn$^{+/+}$ and Pdyn$^{-/-}$ animals after foot shock (stress $F_{3, 219} = 11.71$, $P < 0.0001$; strain $F_{3, 219} = 0.36$, $P =$ ns). This

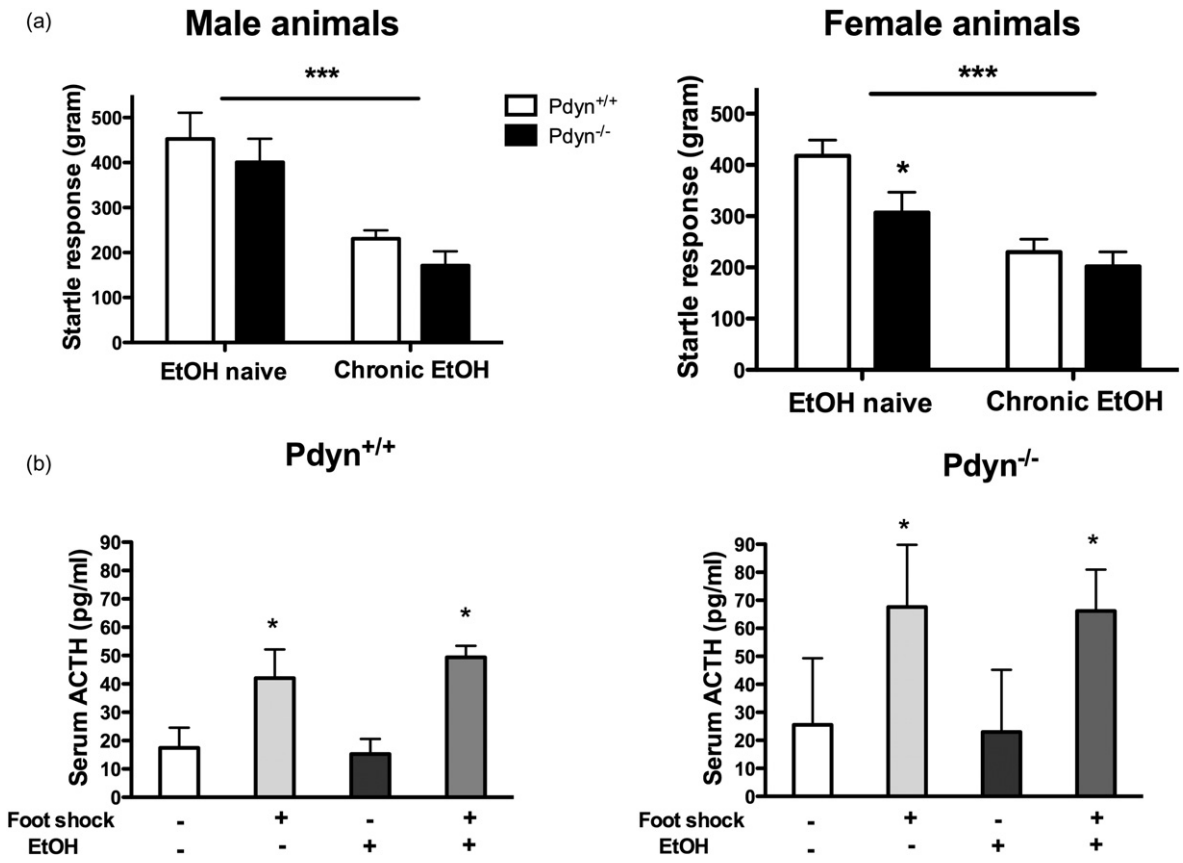

Figure 3 Behavioural and hormonal stress responses of Pdyn$^{-/-}$ and Pdyn$^{+/+}$ animals. (a) The startle response of the Pdyn$^{+/+}$ and Pdyn$^{-/-}$ animals that were not exposed to ethanol was significantly higher compared to animals after long-term alcohol consumption. Pdyn$^{-/-}$ female mice presented significantly lower startle reactions compared with the Pdyn$^{+/+}$ female mice. Chronic ethanol exposure significantly reduced the jumping reaction in both genotypes. (b) Foot shock induced a significant elevation in plasma ACTH level in both strains, independently of the alcohol consumption. $n = 10$; *$P = 0.05$; ***$P < 0.0001$

stress-induced c-Fos expression was more pronounced in the group of wild-type animals that received chronic alcohol (Fig. 4a). In stark contrast almost no c-Fos IR signal was detected in the Pdyn$^{-/-}$ mice that were exposed to stress and chronic alcohol treatment (Fig. 4a). In the PVN of the hypothalamus, which is involved in the regulation of hormonal stress responses, c-Fos reactivity was significantly higher in Pdyn$^{+/+}$ animals than in Pdyn$^{-/-}$ mice (stress $F_{3,219} = 17.37$, $P < 0 < 0001$; strain $F_{3,219} = 6.89$, $P = 0.003$) (Fig. 4b). Alcohol consumption alone elicited c-Fos expression even in non-stressed animals. The mild foot shock also caused an elevation in c-Fos IR that was not affected by ethanol in Pdyn$^{+/+}$ animals, while ethanol exposed Pdyn$^{-/-}$ mice showed no significant c-Fos IR after the foot shock (Fig. 4b). In the periventricular thalamic nucleus, which processes sensory information, we observed significantly higher c-Fos expression after foot shock in the Pdyn$^{+/+}$ animals, which was not influenced by chronic alcohol treatment ($P < 0.001$) (Fig. 4c). In Pdyn$^{-/-}$ animals, the level of basal cellular activity was very high in this brain area and it was significantly decreased by chronic alcohol treatment ($P = 0.01$). Foot shocks caused a further significant elevation independent from the chronic alcohol treatment ($P < 0.001$) (Fig. 4c). We detected significantly higher cellular reaction in Pdyn$^{-/-}$ mice than in wild-type animals ($F_{3,219} = 30.53$, $P < 0.0001$). In hippocampus (dentate gyrus), no elevated c-Fos signals were observed after the foot shock (Fig. 4d).

DISCUSSION

Endogenous opioid peptides modulate chronic ethanol consumption and stress reactivity. Dynorphin, which mediates its effects via activation of KORs, is expressed in brain areas that contribute to mood, motivation and ethanol consumption. Activation of KORs in the limbic system modulates the firing of dopaminergic and glutamatergic neurons and therefore plays an important role in the regulation of emotional and physiological stress responses. Here, we analyzed stress-induced ethanol drinking and long-term ethanol effect on behavioural, hormonal and cellular stress reactivity in dynorphin-deficient mice. Our results indicate that dynorphin

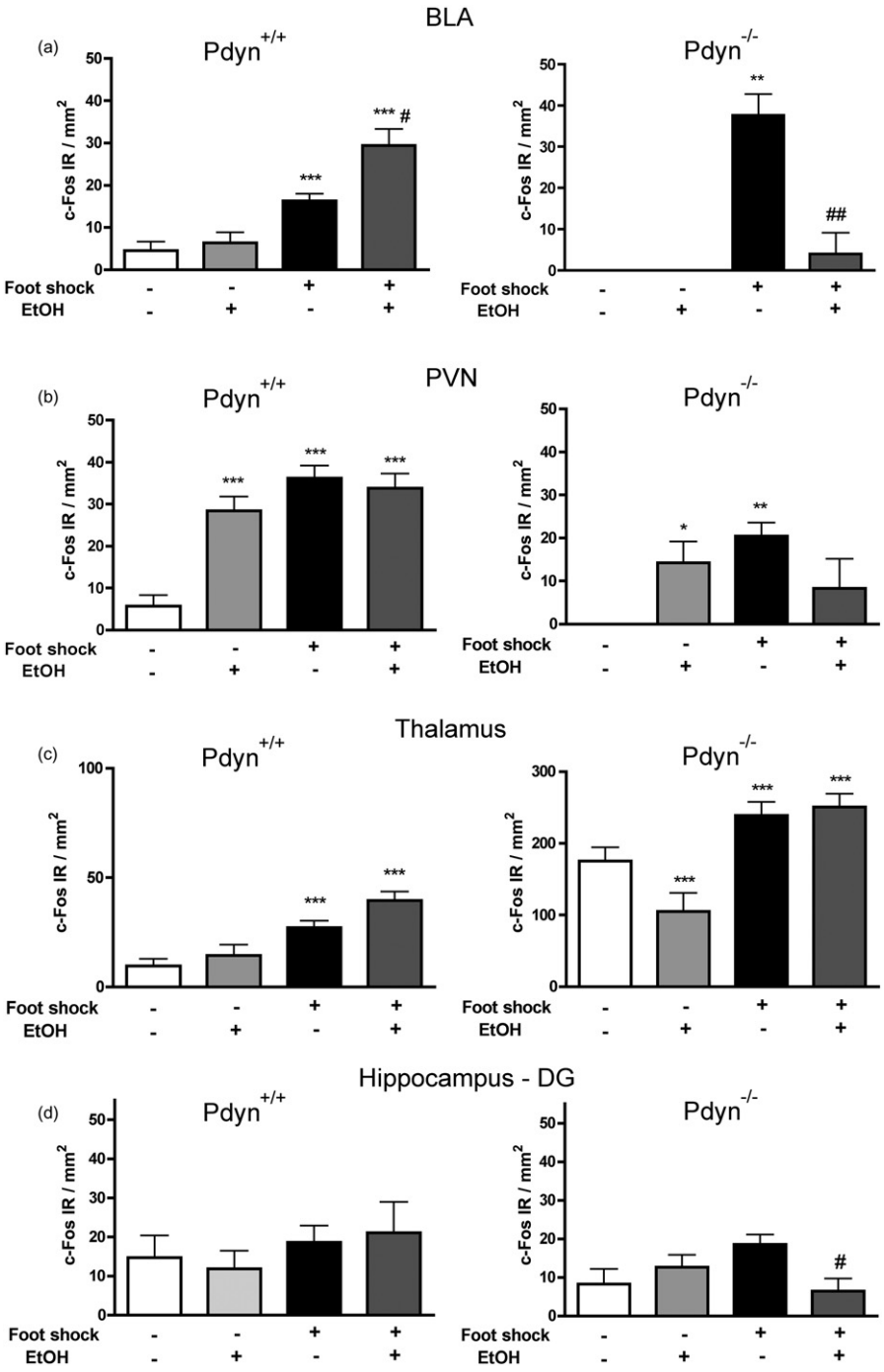

Figure 4 c-Fos immunoreactivity (IR) in different brain areas. (a) In the basolateral amygdala, alcohol naïve Pdyn$^{+/+}$ animals showed a significantly elevated c-Fos IR after the foot shock. The elevation in the c-Fos IR was even more pronounced when the animals were exposed to chronic alcohol treatment before the foot shock. In Pdyn$^{-/-}$ animals, the mild foot shock stress caused a significant elevation in c-Fos IR only in alcohol naïve animals, but not in those exposed to ethanol. (b) In the paraventricular nucleus, ethanol exposure and the stressor both induced c-Fos IR. This was generally higher in Pdyn$^{+/+}$ animals compared with Pdyn$^{-/-}$ mice. (c) In the periventricular thalamic nucleus, we detected a significant elevation of c-Fos IR in the Pdyn$^{+/+}$ animals after stress exposure independent of the EtOH treatment. In Pdyn$^{-/-}$ mice, we detected a very high basal c-Fos IR, which was significantly decreased after 2 months of alcohol drinking. Foot shock stress caused a significant elevation in c-Fos IR in both of alcohol naïve and alcohol-treated groups. Please note that the scale of the *x*-axis differs between the two panels. (d) In the dentate gyrus of the hippocampus, neither alcohol consumption nor stress caused a significant change in the c-Fos IR in the Pdyn$^{+/+}$ and Pdyn$^{-/-}$ animals. BLA, basolateral amygdale; PVN, paraventricular nucleus, DG, dentate gyrus; *Indicates significant difference compared with control groups; #Indicates significant difference between the stressed-EtOH naïve and stressed-EtOH-treated groups; * and #$P < 0.05$; ** and ##$P < 0.001$; ***$P < 0.0001$

modulates ethanol preference and stress-induced alcohol consumption. Lack of dynorphin had no influence on the immediate behavioural and hormonal reactivity to a foot shock stressor. However, the c-Fos expression pattern revealed that the neuronal stress reactivity was strongly affected both by genotype and chronic alcohol treatment. These results suggest that dynorphin modulates the late, long-lasting emotional reactions induced by stress, and this is disturbed by chronic alcohol consumption.

Our finding that Pdyn$^{-/-}$ females exhibited higher ethanol consumption, without increased ethanol preference may be related to the modulatory effects of KOR signalling in diuresis, which is still not completely understood (Wee & Koob 2010). Interestingly, we did not observe increased water consumption in male mice that showed increased ethanol consumption and an enhanced preference for EtOH solution compared with wild-type mice. This suggests that, in males, alcohol is more reinforcing in the absence of dynorphin signalling. Many studies support the existence of sex-difference in addiction processes [for review see Becker & Hu (2008)]. Our findings are in-line with the reported sex-specific differences on the behavioural, physiological and molecular aspects of dynorphin/KOR signalling (Chakrabarti, Liu & Gintzler 2010; Rasakham & Liu-Chen 2011). Sex differences were found in other pharmacological effects of dynorphin/KOR system, like nociception or mood disorders. (Rasakham & Liu-Chen 2011). Numerous studies have examined neuroadaptive changes in the dynorphin/KOR system after chronic exposure of psychostimulant drugs. These studies suggest that male animals are more responsive to KOR agonists than females; however, this effect is dependent on the type of psychoactive drug examined (Rasakham & Liu-Chen 2011). Sershen, Hashim & Lajtha (1998) have shown that acute treatment with spiradoline, a KOR agonist, potentiated cocaine-induced locomotor activity in male C57BL/6J animals, but not in female mice.

It is well known that dopaminergic neurons in the ventral tegmental area are under tonic inhibition of γ-aminobutyric acid (GABA)ergic interneurons. Stimulation of the presynaptic MOR on GABAergic interneurons leads to elevated dopamine release in the nucleus accumbens thus contributing to the reinforcing effect of psychostimulants (Herz 1997), while the activation of KOR in the nucleus accumbens results in a decrease in dopamine level producing an aversive effect in humans and animals (Herz 1997; Zimmer *et al.* 2001; Shippenberg *et al.* 2007). MOR and KOR activation thus have antagonistic effects on the reinforcing properties of alcohol (Herz 1997; Niikura *et al.* 2010). Dynorphin also contributes to the negative emotional state that develops during withdrawal (D'Addario *et al.* 2011). The MOR becomes desensitized in dependent individuals, while the KOR becomes activated or sensitized (Wee & Koob 2010). It has been suggested that these molecular changes contribute to the compulsive drug-seeking behaviour of addicted individuals. Animals lacking dynorphin may thus consume more alcohol because there is no KOR-mediated inhibitory tone over the MOR-mediated reinforcing effect of alcohol.

Our data are consistent with findings in several rat and mouse strains that were selected for a high ethanol preference. Expression studies have demonstrated that dynorphin and KOR levels are lower in those ethanol-preferring strains compared with their low ethanol-drinking counterparts (Winkler & Spanagel 1998; Fadda *et al.* 1999; Marinelli, Kiianmaa & Gianoulakis 2000). Pharmacological studies also support these genetic findings. Thus, administration of the KOR antagonist nor-BNI significantly increased ethanol consumption (Mitchell *et al.* 2005). Conversely, a KOR agonist (U50,488H) dose-dependently reduced ethanol intake in rats (Lindholm *et al.* 2001). Other studies found a contradictory result, where nor-BNI selectively attenuated ethanol self-administration in ethanol-dependent animals, while it did not influence ethanol self-administration in non-dependent animals (Walker & Koob 2008). Naltrexone (an antagonist of MOR) and nalmefene (an antagonist acting on MOR and KOR) reduced ethanol self-administration, but nalmefene was more effective in ethanol-dependent animals. These data suggest that the dynamic interaction of MOR and KOR in the development of addiction processes is important, probably by modulating motivational aspects of addiction.

Blednov *et al.* (2006) found a reduced ethanol preference in female Pdyn$^{-/-}$ animals and no changes in alcohol consumption in male mice. It is conceivable that the different genetic backgrounds of the mice used in our study and the Blednov study contributes to these disparate results, because voluntary alcohol consumption of mice is highly influenced by the genetic background (Crabbe *et al.* 2006). Blednov *et al.* used a mixed C57BL6/6J x 129/SvEv-Tac background strain, whereas our mice had a C57BL/6J genetic background. They also used a different two-bottle choice protocol in which animals received ethanol solution in increasing concentration (the highest dose was 12%).

Stress is one of the main environmental factors that increases the risk for the reinstatement of drug-seeking and drug-taking behaviours in abstinent individuals (Delaney *et al.* 2002; Sinha *et al.* 2009). There is compelling evidence for a role of dynorphin-KOR signalling in this process (Carey *et al.* 2007; Redila & Chavkin 2008; Land *et al.* 2009; Bruchas, Land & Chavkin 2010). Rodents exposed to different types of stressors, like foot shock, immobilization stress and isolation/psychological stress, demonstrated increased ethanol consumption

(Mills, Bean & Hutcheson 1977; Rockman *et al.* 1987; Wolffgramm 1990; Racz *et al.* 2003). This could be due to an elevated rewarding effect of ethanol, or as a form of self-medication to relieve the anxiogenic effects of the stressor (Bruchas *et al.* 2009). Our present results suggest an important role for dynorphin in this process because Pdyn$^{-/-}$ mice did not show any elevation in ethanol consumption or preference after exposure to a foot shock stressor. These data are consistent with findings of Sperling *et al.* (2010), although they used a different stress paradigm. They reported a higher alcohol preference in the two-bottle choice paradigm after repeated exposure to a forced swim stress in C57BL/6J animals. If the mice were treated with a KOR antagonist, or were deficient for dynorphin, they did not display stress-induced increases in alcohol preference (Sperling *et al.* 2010).

For the present experiments, we housed the animals in single cages in order to determine their exact ethanol consumption. Several studies have reported that single housing can function as an environmental stressor and lead to increased ethanol intake in rats, especially in alcohol-preferring rats, which were selectively bred for high alcohol preference (Ehlers *et al.* 2007). However, as all animals were single housed, they were all equally affected by the housing conditions.

It is important to note that Pdyn$^{-/-}$ mice showed normal behavioural (startle) responses to the foot shock stress. Dynorphin had also no influence on the stress-induced activation of the hypothalamic-pituitary-adrenal axis, as Pdyn$^{-/-}$ and Pdyn$^{+/+}$ mice showed similar ACTH hormone levels 5 minutes after the foot shock. The hormonal stress responses were not affected by chronic ethanol treatment, because serum ACTH levels were the same in the ethanol-naïve and in the ethanol-treated mice. However, the startle reactions were significantly attenuated after chronic alcohol drinking in both genotypes. The fact that the foot shock failed to affect ethanol drinking in Pdyn$^{-/-}$ mice is therefore not due to a reduced sensitivity of the animals to this stressor. These data suggest that dynorphin has no modulatory effect in the immediate, early stress responses or the lack of dynorphin in this case can be compensated by the other endogenous opioid peptides.

Interestingly, we found a striking genotype effect on neuronal activation after foot shock exposure, as evaluated by the induction of the immediate early gene c-Fos. This was particularly pronounced in the basolateral amygdala. The foot shock induced a robust c-Fos expression in ethanol-naïve Pdyn$^{+/+}$ mice and an even higher expression in the corresponding Pdyn$^{-/-}$ animals. If wild-type mice were previously exposed to ethanol in the two-bottle choice procedure, the stress-induced elevation of c-Fos expression was further increased, but this was completely absent in the Pdyn$^{-/-}$ mice. Exposure to ethanol also reduced the c-Fos induction in the PVN and hippocampus of Pdyn$^{-/-}$ mice, while it had no effect in Pdyn$^{+/+}$ controls. These data imply that effects of ethanol on neuronal activity after stress exposure are modulated by dynorphin in many brain regions involved in modulation of late stress responses. An exception is the thalamus, where we found a generally higher c-Fos reactivity in Pdyn$^{-/-}$ mice than in Pdyn$^{+/+}$ littermates.

Dynorphin expression is induced after stress exposure (Chartoff *et al.* 2009) in many brain regions involved in the modulation of stress responses, like in amygdala, hippocampus, cortex and hypothalamus (Shirayama *et al.* 2004), through a mechanism that involves the activation of the transcription factor cAMP response element-binding protein (Pliakas *et al.* 2001; Shirayama *et al.* 2004). Several studies have demonstrated an induction of dynorphin/KOR signalling in the basolateral amygdala after stress exposure, probably as a consequence of stress-induced corticotropin-releasing factor (CRF) release. Thus direct injection of CRF into the basolateral amygdala activates KOR signalling and increases anxiety-like behaviour (Bruchas *et al.* 2009). It has been suggested that this may contribute to the reinstatement of drug-seeking behaviour as a means to relieve anxiety.

We also found an elevated cellular activity in the PVN after chronic alcohol treatment in both genotypes. Earlier studies have shown that chronic ethanol consumption increases the expression of dynorphin and enkephalin mRNA in the PVN (Chang *et al.* 2007). In addition, Barson *et al.* (2010) showed that microinjection of morphine, a MOR agonist, into the PVN increased, while injection of a KOR agonist into the same brain region suppressed ethanol intake. These findings confirm the opposing roles of these two opioid peptides on ethanol preference.

Recent studies support a regulatory role of dynorphin/KOR system in chronic alcohol consumption-induced neuronal changes in humans (Bazov *et al.* 2011; Taqi *et al.* 2011). Thus, prodynorphin mRNA and dynorphin level were up-regulated in the dorsolateral prefrontal cortex and the hippocampus in post-mortem analysis of brains of alcoholic patients. These brain areas are implied in cognitive control of drug taking. The studies therefore supported the notion that a dysfunction of the cognitive control of alcohol drinking, a main feature of chronic heavy alcohol consumption, is associated with the activation of dynorphin/KOR signalling (Bazov *et al.* 2011). Interestingly, epigenetic studies showed an altered methylation of prodynorphin CpG dinucleotides overlapping with single-nucleotide polymorphisms (CpG-SNPs), which were associated with alcohol dependence in humans (Taqi *et al.* 2011). These findings indicate that environmental factors contributing to alcohol dependence, which may include stress, affect the expression of prodynorphin through epigenetic mechanisms.

Our present data in animals show that long-term ethanol consumption alters the responsivity of animals to stressors. Ethanol-induced changes were clearly altered in dynorphin-deficient mice thus suggesting a role for dynorphin/KOR signalling in neuroadaptive changes that occur during chronic ethanol treatment.

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Authors Contribution

IR designed the experiments. IR and AZ drafted the manuscript. AM performed the behavioural experiment and contributed to the histological studies. DM contributed to the histological studies. BSW performed the ACTH measurement. All authors critically reviewed content and the approved final version.

References

Barson JR, Carr AJ, Soun JE, Sobhani NC, Rada P, Leibowitz SF, Hoebel BG (2010) Opioids in the hypothalamic paraventricular nucleus stimulate ethanol intake. Alcohol Clin Exp Res 34:214–222.

Bazov I, Kononenko O, Watanabe H, Kuntic V, Sarkisyan D, Taqi MM, Hussain MZ, Nyberg F, Yakovleva T, Bakalkin G (2011) The endogenous opioid system in human alcoholics: molecular adaptations in brain areas involved in cognitive control of addiction. Addict Biol. doi: 10.1111/j.1369-1600.2011.00366.x.

Becker JB, Hu M (2008) Sex differences in drug abuse. Front Neuroendocrinol 29:36–47.

Bilkei-Gorzo A, Racz I, Michel K, Mauer D, Zimmer A, Klingmuller D (2008) Control of hormonal stress reactivity by the endogenous opioid system. Psychoneuroendocrinology 33:425–436.

Blednov YA, Walker D, Martinez M, Harris RA (2006) Reduced alcohol consumption in mice lacking preprodynorphin. Alcohol 40:73–86.

Bruchas MR, Land BB, Chavkin C (2010) The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. Brain Res 1314:44–55.

Bruchas MR, Land BB, Lemos JC, Chavkin C (2009) CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior. Plos ONE 4:e8528.

Carey AN, Borozny K, Aldrich JV, McLaughlin JP (2007) Reinstatement of cocaine place-conditioning prevented by the peptide kappa-opioid receptor antagonist arodyn. Eur J Pharmacol 569:84–89.

Chakrabarti S, Liu NJ, Gintzler AR (2010) Formation of mu-/kappa-opioid receptor heterodimer is sex-dependent and mediates female-specific opioid analgesia. Proc Natl Acad Sci U S A 107:20115–20119.

Chang GQ, Karatayev O, Ahsan R, Avena NM, Lee C, Lewis MJ, Hoebel BG, Leibowitz SF (2007) Effect of ethanol on hypothalamic opioid peptides, enkephalin, and dynorphin: relationship with circulating triglycerides. Alcohol Clin Exp Res 31:249–259.

Chartoff EH, Papadopoulou M, MacDonald ML, Parsegian A, Potter D, Konradi C, Carlezon WA, Jr (2009) Desipramine reduces stress-activated dynorphin expression and CREB phosphorylation in NAc tissue. Mol Pharmacol 75:704–712.

Chefer VI, Czyzyk T, Bolan EA, Moron J, Pintar JE, Shippenberg TS (2005) Endogenous kappa-opioid receptor systems regulate mesoaccumbal dopamine dynamics and vulnerability to cocaine. J Neurosci 25:5029–5037.

Contet C, Kieffer BL, Befort K (2004) Mu opioid receptor: a gateway to drug addiction. Curr Opin Neurobiol 14:370–378.

Crabbe JC, Phillips TJ, Harris RA, Arends MA, Koob GF (2006) Alcohol-related genes: contributions from studies with genetically engineered mice. Addict Biol 11:195–269.

D'Addario C, Caputi FF, Rimondini R, Gandolfi O, Del Borrello E, Candeletti S, Romualdi P (2011) Different alcohol exposures induce selective alterations on the expression of dynorphin and nociceptin systems related genes in rat brain. Addict Biol. doi: 10.1111/j.1369-1600.2011.00326.x

Delaney WP, Grube JW, Greiner B, Fisher JM, Ragland DR (2002) Job stress, unwinding and drinking in transit operators. J Stud Alcohol 63:420–429.

Drolet G, Dumont EC, Gosselin I, Kinkead R, Laforest S, Trottier JF (2001) Role of endogenous opioid system in the regulation of the stress response. Prog Neuropsychopharmacol Biol Psychiatry 25:729–741.

Ehlers CL, Walker BM, Pian JP, Roth JL, Slawecki CJ (2007) Increased alcohol drinking in isolate-housed alcohol-preferring rats. Behav Neurosci 121:111–119.

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

Grisel JE, Mogil JS, Grahame NJ, Rubinstein M, Belknap JK, Crabbe JC, Low MJ (1999) Ethanol oral self-administration is increased in mutant mice with decreased beta-endorphin expression. Brain Res 835:62–67.

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

Job MO, Tang A, Hall FS, Sora I, Uhl GR, Bergeson SE, Gonzales RA (2007) Mu (mu) opioid receptor regulation of ethanol-induced dopamine response in the ventral striatum: evidence of genotype specific sexual dimorphic epistasis. Biol Psychiatry 62:627–634.

Kieffer BL, Gaveriaux-Ruff C (2002) Exploring the opioid system by gene knockout. Prog Neurobiol 66:285–306.

Knoll AT, Carlezon WA, Jr (2010) Dynorphin, stress, and depression. Brain Res 1314:56–73.

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217–238.

Kriegsfeld LJ, Nelson RJ (1998) Short photoperiod affects reproductive function but not dehydroepiandrosterone concentrations in male deer mice (*Peromyscus maniculatus*). J Pineal Res 25:101–105.

Land BB, Bruchas MR, Schattauer S, Giardino WJ, Aita M, Messinger D, Hnasko TS, Palmiter RD, Chavkin C (2009) Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. Proc Natl Acad Sci U S A 106:19168–19173.

Lindholm S, Rosin A, Dahlin I, Georgieva J, Franck J (2007) Ethanol alters the effect of kappa receptor ligands on dopamine release in the nucleus accumbens. Physiol Behav 92:167–171.

Lindholm S, Werme M, Brene S, Franck J (2001) The selective kappa-opioid receptor agonist U50,488H attenuates voluntary ethanol intake in the rat. Behav Brain Res 120:137–146.

Logrip ML, Janak PH, Ron D (2009) Blockade of ethanol reward by the kappa opioid receptor agonist U50,488H. Alcohol 43:359–365.

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.

McLaughlin JP, Marton-Popovici M, Chavkin C (2003) Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J Neurosci 23:5674–5683.

Mills KC, Bean JW, Hutcheson JS (1977) Shock induced ethanol consumption in rats. Pharmacol Biochem Behav 6:107–115.

Mitchell JM, Liang MT, Fields HL (2005) A single injection of the kappa opioid antagonist norbinaltorphimine increases ethanol consumption in rats. Psychopharmacology (Berl) 182:384–392.

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

Niikura K, Narita M, Butelman ER, Kreek MJ, Suzuki T (2010) Neuropathic and chronic pain stimuli downregulate central mu-opioid and dopaminergic transmission. Trends Pharmacol Sci 31:299–305.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA, Jr (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. J Neurosci 21:7397–7403.

Racz I, Bilkei-Gorzo A, Toth ZE, Michel K, Palkovits M, Zimmer A (2003) A critical role for the cannabinoid CB1 receptors in alcohol dependence and stress-stimulated ethanol drinking. J Neurosci 23:2453–2458.

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

Rasakham K, Liu-Chen LY (2011) Sex differences in kappa opioid pharmacology. Life Sci 88:2–16.

Redila VA, Chavkin C (2008) Stress-induced reinstatement of cocaine seeking is mediated by the kappa opioid system. Psychopharmacology 200:59–70.

Roberts AJ, McDonald JS, Heyser CJ, Kieffer BL, Matthes HW, Koob GF, Gold LH (2000) mu-Opioid receptor knockout mice do not self-administer alcohol. J Pharmacol Exp Ther 293:1002–1008.

Rockman GE, Hall A, Hong J, Glavin GB (1987) Unpredictable cold-immobilization stress effects on voluntary ethanol consumption in rats. Life Sci 40:1245–1251.

Sershen H, Hashim A, Lajtha A (1998) Gender differences in kappa-opioid modulation of cocaine-induced behavior and NMDA-evoked dopamine release. Brain Res 801:67–71.

Shippenberg TS, Zapata A, Chefer VI (2007) Dynorphin and the pathophysiology of drug addiction. Pharmacol Ther 116:306–321.

Shirayama Y, Ishida H, Iwata M, Hazama GI, Kawahara R, Duman RS (2004) Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects. J Neurochem 90: 1258–1268.

Sinha R, Fox HC, Hong KA, Bergquist K, Bhagwagar Z, Siedlarz KM (2009) Enhanced negative emotion and alcohol craving, and altered physiological responses following stress and cue exposure in alcohol dependent individuals. Neuropsychopharmacology 34:1198–1208.

Soini SL, Hyytia P, Korpi ER (2002) Brain regional mu-opioid receptor function in rat lines selected for differences in alcohol preference. Eur J Pharmacol 448:157–163.

Spanagel R, Herz A, Shippenberg TS (1992) Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. Proc Natl Acad Sci U S A 89:2046–2050.

Sperling RE, Gomes SM, Sypek EI, Carey AN, McLaughlin JP (2010) Endogenous kappa-opioid mediation of stress-induced potentiation of ethanol-conditioned place preference and self-administration. Psychopharmacology (Berl) 210:199–209.

Taqi MM, Bazov I, Watanabe H, Sheedy D, Harper C, Alkass K, Druid H, Wentzel P, Nyberg F, Yakovleva T, Bakalkin G (2011) Prodynorphin CpG-SNPs associated with alcohol dependence: elevated methylation in the brain of human alcoholics. Addict Biol 16:499–509.

de Waele JP, Gianoulakis C (1997) Characterization of the mu and delta opioid receptors in the brain of the C57BL/6 and DBA/2 mice, selected for their differences in voluntary ethanol consumption. Alcohol Clin Exp Res 21:754–762.

de Waele JP, Kiianmaa K, Gianoulakis C (1995) Distribution of the mu and delta opioid binding sites in the brain of the alcohol-preferring AA and alcohol-avoiding ANA lines of rats. J Pharmacol Exp Ther 275:518–527.

Walker BM, Koob GF (2008) Pharmacological evidence for a motivational role of kappa-opioid systems in ethanol dependence. Neuropsychopharmacology 33:643–652.

Walker BM, Zorrilla EP, Koob GF (2011) Systemic kappa-opioid receptor antagonism by nor-binaltorphimine reduces dependence-induced excessive alcohol self-administration in rats. Addict Biol 16:116–119.

Wee S, Koob GF (2010) The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. Psychopharmacology (Berl) 210:121–135.

Winkler A, Spanagel R (1998) Differences in the kappa opioid receptor mRNA content in distinct brain regions of two inbred mice strains. Neuroreport 9:1459–1464.

Wolffgramm J (1990) Free choice ethanol intake of laboratory rats under different social conditions. Psychopharmacology (Berl) 101:233–239.

Zimmer A, Valjent E, Konig M, Zimmer AM, Robledo P, Hahn H, Valverde O, Maldonado R (2001) Absence of delta-9-tetrahydrocannabinol dysphoric effects in dynorphin-deficient mice. J Neurosci 21:9499–9505.

