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

Ildikó Rácz¹, Astrid Markert¹, Daniela Mauer¹, Birgit Stoffel-Wagner² & Andreas Zimmer¹

Institute of Molecular Psychiatry, University of Bonn, Bonn, Germany¹ and Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany²

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.

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 [µ-(MOR), δ- (DOR), κ-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, Kilanmää & Gianoulakis 1995; de Waele & Gianoulakis 1997; Soini, Hyttia & 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 adrenocorticotrophic hormone (ACTH) measurement and 3 animals were used for the immunohistochemistry. 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% H₂O₂, 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 NH₄Ni-sulphate in 50 mM (pH 7.3) Tris-buffer. The reaction was started by adding 30% H₂O₂ (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 mm² 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 peri-ventricular 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 ($F_{1,154} = 13.7, P < 0.0001$), 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
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 (\(F_{3, 108} = 57.45, P < 0.0001\)). 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 (\(F_{1, 108} = 3.04, P = \text{ns}\)).

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 \(F_{3, 219} = 0.36, P = \text{ns}\)). This
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 ($P = 0.01$). 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<sup>+/−</sup> 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<sup>−/−</sup> 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<sup>+/−</sup> animals compared with Pdyn<sup>−/−</sup> mice. (c) In the periventricular thalamic nucleus, we detected a significant elevation of c-Fos IR in the Pdyn<sup>+/−</sup> animals after stress exposure independent of the EtOH treatment. In Pdyn<sup>−/−</sup> 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<sup>+/−</sup> and Pdyn<sup>−/−</sup> animals. BLA, basolateral amygdala; 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; Nikura 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.
Reduced the c-Fos induction in the PVN and hippocampus and absent in the Pdyn mice were previously exposed to ethanol in the two-bottle induced activation of the hypothalamic-pituitary-adrenal stress. Dynorphin had also no influence on the stress-normal behavioural (startle) responses to the 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-prefering 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 behavourial (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.

Acknowledgement

This work was supported by grants from the Bundesministerium für Forschung und Bildung (NGFN2, FKZ 01GS08156) and the Deutsche Forschungsgemeinschaft (FOR926). We thank Friederike Hinrichsen for technical assistance during the early phase of this study and Dr. Sophie Imbeault for reading and correcting the manuscript.

Authors Contribution

IR designed the experiments. IR and AZ drafted the manuscript. AM performed the behaviour 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


Ethanol in dynorphin knockouts


