Published in final edited form as:

Pharmacol Biochem Behav. 2012 July ; 102(1): 44–47. doi:10.1016/j.pbb.2012.03.019.

Kappa opioid regulation of anxiety-like behavior during acute ethanol withdrawal

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Abstract

Withdrawal is one of the defining characteristics of alcohol dependence, and is often characterized by impaired physiological function and enhanced negative affect. Recent evidence suggests that the dynorphin (DYN)/kappa opioid receptor (KOR) system may be a key mediator in the negative affect often associated with drugs of abuse. The objective of the present experiments was to determine the role of the DYN/KOR system in the regulation of anxiety-related behavior during acute withdrawal from ethanol. Rats were fed an ethanol liquid diet and following removal, the ability of the KOR antagonist nor-BNI to attenuate the increased anxiogenic-like response characteristic of ethanol withdrawal was investigated using the elevated plus maze. A comparison study was also conducted examining anxiety-related behavior following direct activation of KORs via injections of the KOR agonist U50,488. Rats experiencing ethanol withdrawal showed a significant decrease in open arm exploration compared to controls, an effect that was blocked by nor-BNI. Similar decreases in open arm exploration were observed following injections with the KOR agonist, U50,488, an effect also reversed by pretreatment with nor-BNI. These results suggest that similar mechanisms are involved in the regulation of ethanol withdrawal- and KOR agonist-induced changes in behavior. Given the potential role of enhanced negative affect in persistent ethanol drinking, understanding the role of the DYN/KOR system in regulating anxiety associated with withdrawal may be critical in understanding the factors associated with the nature of alcohol dependence.

Keywords

Ethanol; Kappa Opioid; Stress; Withdrawal; Elevated Plus Maze; Anxiety

1. Introduction

Withdrawal is one of the defining characteristics of alcohol dependence, and is often characterized by impaired physiological function and enhanced negative affect. Recent findings implicate the dynorphin (DYN)/kappa opioid receptor (KOR) system as one of the neurobiological mechanisms underlying increased responsiveness to stress and negative affective-like states in laboratory animals (Carlezon et al., 2006; Land et al., 2008; McLaughlin et al., 2003; Todtenkopf et al., 2004). Evidence has also emerged suggesting a role for the DYN/KOR system in the regulation of the stress-related effects of alcohol

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(Walker et al., in press). For example, KOR antagonism reduces the characteristic increase in ethanol self-administration in rats following exposure to chronic ethanol vapor without affecting behavior in control rats (Nealey et al., 2011; Walker and Koob, 2008; Walker et al., 2011), suggesting that kappa opioid receptors may regulate the negative reinforcing properties of ethanol. In addition, the KOR antagonist norbinaltorphimine (nor-BNI) reduces forced swim stress-induced increases in ethanol intake, and the KOR agonist U50,488 potentiates ethanol conditioned place preference and ethanol drinking in mice (Sperling et al., 2010). The role of the DYN/KOR system in stress-related behaviors during ethanol withdrawal, however, has yet to be explored.

The stress-related component of withdrawal seems to be critical in the development of alcoholism because anxiety and depression, which appear to be manifestations of alcohol stress, are thought to set the stage for relapse in many alcoholics (Cloninger, 1987). When examining animal models, rats chronically exposed to ethanol show increased anxiety-like behaviors when tested during withdrawal (Baldwin et al., 1991; Höltter et al., 1998; Rasmussen et al., 2001; Rassnick et al., 1993; Valdez et al., 2003b; 2004). Under similar conditions, increases in ethanol self-administration are also observed (O'Dell et al., 2004; Roberts et al., 2000; Valdez et al., 2002b; Walker and Koob, 2008).

The objective of the present experiments is to determine the role of the DYN/KOR system in the regulation of anxiety-related behaviors characteristic of acute withdrawal from ethanol. The ability of nor-BNI to attenuate the anxiogenic-like response in the elevated plus-maze was investigated in ethanol dependent rats. A comparison study was also conducted examining anxiety-like behavior following direct activation of KORs via injections of the KOR agonist U50,488. These experiments aim to provide further insights to the interaction between kappa opioids and negative affective states related to alcohol withdrawal.

2. Materials and Methods

2.1 Animals

Male Wistar rats (Charles River, Kingston, NY; $n=111$) were used in this experiment. Body weights were 200-250 g and age was approximately 60 days at the start of the experiments. Rats were group housed (3 per cage) with food and water available *ad libitum* except during liquid diet administration, and were weighed daily. Rats were maintained on a 12-hr light/dark cycle (lights on at 22:00h). Procedures met the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication number 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Grand Valley State University.

2.2 Ethanol liquid diet procedure

Details of a similar (8.7% v/v) liquid diet procedure have been reported previously (Schulteis et al., 1996). Briefly, food and water were removed and replaced with a nutritionally complete liquid diet. The ethanol diet consisted of a chocolate flavored, liquid nutritional supplement (Mead Johnson, Inc., Evansville, IN), vitamin fortification mixture and mineral salt (0.3 and 0.5 g/100 ml respectively; ICN Nutritional Biochemicals, Aurora, OH), distilled water and 95% ethanol diluted to a final concentration of 10% (v/v). Sucrose was isocalorically substituted for ethanol in the control diet. Rats in the ethanol condition received *ad libitum* access to the diet. In order to control for caloric intake, control rats were pair-fed a daily ration equal to the prior mean daily intake of the ethanol group. Diets were prepared and administered at the onset of the dark cycle for 28 days.

2.3 Elevated Plus-Maze

The elevated plus-maze (Med Associates, St. Albans, VT) was made of dark Plexiglas and consisted of four arms (50 cm long × 10 cm wide). The two enclosed arms had 40 cm high dark walls while the two open arms had 0.5 cm high ledges. Lighting on the open arms was 1.5-2.0 lux. The maze was elevated to a height of 50 cm. Individual animals were handled for approximately 10-20 minutes daily prior to testing. Because exposure to the elevated plus-maze itself has been proposed to be a stressor (Lister, 1987), this handling procedure was used to prevent a floor effect under which anxiogenic-like effects of ethanol withdrawal or U50,488 may not be detected. Testing occurred 2-3 hours after the onset of the dark cycle. White noise (70 dB) was present throughout habituation and testing.

Rats were placed individually onto the center of the apparatus facing a closed arm, and time spent on and entries onto each arm were recorded automatically by photocell beams and monitored by a computer for 5 minutes. The maze was cleaned with water and dried after each trial. Open arm preference, as indicated by the ratio of open arm to total arm time and entries, has been proposed to relate inversely to anxiety (Cruz et al., 1994). Arm entries have been proposed as an index of locomotor activity (Cruz et al., 1994). Each rat was naive to the apparatus.

2.4 Drugs and Injections

U50,488 ((*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclo-hexyl] benzeneacetamide; Tocris Biosciences, Ellisville, MO) and nor-binaltorphimine (nor-BNI; Tocris Biosciences, Ellisville, MO) were dissolved in 0.9% saline solution for intraperitoneal (i.p.) injections.

2.6 Experimental Procedure

To examine the regulation of anxiety-like behavior via KORs during ethanol withdrawal, rats were given access to an ethanol or control liquid diet. Immediately following removal of the diet, rats received i.p. injections of nor-BNI (10 or 20 mg/kg) or saline. They were then examined in the elevated plus-maze 24-30 hours following injections and removal of the liquid diet. This pretreatment period was chosen because previous research has shown that nor-BNI is most selective for KORs at 24 hours after administration as opposed to earlier time points (Endoh et al., 1992).

A second experiment was conducted examining behavior in the elevated plus-maze following injections of the KOR agonist U50,488. Rats received i.p. injections of nor-BNI (20 mg/kg) or saline 24-30 hours prior to testing in the elevated plus-maze. Based on the results of the previous experiment, only the 20 mg/kg dose of nor-BNI was tested. This dose has also been shown to decrease ethanol self-administration in dependent rats (Walker et al., 2011). On the day of testing, rats were further divided into groups receiving U50,488 (10 mg/kg) or saline. Injections of U50,488 or saline took place 10 minutes prior to testing in the elevated plus-maze.

2.7 Data Analysis

Data were analyzed by using a two-way analysis of variance (ANOVA) with diet (ethanol or control), agonist dose (10 mg/kg U50,488 or vehicle), or antagonist dose (10 or 20 mg/kg nor-BNI or vehicle) as between-subjects factors. Tukey's test was used for post hoc analysis as warranted.

3. Results

Rats fed the ethanol liquid diet consumed an average of 14.6 $\pm$ 1.3 g/kg for 28 days. This amount of ethanol intake has previously been shown to induce physical withdrawal signs (Macey et al., 1996) and pharmacologically relevant blood ethanol levels in rats (122-145 mg%; see Valdez et al., 2003b; 2004). Although the average per animal intake was based on total intake per cage, there were no significant differences in body weights suggesting that wide variability in consumption between animals housed in the same cages was unlikely. There were also no significant differences between pair fed control rats and ethanol liquid diet exposed rats in body weight and the amount of fluid consumed.

There was a significant interaction between diet and dose of nor-BNI on the percentage of open arm time, $F(2,60)=20.91$, $p<0.0001$. Rats fed the ethanol diet showed a characteristic decrease in the percentage of time spent exploring the open arms compared to controls. nor-BNI attenuated this effect, as rats injected with a 20 mg/kg dose explored the open arms at a level comparable to that of controls (Figure 1A). Rats fed the ethanol diet also had fewer arm entries compared to controls, $F(1,60)=10.19$, $p<0.0001$ (Figure 1B). There was no significant main effect of nor-BNI dose, nor a significant interaction between diet and nor-BNI dose on total arm entries.

There was a significant interaction between U50,488 dose and nor-BNI dose on the percentage of time spent exploring the open arms of the elevated plus-maze, $F(1,44)=6.10$, $p<0.05$ (Figure 2A). Injections of 10 mg/kg U50,488 significantly decreased percent open arm time compared to controls, an effect reversed by pretreatment with nor-BNI. In addition, nor-BNI was found to increase the total number of arm entries, $F(1,44)=11.73$, $p<0.05$ (Figure 2B).

4. Discussion

The results of these experiments further support the hypothesis that the DYN/KOR system is involved in regulating certain stress-related effects of drugs of abuse, including chronic exposure to ethanol. Chronic exposure to an ethanol liquid diet led to an increased anxiogenic-like effect in rats examined in the elevated plus maze, as evidenced by the reduction in open arm exploration. This effect has been previously linked to neurobiological regulators of stress, such as corticotropin releasing factor (CRF) (Rassnick et al., 1993; Valdez et al., 2003b) and neuropeptide Y (NPY) (Badia-Elder et al., 2003; Sparta et al., 2007). The present results suggest that the DYN/KOR system is also involved in the regulation of the anxiogenic-like response characteristically observed during ethanol withdrawal, as the KOR antagonist nor-BNI significantly attenuated decreased open arm exploration. A similar decrease in open arm exploration was observed following injections of the KOR agonist U50,488, an effect also reversed by pretreatment with nor-BNI. Taken together, it appears that similar mechanisms are involved in the regulation of ethanol withdrawal- and kappa agonist-induced changes in behaviors.

One issue to consider is whether the effect of nor-BNI on open arm exploration is selective to ethanol dependence. Although control rats injected with 20 mg/kg nor-BNI spent approximately 15% more time exploring the open arms compared to those receiving saline injections, this difference was not statistically significant and may simply be due to individual variability. In addition, nor-BNI pretreatment did not enhance open arm exploration in rats injected with saline in the second experiment, but significantly increased percent open arm time in those receiving U50,488. Although it is difficult to interpret if the actions of nor-BNI are specific to ethanol withdrawal in the present study, the results of the current experiments support the hypothesis that behavioral changes due to KOR antagonism

may be selective to exposure to stressors. Withdrawal from ethanol has been shown to have anxiogenic-like effects (Baldwin et al., 1991; Höltér et al., 1998; Rasmussen et al., 2001), and stress-related biological mechanisms appear to mediate both ethanol withdrawal (Rassnick et al., 1993; Valdez et al., 2002b; 2003b) and KOR agonist (Valdez et al., 2007) induced changes in behavior. Further study of the role of the DYN/KOR system in the regulation of anxiety-related behaviors will aid in clarifying this issue. Nonetheless, these data demonstrate that the anxiogenic-like effects of withdrawal from ethanol are, in part, mediated by DYN/KOR mechanisms.

In contrast to the findings of the current experiments, KOR antagonists have previously been shown to increase open arm exploration of the elevated plus maze under baseline conditions (Knoll et al., 2011). Because exposure to the elevated plus maze itself has been proposed as a stressor that may also decrease the percentage of time spent in the open arms (Lister, 1987), individual rats were handled extensively prior to testing in order to avoid a possible floor effect. Prior handling history has been shown to affect open arm exploration under baseline conditions (Andrews and File, 1993; Valdez et al., 2002a). The level of open arm exploration observed under control conditions in the current experiments were similar to those previously seen following treatment with compounds shown to have anxiolytic-like effects, such as benzodiazepines, CRF antagonists and CRF₂ receptor agonists (Heinrichs et al, 1994; Valdez et al., 2002a; 2003a), suggesting that prior handling experience may have also reduced the anxiogenic properties of exposure to the elevated plus maze.

It should be noted that rats experiencing ethanol withdrawal showed a significant decrease in the number of total arm entries, an indication of general locomotor activity (Cruz et al., 1994). KOR activation has previously been shown to have motor suppressive effects (Negus, 2004). It is possible that the decreases in open arm exploration observed in these animals may have been due to a general decrease in motor activity. The elevated plus-maze, however, examines behavior in a novel context, and exposure to stress in novel environments has been shown to increase measures of anxiety-like behavior such as freezing and immobility (Pellow et al., 1985). As stated previously, withdrawal from ethanol can act as a stressor (Rassnick et al., 1993; Valdez et al., 2003b), and examination of stress-related behaviors following withdrawal in other animal models of anxiety would aid in clarifying this issue. Nonetheless, nor-BNI alone did not have any significant effects on the number of arm entries in ethanol-exposed rats and increased total arm entries in rats injected with U50,488, suggesting that its ability to enhance open arm exploration in the elevated plus-maze is unlikely due to nonspecific actions.

5. Conclusions

Consistent with recent evidence, the current data further support the hypothesis that the DYN/KOR system may be a key mediator in stress-related behavior associated with drugs of abuse. The present experiment extends this hypothesis, demonstrating that the DYN/KOR system is also involved in the regulation of stress-related behaviors associated with ethanol. Results of the current study demonstrated that KOR antagonism blocks the increases in anxiety-like behavior observed during withdrawal from ethanol. Similar results were observed in rats injected with the KOR agonist, U50,488, suggesting that similar mechanisms are involved in the regulation of ethanol withdrawal- and KOR agonist-induced changes in behavior. Given the potential role of enhanced negative affect in persistent ethanol drinking, understanding the role of DYN/KOR system in regulating emotional states associated with withdrawal may be critical in understanding the factors associated with the nature of dependence.

Acknowledgments

Supported was provided by R15 AA018213 from the National Institute on Alcohol Abuse and Alcoholism (GRV) and the Center for Scholarly and Creative Excellence at Grand Valley State University. We gratefully recognize the technical assistance of Kristen Thomas, Erica Goodrich and Katelyn Dekker. We also thank Dr. Brendan Walker of Washington State University for helpful discussions.

Abbreviations

DYN	dynorphin
KOR	kappa opioid receptor
nor-BNI	norbinaltorphimine
U50,488	(<i>trans</i>)-3,4-dichloro- <i>N</i> -methyl- <i>N</i> -[2-(1-pyrrolidinyl)-cyclo-hexyl] benzeneacetamide
CRF	corticotropin-releasing factor
NPY	neuropeptide Y

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Highlights

- Ethanol withdrawal enhanced the anxiety-like behavior in rats, an effect blocked by KOR antagonism
- Similar results found following injections with a KOR agonist.
- Similar mechanisms involved in ethanol withdrawal- and KOR agonist-induced behavioral changes

Figure 1.

A. nor-BNI attenuates the decrease in the percentage of time exploring the open arms during ethanol withdrawal. Rats ($n=9-13$ per group) received i.p. injections of 0-20 mg/kg of nor-BNI at 24-25 hours prior to testing and were then examined in the elevated plus maze. The percentage of time spent exploring the open arms, as indicated by the ratio of open arm to total arm time and entries, has been proposed to relate inversely to anxiety. $*p < 0.05$ Tukey's test compared to ethanol-exposed rats receiving 20 mg/kg nor-BNI and controls. B. Effects of nor-BNI on total arm entries in the elevated plus maze. Rats received i.p. injections of 0-20 mg/kg of nor-BNI at least 24 hours prior to testing and were then

examined in the elevated plus maze. Arm entries have been proposed as an index of locomotor activity. $*p < 0.05$ Tukey's test compared to controls.

Figure 2.

A. nor-BNI attenuates U50,488-induced decreases in the percentage of time spent on the open arms. Rats ($n=10-12$ per group) were pretreated with nor-BNI (20 mg/kg, i.p.) or vehicle at least 24 hours prior to injection of U50,488 (0 or 10 mg/kg). Injections of U50,488 occurred 10 minutes before testing in the elevated plus maze. * $p < 0.05$ compared to vehicle, 0 mg/kg U50,488 group, Tukey's test. B. Effects of U50,488 and nor-BNI on total arm entries. Rats were pretreated with nor-BNI or vehicle at least 24 hours prior to injection of U50,488 (0 or 10 mg/kg). Injections of U50,488 occurred 10 minutes before testing in the elevated plus-maze. Arm entries have been proposed as an index of locomotor activity. * $p < 0.05$ compared to nor-BNI, Tukey's test.