Published in final edited form as:

Neuropharmacology. 2016 November ; 110(Pt A): 190–197. doi:10.1016/j.neuropharm.2016.07.022.

Switch from excitatory to inhibitory actions of ethanol on dopamine levels after chronic exposure: Role of kappa opioid receptors

Anushree N. Karkhanis^{1,2}, Kimberly N. Huggins¹, Jamie H. Rose¹, and Sara R. Jones^{1,2}

¹Department of Physiology and Pharmacology, Wake Forest School of Medicine, Winston-Salem, NC, USA

²Translational Center for the Neurobehavioral Study of Alcohol, Wake Forest School of Medicine, Winston-Salem, NC, USA

Abstract

Acute ethanol exposure is known to stimulate the dopamine system; however, chronic exposure has been shown to downregulate the dopamine system. In rodents, chronic intermittent exposure (CIE) to ethanol also increases negative affect during withdrawal, such as, increases in anxiety- and depressive-like behavior. Moreover, CIE exposure results in increased ethanol drinking and preference during withdrawal. Previous literature documents reductions in CIE-induced anxiety-, depressive-like behaviors and ethanol intake in response to kappa opioid receptor (KOR) blockade. KORs are located on presynaptic dopamine terminals in the nucleus accumbens (NAc) and inhibit release, an effect which has been linked to negative affective behaviors. Previous reports show an upregulation in KOR function following extended CIE exposure; however it is not clear whether there is a direct link between KOR upregulation and dopamine downregulation during withdrawal from CIE. This study aimed to examine the effects of KOR modulation on dopamine responses to ethanol of behaving mice exposed to air or ethanol vapor in a repeated intermittent pattern. First, we showed that KORs have a greater response to an agonist after moderate CIE compared to air exposed mice using *ex vivo* fast scan cyclic voltammetry. Second, using *in vivo* microdialysis, we showed that, in contrast to the expected increase in extracellular levels of dopamine following an acute ethanol challenge in air exposed mice, CIE exposed mice exhibited a robust decrease in dopamine levels. Third, we showed that blockade of KORs reversed the aberrant inhibitory dopamine response to ethanol in CIE exposed mice while not affecting the air exposed mice demonstrating that inhibition of KORs “rescued” dopamine responses in CIE exposed mice. Taken together, these findings indicate that augmentation of dynorphin/KOR system activity drives the reduction in stimulated (electrical and ethanol) dopamine release in the NAc. Thus, blockade of KORs is a promising avenue for developing pharmacotherapies for alcoholism.

Corresponding Author: Sara R. Jones, Ph.D., Department of Physiology and Pharmacology, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC, 27157, Phone number: 336-716-8533, srjones@wakehealth.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

chronic ethanol; kappa opioid receptors; dopamine; microdialysis; voltammetry

1. INTRODUCTION

Drug addiction is a chronically relapsing disorder that progresses from positive reinforcement, drug induced elevation in mood, to negative reinforcement, wherein drugs are used to alleviate negative emotional states (Koob et al., 2014; Wee and Koob, 2010). Escalation in consumption and repeated cycles of drug intake and withdrawal result in neuroadaptive changes leading to an emergence of negative affect such as anxiety, anhedonia, and depression (Weiss et al., 2001). Both cocaine (Buffalari et al., 2012) and ethanol (Rose et al., 2015) withdrawal has been shown to increase anxiety-like behavior in rodents. Withdrawal-induced increased anxiety in turn results in enhancement of drug seeking behavior to reduce negative affect (Koob, 2013). Repeated exposure to ethanol develops tolerance to many of ethanol's effects, such as sedation (Broadwater et al., 2011), locomotion (Zapata et al., 2006), and social activity (Varlinskaya and Spear, 2007). Thus it is possible that animals increase drug consumption in order to achieve the pre-tolerant positive reinforcing effects and to alleviate negative affective symptoms, leading to greater withdrawal-induced anxiety, and so on. This vicious cycle leads to excessive and compulsive abuse of drugs such as alcohol, leading ultimately to addiction.

Ethanol has multiple pharmacological targets of action in the brain, including the dopamine and dynorphin/kappa opioid receptor (KOR) systems. Acute administration of ethanol results in a transient elevation in extracellular accumbal dopamine (Imperato and Di Chiara 1986; Yim et al., 1998) likely driven primarily by an increased firing rate of dopaminergic neurons in the ventral tegmental area (VTA; Brodie et al, 1999). Conversely, chronic ethanol administration results in a functional decrease in dopamine system function with attenuated firing rates (Bailey et al, 2001; Shen 2003), reduced electrically stimulated dopamine release, increased uptake rates (Karkhanis et al., 2015; Rose et al., 2015), and attenuated ethanol-evoked dopamine responses (Zapata and Shippenberg, 2006; Zapata et al., 2006). With respect to the dynorphin/KOR system, at high doses acute ethanol exposure increases extracellular levels of dynorphin in the NAc (Marinelli et al., 2006). Recent studies have shown that chronic ethanol exposure enhances KOR responsiveness to agonist (Rose et al., 2015), augments KOR coupling with G-protein (Kissler et al., 2014) and alters dynorphin expression and levels, although the direction and magnitude of change is variable (Lindholm et al., 2001; Kissler et al., 2014; Rose et al., 2015).

Within the NAc, KORs are expressed presynaptically on dopamine terminals and suppress dopamine release when activated (Werling et al., 1988; Svingos et al, 2001; Ebner et al., 2010). The KOR system appears to exert tonic control on dopamine levels in the NAc, because infusion of KOR antagonists and genetic deletion of KORs results in increased basal dopamine levels in the NAc (Spanagel et al, 1992; Chefer et al, 2005). Conversely, acute administration of KOR agonists reduces basal dopamine levels (Spanagel et al, 1990) and opposes the increase in dopamine following administration of drugs of abuse including

heroin (Xi et al, 1998), cocaine (Maisonneuve et al, 1994), amphetamine (Gray et al, 1999) and ethanol (Lindholm et al, 2007). It is likely that interactions between dopamine and dynorphin/KOR systems contribute to neurochemical and behavioral changes following chronic intermittent ethanol (CIE) exposure. Thus, determining the impact of KOR system activity on dopamine signaling after CIE offers a potential target for therapeutic treatments of alcohol abuse and dependence.

The goal of these studies was to test the hypothesis that CIE produces an overall attenuation of dopamine responses, and that these changes are mediated, at least in part, by KOR activation. We exposed adult mice to CIE vapor and measured dopamine activity with *in vivo* microdialysis and *ex vivo* voltammetry. We further examined the responsiveness of KORs to agonist using voltammetry to confirm the hypothesis that KOR function was enhanced following moderate (3 cycle) CIE exposure. Additionally, we examined the effect of acute ethanol challenge on dopamine responses in the NAc of air and CIE exposed mice using microdialysis. While there was no difference in tonic baseline levels of dopamine between air and CIE exposed mice, KORs showed increased activity in CIE compared to air exposed mice, and acute ethanol unexpectedly decreased extracellular dopamine levels, an effect that was reversed by a KOR antagonist. These data suggest that chronic ethanol exposure-induced tolerance of dopamine responses to ethanol is potentially driven via elevated KOR signaling. The reversal of ethanol-induced dopamine response following KOR blockade confirm increased dynorphin/KOR system activity further suggests that drugs targeting this system may prove to be promising pharmacotherapies to treat alcoholism.

2. METHODS

2.1 Animals

Male C57BL/6 mice (8 -12 weeks; Jackson Laboratories, Bar Harbor, ME) were used for all experiments. Animals were housed individually with food and water *ad libitum* (12-hr light-dark cycle). Experimental protocols adhered to National Institutes of Health Animal Care Guidelines and were approved by the Wake Forest School of Medicine Institutional Animal Care and Use Committee.

2.2 CIE and Withdrawal

The design of the repeated ethanol exposure and withdrawal paradigm was adapted from Becker and colleagues (Becker, 1994; Becker et al, 1997) with minor modifications. Mice were assigned to either control/air or ethanol exposure groups. The ethanol group underwent 16 hours of continuous ethanol vapor exposure followed by 8 hours off in room air each day for four days, followed by three days of abstinence (1 cycle of CIE; Fig. 1A), this was repeated three times for a total of 3 cycles of CIE. A loading dose of 1 g/kg ethanol (20 % w/v) and the ethanol dehydrogenase inhibitor, pyrazole (85 mg/kg) in 0.9% saline was administered i.p. to the mice each day prior to entering the ethanol vapor inhalation chamber. Following the injections, mice were placed inside the ethanol vapor chamber (within their home cages). Ethanol was delivered to the chamber by volatilizing 190 proof ethanol. The ethanol concentration was maintained by mixing the ethanol vapor with fresh air at a rate of 10 L/min. The control group was treated identically to the ethanol group, with

administration of i.p. pyrazole only before they were placed in chambers flowing fresh air. Blood samples were collected and analyzed as described before (Karkhanis et al., 2015). The average blood ethanol concentration for the mice exposed to three cycles of the ethanol vapor was 180 ± 15 mg/dL (mean $\pm$ SEM).

2.3 Brain slice preparation

Immediately upon completion of the vapor exposure, mice were sacrificed by decapitation and brains were rapidly removed and transferred into ice-cold, pre-oxygenated (95% O₂/5% CO₂) artificial cerebral spinal fluid (aCSF) consisting of (in mM): NaCl (126), KCl (2.5), NaH₂PO₄ (1.2), CaCl₂ (2.4), MgCl₂ (1.2), NaHCO₃ (25), glucose (11), L-ascorbic acid (0.4) and pH was adjusted to 7.4. The brain was sectioned into 400 μ m-thick coronal slices containing the striatum with a vibrating tissue slicer (Leica VT1000S, Vashaw Scientific, Norcross, GA) and transferred to a submersion recording chamber perfused at 1 ml/min at 32 °C with oxygenated aCSF.

2.4 Fast Scan Cyclic Voltammetry

Following an equilibration period (30-min), a carbon fiber microelectrode (approximately 150 μ m length, 7 μ m radius) and a bipolar stimulating electrode were placed in close proximity to each other (approximately 100 μ m apart) into the NAc core (Fig. 1B). DA was evoked by a single, rectangular, electrical pulse (300 μ A, 2 ms) applied every 5 min. Extracellular DA was recorded every 100 ms using fast-scan cyclic voltammetry (Calipari et al, 2012) by applying a triangular waveform (-0.4 to $+1.2$ to -0.4 V vs Ag/AgCl, 400 V/s). One slice was used per animal (air, $n = 5$; CIE, $n = 5$). After achieving a stable dopamine response, cumulative concentration-response curve was obtained for U69,593 (30, 100, and 300 nM), a KOR agonist, with each dose added after signal stability was reached (approximately 45 min). After dopamine response was stabilized for the final concentration of U69,593, norbinaltorphimine (norBNI; 10 μ M), a KOR antagonist, was bath applied to the slices to verify that the effect of U69,593 on dopamine release was in fact due to KOR activation. Immediately following the completion of each experiment, recording electrodes were calibrated by recording their response (in current; nA) to 3 μ M dopamine in aCSF using a flow-injection system.

To determine kinetic parameters, evoked levels of dopamine were modeled using Michaelis–Menten kinetics, as a balance between release and uptake (Wightman et al., 1988). Michaelis–Menten modeling of baseline dopamine signals provides parameters that describe the amount of dopamine released following stimulation (i.e., the peak height of the signal) and the maximal rate of dopamine uptake ($V_{\max}$). For baseline modeling, we followed standard voltammetric modeling procedures by setting the apparent K_m value to 160 nM for each animal based on well-established research on the affinity of dopamine for the dopamine transporter (Wu et al., 2001), whereas baseline $V_{\max}$ values were allowed to vary as the maximal rate of dopamine uptake. All voltammetry data were collected and modeled using Demon Voltammetry and Analysis Software (Yorgason et al., 2011).

2.5 Microdialysis

For microdialysis experiments, surgeries were conducted 2 days prior to starting the last air/ethanol vapor exposure regimen (cycle 3). Mice were anesthetized with a combination of ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip). Guide cannulae (P000138; CMA Microdialysis, Harvard Apparatus, Holliston, MA) for probes were directed at the NAc (Fig. 1C) using the following coordinates from bregma: anterior +1.7 mm; lateral -0.8 mm; and ventral -3.0 mm (Paxinos and Watson, 2008). Animals were administered with saline or norBNI (10 mg/kg; s.c.), the KOR antagonist, immediately before being placed in the vapor chamber before the last exposure. Following the final 16 hour vapor exposure mice were immediately transported to the microdialysis procedure room. Mice were placed in a microdialysis test chamber (CMA/Microdialysis, Chelmsford, MA) housed within a sound-attenuating exterior chamber equipped with a light and fan immediately after cessation of the last vapor exposure. Microdialysis probes (P000082; CMA Microdialysis, Harvard Apparatus, Holliston, MA) were inserted into the cannula. A 10 mL syringe containing artificial cerebrospinal fluid (aCSF; in mM: 148 NaCl, 2.7 KCl, 1.2 CaCl₂ and 0.85 MgCl₂; pH= 7.4 with NaH₂PO₄) was inserted in an infusion pump set initially to flow at a rate of 1.0 µL/min with at least a two hour period of equilibration prior to the start of the experiment. Although this seems like a short time for the microdialysis probes and dopamine levels to equilibrate, other studies in the past have sampled dopamine levels shortly (approximately two hours) after implantation of microdialysis probes (Jamal et al., 2016; Podurgeil et al., 2016; Tammimaki et al., 2016). For all microdialysis experiments, samples (20 µL) were collected at 20 min intervals. Each sample was analyzed immediately by high performance liquid chromatography with electrochemical detection (ESA, Chelmsford, MA). All samples were analyzed using a mobile phase described previously (Karkhanis et al., 2014). Experiments commenced once a stable baseline was apparent (i.e., 3 consecutive samples with consistent, stable peaks). Following stable baselines, all mice were injected with ethanol (2 g/kg) and samples were collected for an additional 80 mins. Immediately after the completion of the experiment, mice were sacrificed by inhalation of isoflurane and cervical dislocation; brains were then removed for probe placement confirmation. There were a total of four groups for the microdialysis experiment: air-saline, air-norBNI, CIE-saline, and CIE-norBNI; *n* = 5 in each group.

Separate groups of mice exposed to air or CIE were used to examine the effects of U69,593 *in vivo* on dopamine levels in the NAc. All surgical and ethanol vapor exposure procedures were as described above. Immediately after the cessation of the last vapor exposure (last 16 hour exposure of the 3rd cycle), animals were transported to the microdialysis procedure room and connected to the apparatus. Following the two-hour equilibration period and after stable baselines were obtained, saline was administered intraperitoneally. As described before, samples were collected at 20 min intervals. After three sample collections (one hour), mice were administered with U69,593 (0.56 mg/kg; i.p.) and samples were collected for an additional 80 mins. There were a total of two groups for this experiment: air and CIE; *n* = 6 in each group.

2.6 Chemicals and Drugs

Components of the mobile phase, artificial cerebrospinal fluid and neurotransmitter standards were of HPLC grade or the highest quality obtainable from Sigma-Aldrich (St. Louis, MO). The KOR agonist, U69,593 was obtained from Sigma-Aldrich (St. Louis, MO). The KOR antagonist, norBNI, was generously provided by the National Institutes of Drug Abuse.

2.7 Statistics

Graph Pad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used to statistically analyze data sets and create graphs. Data collected in microdialysis and voltammetry studies, were analyzed using repeated measures (RM) two-way analysis of variance (ANOVA). For the microdialysis experiments, treatment (Air vs. CIE or saline vs. norBNI) and time were the two independent variables, with the measure of extracellular levels of dopamine being the dependent variable. For the U69,593 voltammetry experiments, the two independent variables were, treatment (Air vs. CIE) and concentration U69,593 and dopamine release was the dependent variable. If significant interactions or main effects were obtained, group differences were tested using Bonferroni's *post hoc* analysis.

3. RESULTS

3.1 KOR response to agonist is augmented following CIE exposure

Previously, we have shown that 5-cycles of ethanol vapor exposure increased KOR function (Rose et al., 2015). Here, we used *ex vivo* voltammetry to show that 3-cycle vapor exposure downregulated the dopamine system and augmented KORs functionality. Additionally, in the current study we confirmed that the KOR agonist-induced reduction in dopamine release is indeed via activation of KORs by reversing this effect with a KOR-specific antagonist (norBNI). Electrically stimulated dopamine release at baseline was observed to be lower in CIE exposed compared to air exposed mice (Fig. 2A; $t_8=5.33$, $p<0.001$). Furthermore, dopamine reuptake rates were greater in CIE exposed mice (Fig. 2B; $t_8=2.75$, $p<0.05$). Activation of KORs using U69,593 revealed an increased responsiveness of KORs following CIE exposure (Fig 2C). There was a significant interaction between concentration of U69,593 and exposure treatment ($F_{(3,24)}=10.27$, $p<0.001$). Additionally, main effects of concentration ($F_{(3,24)}=43.0$, $p<0.001$) and exposure treatment ($F_{(1,24)}=10.38$, $p<0.05$) were observed. *Post-hoc* analysis revealed a significant difference between the exposure groups at the 100 and 300 nM concentrations of U69,593. Following the U69,593 experiments, bath application of norBNI returned electrically stimulated dopamine release back to baseline levels in both air (Fig. 2D; air-baseline vs. airnorBNI; $t_4=1.10$, $p>0.05$) and CIE (Fig. 2D; CIE-baseline vs. CIE-norBNI; $t_4=2.34$, $p>0.05$) exposed animals.

In order to examine the responsivity of KORs to the agonist *in vivo*, microdialysis experiments were conducted in mice immediately after the cessation of the last vapor exposure. Baseline levels of dopamine were not significantly different between the two groups (Fig 2E). We administered saline to examine the effect of an i.p. injection on dopamine in air- and CIE-exposed mice. Saline administration did not have any effects on dopamine levels in either group (Fig. 2F). Next we examined the effects of U69,593 on

dopamine levels and observed a significant decline in dopamine levels, approximately 45% from baseline in CIE-exposed mice and a 5% decline in air exposed mice. There was a interaction between exposure group and time following the U69,593 administration (Fig. 2F; $F_{(9,90)}=5.36$, $p<0.0001$). Additionally, we found a main effect of exposure (Fig. 2F; $F_{(1,90)}=20.19$, $p<0.01$) and time (Fig. 2F; $F_{(9,90)}=10.17$, $p<0.0001$). *Post hoc* analysis revealed significant differences at the 80, 100, 120, and 140 mins post-U69,593 challenge time points ($p<0.01$).

3.2 Tonic dopamine response to acute ethanol challenge is reversed in CIE exposed mice

In vivo microdialysis experiments did not show any group differences in extracellular levels of dopamine between air and CIE exposed mice at baseline (Fig. 3A; air exposed, solid blue bar; CIE exposed, solid red bar). Following an acute ethanol challenge, air exposed mice exhibited the expected increase in extracellular dopamine levels (Fig. 3B; solid blue circles; $F_{(4,20)}=4.96$, $p<0.01$). *Post-hoc* analysis showed that a significant increase in dopamine occurred at the 20 min post-challenge time point. Interestingly, CIE exposure reversed the response of dopamine to acute ethanol challenge (Fig. 3B; solid red circles; $F_{(4,20)}=7.42$, $p<0.001$). A significant reduction in extracellular dopamine levels was observed at the 20, 40, 60, and 80 min post-challenge time points. A two-way ANOVA comparing the air and CIE exposed mice showed a significant difference between the two groups. There was an interaction between the exposure group and time (Fig. 3B; $F_{(6,48)}=14.9$, $p<0.001$). Furthermore, there was a main effect of the exposure ($F_{(1,48)}=42.2$, $p<0.005$) and time ($F_{(6,48)}=3.23$, $p<0.01$). *Post hoc* group comparisons revealed significant differences at the 20, 40, 60, and 80 min post-challenge time points ($t = 3.917$, $p<0.01$). This novel finding, that acute ethanol (2 g/kg, ip) increased dopamine efflux in air exposed mice while decreasing dopamine in CIE exposed mice, indicates that a dependence-inducing vapor exposure regimen markedly alters DA regulation mechanisms.

3.3 Effect of KOR blockade on accumbal DA response to acute ethanol challenge

Pretreatment with norBNI did not change baseline dopamine levels in the NAc measured by *in vivo* microdialysis in air exposed mice (Fig. 3A; air-saline, solid blue bar; air-norBNI, shaded blue bar). Acute ethanol challenge resulted in a significant and similar elevation of dopamine levels in air exposed mice pretreated with saline and norBNI (Fig. 3C). Therefore, both groups showed a significant increase in ethanol-induced dopamine levels (Fig. 3C; air-saline, solid blue circles, $F_{(4,20)}=4.96$, $p<0.01$; air-norBNI, shaded blue circles, $F_{(4,20)}=5.80$, $p<0.01$). For both groups, a significant elevation of dopamine occurred at the 20 min post-ethanol time point. There were no significant differences between air exposed mice pretreated with saline and those pretreated with norBNI.

CIE-exposed animals pretreated with norBNI did not exhibit different baseline extracellular dopamine levels compared to CIE exposed mice pretreated with saline (Fig. 3A; CIE-saline, solid red bar; CIE-norBNI, shaded red bar). As reported above, acute ethanol administration resulted in a reduction in dopamine levels in CIE-exposed mice (Fig. 3D; solid red circles; $F_{(4,20)}=7.42$, $p<0.001$). NorBNI pretreatment reversed this ethanol-induced dopamine response in CIE exposed mice such that acute ethanol induced a significant augmentation in extracellular levels of dopamine (Fig. 3D; shaded red circles; $F_{(4,20)}=6.97$, $p<0.01$) at the 20

and 40 min post-ethanol challenge time points. An interaction between time and norBNI-pretreatment parameters ($F_{(6,48)}=17.6$, $p<0.001$), a main effect of norBNI-pretreatment ($F_{(1,48)}=96.2$, $p<0.01$), and a main effect of time ($F_{(6,48)}=3.06$, $p<0.05$) was observed. *Post-hoc* tests revealed a significant difference between dopamine response to ethanol challenge in the presence and absence of nor-BNI in CIE exposed mice at 20, 40, 60, and 80 min post-ethanol administration time points.

4. DISCUSSION

The results of this study showed an interaction between KOR system activation and dopamine responses following CIE exposure. Chronic ethanol exposure augments KORs' responsiveness to activation with the agonist, U69,593. While baseline extracellular dopamine levels were not different between air and CIE exposed mice, an acute ethanol (2 g/kg) challenge increased dopamine levels in air exposed mice but decreased dopamine levels in CIE exposed mice. In addition, blockade of KORs restored normal dopamine increases in response to acute ethanol administration in CIE exposed animals, with no change in air exposed mice. These data suggest that the overactive KORs contribute to decreased dopamine responses (from baseline) to salient stimuli, such as acute exposure to ethanol, which could potentially lead to anxiety further leading to increased drug consumption.

4.1 CIE-induced exacerbation of KOR responsiveness

Data from the current study showing exacerbated KOR responsiveness to U69,593 following moderate CIE exposure reinforce previous data from our group utilizing an extended CIE exposure paradigm (5-cycles) and others utilizing rats (Kissler et al., 2014; Rose et al., 2015). In the current study we further demonstrated that the U69,593-induced reduction in dopamine release was in fact mediated by KORs, as norBNI application reversed the agonist-induced decreases in dopamine signals. Furthermore, we showed that U69,593 administration reduced dopamine levels below baseline by about 45% in CIE-exposed animals compared to a 5% reduction in air-exposed animals, further demonstrating that KORs have increased responsivity to agonist following CIE-exposure. The upregulation in KOR response and the ensuing reductions in mesolimbic dopamine may contribute to negative affective state that plays a role in vulnerability to relapse. These alterations in the KOR system following CIE exposure, however, are not limited to receptor hyperactivity alone. Acute and protracted ethanol withdrawal results in increased prodynorphin mRNA levels as well as increased prodynorphin-derived peptide release in the NAc (Lindholm et al., 2000; Przewlocka et al., 1997). These neurochemical changes correspond to behavioral changes following chronic ethanol including excessive ethanol self-administration, increased anxiety-like behavior, and increased depressive-like behaviors (Nealey et al., 2011; Rose et al., 2015). These behaviors can be reversed via KOR blockade selectively in rodents with chronic exposure to ethanol, while having no effect on controls (Nealey et al., 2011; Rose et al., 2015).

4.2 Augmented KOR function-associated reduction in dopamine system function

While no significant reductions in baseline levels of dopamine have been found in mice following CIE exposure, a functional hypodopaminergic response has been well characterized (Karkhanis et al., 2015; Rose et al., 2015). An acute ethanol challenge has been repeatedly shown to increase dopamine levels in the NAc in ethanol naïve rodents (Imperato and Di Chiara, 1986; Yim et al., 1998; Karkhanis et al., 2014). Likewise, current observations showed that acute ethanol challenge increased extracellular dopamine levels in air exposed mice. However, when ethanol was administered to CIE exposed mice, decreases (from baseline) in dopamine levels were observed in the NAc. Acute ethanol exposure elevates dynorphin levels in the NAc (Marinelli et al., 2006) and thus, it is possible that the complete reversal in dopamine response to acute ethanol challenge observed in the current study is driven by hyperactivity of KORs on presynaptic dopamine terminals. This reduction in ethanol-induced extracellular dopamine levels is “rescued” by KOR blockade as, in CIE exposed mice pretreated with norBNI, an acute ethanol challenge increased dopamine levels. NorBNI pretreatment in air exposed mice did not alter the ethanol induced dopamine response. In a previous study, pretreatment with norBNI resulted in an augmentation of ethanol-induced increase dopamine levels compared to air animals not pretreated with norBNI (Zapata and Shippenberg, 2006); however, this difference is potentially due to the dose and treatment time of norBNI. This previous study used a dose of 20 mg/kg, s.c. (vs. 10 mg/kg, s.c., used in the current study) and administered norBNI six days before acute ethanol challenge (vs. 20 hours before ethanol challenge in the current study). Wee and Koob (2010) suggest that KORs are not actively involved in mediating reinforcing aspects of drug administration without the condition of dependence, but alter several parameters in dependent (or chronically exposed) animals. For example, in ethanol dependent rats (chronic drinking > 16 months), blockade of KORs with norBNI decreased withdrawal-induced ethanol consumption, an effect not seen in nondependent animals (Walker and Koob, 2008). In a free access ethanol drinking paradigm, the acute activation of KORs regulates palatability (Beczowska et al., 1993; Woolley et al., 2007), attenuates ethanol consumption (Lindholm et al., 2001), decreases stress-induced ethanol place preference (Matsuzawa et al., 1998), and antagonizes the rewarding effects of ethanol, although this is thought to be through direct aversive effects of KOR activation, as opposed to modulation of ethanol reward (Hölter et al., 2000).

4.3 Effects of KOR blockade on reducing negative affective state

A low-dopamine state of an animal is often correlated with negative affective signs such as anxiety and anhedonia (Radke and Gewirtz, 2012). The intensity of these symptoms increases with repeated drug exposure, eventually leading to dependence and a shift in hedonic set point to a more negative state (Koob and Le Moal, 1997; Koob, 2013). The dynorphin/KOR system is involved in mediating negative affective states (Bruchas et al., 2010; Berger et al., 2013). For example, elevations in intracranial self-stimulation thresholds, which model anhedonia, are found after prolonged activation of KORs (Chartoff et al., 2015). The current data and previous studies have shown that excessive exposure to drugs of abuse results in an upregulated dynorphin/KOR system (Lindholm et al., 2000; Kissler et al., 2014; Rose et al., 2015). Moreover, it has been postulated that a shift in hedonic set point is driven, in part, by hyperactivity of the dynorphin/KOR system in

dependent animals (Bruchas et al., 2010). Thus it is possible that KOR blockade, which decreases ethanol intake selectively in dependent animals (Walker and Koob, 2008; Walker et al., 2011; Rose et al., 2015) and in animals exposed to chronic early-life stress (Karkhanis et al., 2016), relieves the negative affective state that the animals is in, potentially by normalizing dopamine system function.

4.4 Conclusions

Data in the current study demonstrate that excessive exposure to ethanol results in a functionally upregulated KOR system and a downregulated dopamine system. The upregulation hyperactivity of the dynorphin/KOR receptor system may in fact be a primary driver of the downregulation of the dopamine system, because current data showed that KOR blockade reversed the ethanol-induced dopamine response in dependent animals back to normal. These data, in conjunction with other studies showing the effectiveness of KOR blockade in decreasing ethanol intake in dependent animals suggest that the dynorphin/KOR system is a promising therapeutic target to treat alcoholism.

ACKNOWLEDGEMENTS

ANK, KNH, and SRJ were responsible for the study concept and design. JHR conducted the air/ethanol vapor exposure protocol. ANK and KNH conducted the microdialysis data and KNH conducted the voltammetry experiments. ANK analyzed these data and drafted the manuscript. JHR and SRJ provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication. Authors thank Jason Locke and Joanne Konstantopoulos for their technical support. This research was funded by the following grants awarded by the National Institutes of Health: T32 AA007565-21 (ANK); F31 DA03558 (JHR); P01 AA021099 (SRJ); U01 AA014091 (SRJ).

REFERENCES CITED

- Bailey CP, O'Callaghan MJ, Croft AP, Manley SJ, Little HJ. Alteration in mesolimbic dopamine function during the abstinence period following chronic ethanol consumption. *Neuropharmacology*. 2001; 41:989–999. [PubMed: 11747903]
- Becker HC. Positive relationship between the number of prior ethanol withdrawal episodes and the severity of subsequent withdrawal seizures. *Psychopharmacology*. 1994; 116:26–32. [PubMed: 7862927]
- Becker HC, Diaz-Granados JL, Weathersby RT. Repeated ethanol withdrawal experience increases the severity and duration of subsequent withdrawal seizures in mice. *Alcohol*. 1997; 14:319–326. [PubMed: 9209546]
- Beczowska IW, Koch JE, Bostock ME, Leibowitz SF, Bodnar RJ. Central opioid receptor subtype antagonists differentially reduce intake of saccharin and maltose dextrin solutions in rats. *Brain Res*. 1993; 618:261–270. [PubMed: 8397050]
- Berger AL, Williams AM, McGinnis MM, Walker BM. Affective cue-induced escalation of alcohol self-administration and increased 22-kHz ultrasonic vocalizations during alcohol withdrawal: role of kappa-opioid receptors. *Neuropsychopharmacology*. 2013; 38:647–654. [PubMed: 23212453]
- Broadwater M, Varlinskaya EI, Spear LP. Chronic intermittent ethanol exposure in early adolescent and adult male rats: effects on tolerance, social behavior, and ethanol intake. *Alcohol Clin Exp Res*. 2011; 35:1392–1403. [PubMed: 21352250]
- Brodie MS, Pesold C, Appel SB. Ethanol directly excites dopaminergic ventral tegmental area reward neurons. *Alcohol Clin Exp Res*. 1999; 23:1848–52. [PubMed: 10591603]
- Bruchas MR, Land BB, Chavkin C. The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Res*. 2010; 1314:44–55. [PubMed: 19716811]

- Buffalari DM, Baldwin CK, See RE. Treatment of cocaine withdrawal anxiety with guanfacine: relationships to cocaine intake and reinstatement of cocaine seeking in rats. *Psychopharmacology*. 2012; 223:179–190. [PubMed: 22526535]
- Calipari ES, Ferris MJ, Melchior JR, Bermejo K, Salahpour A, Roberts DC, Jones SR. Methylphenidate and cocaine self-administration produce distinct dopamine terminal alterations. *Addict Biol*. 2012; 19:145–155. [PubMed: 22458761]
- Chartoff EH, Ebner SR, Sparrow A, Potter D, Baker PM, Ragozzino ME, Roitman MF. Relative Timing Between Kappa Opioid Receptor Activation and Cocaine Determines the Impact on Reward and Dopamine Release. *Neuropsychopharmacology*. 2015 [Epub ahead of print].
- Chefer VI, Czyzyk T, Bolan EA, Moron J, Pintar JE, Shippenberg TS. Endogenous kappa-opioid receptor systems regulate mesoaccumbal dopamine dynamics and vulnerability to cocaine. *J Neurosci*. 2005; 25:5029–37. [PubMed: 15901784]
- Ebner SR, Roitman MF, Potter DN, Rachlin AB, Chartoff EH. Depressive-like effects of the kappa opioid receptor agonist salvinorin A are associated with decreased phasic dopamine release in the nucleus accumbens. *Psychopharmacology*. 2010; 210:241–252. [PubMed: 20372879]
- Gray AM, Rawls SM, Shippenberg TS, McGinty JF. The kappa-opioid agonist, U-69593, decreases acute amphetamine-evoked behaviors and calcium-dependent dialysate levels of dopamine and glutamate in the ventral striatum. *J Neurochem*. 1999; 73:1066–1074. [PubMed: 10461896]
- Hölter SM, Henniger MS, Lipkowski AW, Spanagel R. Kappa-opioid receptors and relapse-like drinking in long-term ethanol-experienced rats. *Psychopharmacology*. 2000; 153:93–102. [PubMed: 11255932]
- Imperato A, Di Chiara G. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *J Pharmacol Exp Ther*. 1986; 239:219–28. [PubMed: 3761194]
- Jamal M, Ameno K, Miki T, Tanaka N, Ito A, Ono J, Takakura A, Kumihashi M, Kinoshita H. Ethanol and acetaldehyde differentially alter extracellular dopamine and serotonin in Aldh2-knockout mouse dorsal striatum: A reverse microdialysis study. *Neurotoxicology*. 2016; 52:204–209. [PubMed: 26711020]
- Karkhanis AN, Locke JL, McCool BA, Weiner JL, Jones SR. Social isolation rearing increases nucleus accumbens dopamine and norepinephrine responses to acute ethanol in adulthood. *Alcohol Clin Exp Res*. 2014; 38:2770–2779. [PubMed: 25421514]
- Karkhanis AN, Rose JH, Huggins KN, Konstantopoulos JK, Jones SR. Chronic intermittent ethanol exposure reduces presynaptic dopamine neurotransmission in the mouse nucleus accumbens. *Drug Alcohol Depend*. 2015; 150:24–30. [PubMed: 25765483]
- Karkhanis AN, Rose JH, Weiner JL, Jones SR. Early-life social isolation stress increases kappa opioid receptor responsiveness and downregulates the dopamine system. *Neuropsychopharmacology*. 2016 [Epub ahead of print].
- Kissler JL, Sirohi S, Reis DJ, Jansen HT, Quock RM, Smith DG, Walker BM. The one two punch of alcoholism: role of central amygdala dynorphins/kappa-opioid receptors. *Biol Psychiatry*. 2014; 75:774–782. [PubMed: 23611261]
- Koob GF. Addiction is a Reward Deficit and Stress Surfeit Disorder. *Front Psychiatry*. 2013; 4:72. [PubMed: 23914176]
- Koob GF, Buck CL, Cohen A, Edwards S, Park PE, Schlosburg JE, Schmeichel B, Vendruscolo LF, Wade CL, Whitfield TW Jr, George O. Addiction as a stress surfeit disorder. *Neuropharmacology*. 2014; 76:370–382. [PubMed: 23747571]
- Koob GF, Le Moal M. Drug abuse: hedonic homeostatic dysregulation. *Science*. 1997; 278:52–58. [PubMed: 9311926]
- Lindholm S, Ploj K, Franck J, Nylander I. Repeated ethanol administration induces short and long-term changes in enkephalin and dynorphin tissue concentrations in rat brain. *Alcohol*. 2000; 22:165–171. [PubMed: 11163124]
- Lindholm S, Werme M, Brené S, Franck J. The selective kappa-opioid receptor agonist U50,488H attenuates voluntary ethanol intake in the rat. *Behav Brain Res*. 2001; 120:137–146. [PubMed: 11182162]

- Lindholm S, Rosin A, Dahlin I, Georgieva J, Franck J. Ethanol alters the effect of kappa receptor ligands on dopamine release in the nucleus accumbens. *Physiol Behav.* 2007; 92:167–171. [PubMed: 17610914]
- Maisonneuve IM, Archer S, Glick SD. U50,488, a kappa opioid receptor agonist, attenuates cocaine-induced increases in extracellular dopamine in the nucleus accumbens of rats. *Neurosci Lett.* 1994; 181:57–60. [PubMed: 7898771]
- Marinelli PW, Lam M, Bai L, Quirion R, Gianoulakis C. A microdialysis profile of dynorphin A(1-8) release in the rat nucleus accumbens following alcohol administration. *Alcohol Clin Exp Res.* 2006; 30:982–990. [PubMed: 16737456]
- Matsuzawa S, Suzuki T, Misawa M, Nagase H. Involvement of mu- and delta-opioid receptors in the ethanol-associated place preference in rats exposed to foot shock stress. *Brain Res.* 1998; 803:169–177. [PubMed: 9729364]
- Nealey KA, Smith AW, Davis SM, Smith DG, Walker BM. κ -opioid receptors are implicated in the increased potency of intra-accumbens nalmefene in ethanol-dependent rats. *Neuropharmacology.* 2011; 61:35–42. [PubMed: 21338616]
- Podurgiel SJ, Yohn SE, Dortche K, Correa M, Salamone JD. The MAO-B inhibitor deprenyl reduces the oral tremor and the dopamine depletion induced by the VMAT-2 inhibitor tetrabenazine. *Behav Brain Res.* 2016; 298:188–191. [PubMed: 26590367]
- Przewłocka B, Turchan J, Laso W, Przewłocki R. Ethanol withdrawal enhances the prodynorphin system activity in the rat nucleus accumbens. *Neurosci Lett.* 1997; 238:13–16. [PubMed: 9464643]
- Radke AK, Gewirtz JC. Increased dopamine receptor activity in the nucleus accumbens shell ameliorates anxiety during drug withdrawal. *Neuropsychopharmacology.* 2012; 37:2405–2415. [PubMed: 22692565]
- Rose JH, Karkhanis AN, Chen R, Gioia D, Lopez MF, Becker HC, McCool BA, Jones SR. Supersensitive Kappa Opioid Receptors Promotes Ethanol Withdrawal-Related Behaviors and Reduce Dopamine Signaling in the Nucleus Accumbens. *Int J Neuropsychopharmacol.* 2015 [Epub ahead of print].
- Shen RY. Ethanol withdrawal reduced the number of spontaneously active ventral tegmental area dopamine neurons in conscious animals. *J Pharmacol Exp Ther.* 2003; 307:566–572.
- Spanagel R, Herz A, Shippenberg TS. The effects of opioid peptides on dopamine release in the nucleus accumbens: an in vivo microdialysis study. *J Neurochem.* 1990; 55:1734–1740. [PubMed: 1976759]
- Spanagel R, Herz A, Shippenberg TS. Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proc Natl Acad Sci U S A.* 1992; 89:2046–50. [PubMed: 1347943]
- Svingos AL, Chavkin C, Colago EE, Pickel VM. Major coexpression of kappa-opioid receptors and the dopamine transporter in nucleus accumbens axonal profiles. *Synapse.* 2001; 42:185–192. [PubMed: 11746715]
- Tammimäki A, Aonurm-Helm A, Kaenmäki M, Mannisto. Elimination of extracellular dopamine in the medial prefrontal cortex of conscious mice analysed using selective enzyme and uptake inhibitors. *J Physiol Pharmacol.* 2016; 67:301–309. [PubMed: 27226189]
- Varlinskaya EI, Spear LP. Chronic tolerance to the social consequences of ethanol in adolescent and adult Sprague-Dawley rats. *Neurotoxicol Teratol.* 2007; 29:23–30. [PubMed: 17055219]
- Walker BM, Koob GF. Pharmacological evidence for a motivational role of kappa-opioid systems in ethanol dependence. *Neuropsychopharmacology.* 2008; 33:643–652. [PubMed: 17473837]
- Walker BM, Zorrilla EP, Koob GF. Systemic κ -opioid receptor antagonism by nor binaltorphimine reduces dependence-induced excessive alcohol self-administration in rats. *Addict Biol.* 2011; 16:116–119. [PubMed: 20579007]
- Wee S, Koob GF. The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacology.* 2010; 210:121–35. [PubMed: 20352414]
- Weiss F, Ciccocioppo R, Parsons LH, Katner S, Liu X, Zorrilla EP, Valdez GR, Ben-Shahar O, Angeletti S, Richter RR. Compulsive drug-seeking behavior and relapse. Neuroadaptation, stress, and conditioning factors. *Ann N Y Acad Sci.* 2001; 937:1–26. [PubMed: 11458532]

- Werling LL, Frattali A, Portoghese PS, Takemori AE, Cox BM. Kappa receptor regulation of dopamine release from striatum and cortex of rats and guinea pigs. *J Pharmacol Exp Ther*. 1988; 246:282–286. [PubMed: 2839666]
- Wightman RM, Amatore C, Engstrom RC, Hale PD, Kristensen EW, Kuhr WG, May LJ. Real-time characterization of dopamine overflow and uptake in the rat striatum. *Neuroscience*. 1988; 25:513–523. [PubMed: 3399057]
- Woolley JD, Lee BS, Kim B, Fields HL. Opposing effects of intra-nucleus accumbens mu and kappa opioid agonists on sensory specific satiety. *Neuroscience*. 2007; 146:1445–1452. [PubMed: 17445988]
- Wu Q, Reith ME, Wightman RM, Kawagoe KT, Garriss PA. Determination of release and uptake parameters from electrically evoked dopamine dynamics measured by real-time voltammetry. *J Neurosci Methods*. 2001; 112:119–133. [PubMed: 11716947]
- Xi ZX, Fuller SA, Stein EA. Dopamine release in the nucleus accumbens during heroin self-administration is modulated by kappa opioid receptors: an in vivo fast-cyclic voltammetry study. *J Pharmacol Exp Ther*. 1998; 284:151–161. [PubMed: 9435173]
- Yim HJ, Schallert T, Randall PK, Gonzales RA. Comparison of local and systemic ethanol effects on extracellular dopamine concentration in rat nucleus accumbens by microdialysis. *Alcohol Clin Exp Res*. 1998; 22:367–374. [PubMed: 9581642]
- Yorgason JT, Espana RA, Jones SR. Demon voltammetry and analysis software: analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *J. Neurosci. Methods*. 2011; 202:158–164. [PubMed: 21392532]
- Zapata A, Shippenberg TS. Endogenous kappa opioid receptor systems modulate the responsiveness of mesoaccumbal dopamine neurons to ethanol. *Alcohol Clin Exp Res*. 2006; 30:592–7. [PubMed: 16573576]
- Zapata A, Gonzales RA, Shippenberg TS. Repeated ethanol intoxication induces behavioral sensitization in the absence of a sensitized accumbens dopamine response in C57BL/6J and DBA/2J mice. *Neuropsychopharmacology*. 2006; 31:396–405. [PubMed: 16034441]

HIGHLIGHTS

- 3 weeks of CIE vapor exposure resulted in functional hyperactivity of KORs in the NAc, a reduction in dopamine release, and a facilitation of dopamine uptake.
- Acute ethanol challenge reversed dopamine response in CIE vapor exposed mice.
- KOR blockade normalized this acute ethanol-induced dopamine response in CIE vapor exposed mice, with no change in control mice.

Figure 1.

(A) Schematic of the experimental paradigm. Mice were exposed to three cycles of air or ethanol vapor exposure. Each cycle consisted of 16 hours of air/ethanol vapor exposure followed of 8 hours of room air for four consecutive days, followed by three days of abstinence. This cycle was repeated three times. Microdialysis cannulation surgeries were conducted after termination of cycle two and two days before the beginning of cycle three. Voltammetry and microdialysis experiments were conducted at the cessation of the last 16 hour exposure. (B) Coronal sections showing locations of voltammetric measurements. Blue and red dots represent recording sites in air- and CIE-exposed mice, respectively. (C) Coronal sections showing microdialysis probe locations. Blue and red lines represent probe tracks in air- and CIE-exposed mice, respectively.

Figure 2.

Voltammetric measurements of baseline stimulated dopamine release and reuptake, and KOR activity. (A) Electrically stimulated baseline dopamine release is significantly lower in CIE (red bar) compared to air (blue bar) exposed mice. (B) The uptake rate of dopamine is faster in CIE (red bar) compared to air (blue bar) exposed animals. (C) Cumulative dose response curve of U69,593, a KOR agonist followed by norBNI, a KOR antagonist mediated reversal. The KOR activation-mediated reduction in electrically stimulated dopamine release was augmented in CIE exposed mice (red circle) in comparison to air exposed mice (blue circle). Bath application of norBNI increased the evoked dopamine release to the baseline levels in both air and CIE exposed mice. (D) In air exposed mice, the comparison between baseline (solid blue bar) and post-norBNI application (shaded blue bar) evoked dopamine release showed no differences. Similarly, in CIE exposed mice evoked dopamine release post-norBNI application (shaded red bar) matched the baseline dopamine release (solid red bar). (E) No differences were observed in baseline dopamine levels in air (blue bar) and CIE (red bar) exposed mice. (F) Saline injection (i.p.) did not increase dopamine levels in air and CIE exposed mice. U69,593 resulted in a significant reduction in dopamine (below baseline) in CIE (red circle) compared to air (blue circles) exposed mice. Voltammetry experiments: Air ($n = 5$); Chronic intermittent ethanol (CIE; $n = 5$). Microdialysis experiments: Air ($n = 6$); CIE ($n = 6$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 3.

Microdialysis measurements of baseline and ethanol-induced extracellular levels of dopamine in the absence and presence of norBNI. (A) Baseline extracellular levels of dopamine were not different between air (solid red bar) and CIE (solid blue bar) exposed mice pretreated with saline. Furthermore, administration of norBNI did not alter the dopamine levels in air (shaded red bar) and CIE (shaded blue bar) exposed mice. (B) Acute ethanol administration resulted in the expected increase in dopamine levels in air exposed mice pretreated with saline (solid blue circles). However, in the CIE exposed mice pretreated with saline (solid red circles), ethanol administration resulted in a reduction in dopamine levels compared to baseline. (C) The effect of acute ethanol administration on extracellular dopamine levels was not different in air exposed mice pretreated with saline (solid blue circles) and norBNI (shaded blue circles). (D) NorBNI pretreatment in CIE exposed mice (shaded red circles) reversed the ethanol-mediated reduction in dopamine levels observed in saline pretreated CIE exposed mice (solid red circles). Air-saline ($n = 5$); Air-norBNI ($n = 5$); CIE-saline ($n = 5$); CIE-norBNI ($n = 5$). ### $p < 0.001$; ## $p < 0.01$; comparison of ethanol-induced elevation/reduction in dopamine levels to baseline dopamine levels within a group. Comparison between air exposed and CIE exposed groups. *** $p < 0.001$; ** $p < 0.01$; comparison between air exposed and CIE exposed groups.