JPET Fast Forward. Published on April 15, 2013 as DOI: 10.1124/jpet.112.202903

JPET Fast Forward. Published on April 15, 2013 as DOI:10.1124/jpet.112.202903

Title Page

Kappa opioid receptors in the central amygdala regulate ethanol actions at presynaptic GABAergic sites

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Running Title Page

a) Running title: Kappa opioid receptors regulate ethanol-GABA interactions

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c) text pages: 28
tables: 0
figures: 4; 1 supplemental figure
references: 59
words in Abstract: 230
words in Introduction: 717
words in Discussion: 1217

JPET Fast Forward. Published on April 15, 2013 as DOI: 10.1124/jpet.112.202903
This article has not been copyedited and formatted. The final version may differ from this version.

d) Abbreviations:

KO	knock-out
KOR	kappa opioid receptor
GABA	gamma aminobutyric acid
WT	wild-type
CeA	central nucleus of the amygdala
eIPSC	evoked inhibitory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
CGP 55845	3-*N*[1-(*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphinic acid
ACSF	artificial cerebrospinal fluid
AP-5	d-2-amino-5-phosphonovalerate
BMI	bicuculline methiodide
DNQX	6,7-dinitroquinoxaline-2,3-dione (DNQX)
NAcc	nucleus accumbens
TTX	tetrodotoxin

e) Recommended section: Neuropharmacology

Abstract

Human and animal studies indicate that kappa opioid receptors (KORs) are involved in ethanol drinking and dependence (Xuei et al., 2006; Walker and Koob, 2008; Walker et al., 2011). Using *in vitro* single-cell recording techniques in mouse brain slices, we examined the physiological effects of KOR activation in the central amygdala (CeA) on GABAergic neurotransmission and its interaction with acute ethanol. A selective KOR agonist (U69593, 1 μM) diminished evoked GABAergic inhibitory postsynaptic currents (IPSCs) by 18% ($n = 10$), whereas blockade of KORs with a selective antagonist (nor-binaltorphimine, 1 μM) augmented the baseline evoked GABAergic IPSCs by 14% ($p < 0.01$; $n = 34$), suggesting that the KOR system contributes to tonic inhibition of GABAergic neurotransmission in the CeA. In addition, the enhancement by acute ethanol of GABAergic IPSC amplitudes was further augmented by pharmacological blockade of KORs, from 14 % ($n = 36$) to 27% ($n = 26$; $p < 0.01$), or by genetic deletion of KORs, from 14 % in WT mice ($n = 19$) to 34 % in KOR knockout (KO) mice ($n = 13$, $p < 0.01$). Subsequent experiments using tetrodotoxin to block activity-dependent neurotransmission suggest that KORs regulate GABA release at presynaptic sites. Our data support the idea that KORs modulate GABAergic synaptic responses and ethanol effects as one of multiple opioid system-dependent actions of ethanol in the CeA, possibly in a circuit-specific manner.

Introduction

The kappa opioid receptor (KOR) and its putative endogenous agonist, the neuropeptide dynorphin, have been identified as playing a critical role in alcohol abuse and dependence. Recent studies of humans of European American descent demonstrate that variations in the genes encoding KOR and dynorphin, *OPRK1* and *PDYN*, are associated with alcohol dependence (Xuei et al., 2006; Edenberg et al., 2008; Karpyak et al., 2012). In addition, animal studies using rats show that activation of KORs with a selective KOR agonist reduces voluntary ethanol intake (Lindholm et al., 2001), while a selective KOR antagonist increases alcohol self-administration (Mitchell et al., 2005). However, chronic treatment with a KOR agonist enhanced ethanol intake during alcohol deprivation in rats that had long-term exposure to ethanol (Hölter et al., 2000). Furthermore, a KOR antagonist selectively reduced ethanol self-administration in rats made dependent on ethanol but not in non-dependent rats (Walker and Koob, 2008; Walker et al., 2011). Mice lacking KORs drink half as much ethanol as either wild-type or heterozygous mice (Kovacs et al., 2005), and mice lacking dynorphin also show reduced voluntary ethanol consumption (Blednov et al., 2006). While the mechanistic role of the dynorphin/KOR system in ethanol dependence is not clear, involvement in ethanol abuse and dependence is in line with the well-established role of the dynorphin/KOR system in stress-induced depression-like behaviors and relapse to drug-seeking behaviors in both rats and mice (Beardsley et al., 2005; Carey et al., 2007; Land et al., 2008; Carey et al., 2009).

The activation of KOR by agonists generally inhibits neurons through coupling of inhibitory G-proteins (Gi/Go) either through enhanced potassium conductance (Madamba et al., 1999) or inhibition of N-type calcium ion channels (Simmons and Chavkin, 1996; Hjelmstad and Fields, 2003). KORs are localized on axon terminals as well as neuronal cell bodies, and may act through two mechanisms: the inhibition of neurotransmission directly at terminal release sites (Svingos et al., 1999; Li et al., 2012) as well as direct hyperpolarization of cell bodies (Margolis et al., 2003). An example of such modulation of neurotransmission, a decrease in dopamine release by the dynorphin/KOR system in the nucleus accumbens (NAcc), was proposed as one mechanism underlying the effect on alcohol consumption (Lindholm et al., 2007). As with other drugs of abuse, ethanol acutely induces dopamine release in NAcc (Weiss et al., 1993; Gonzales et al., 2004), whereas stimulation of KORs reduces the release of dopamine in NAcc (Spanagel et al., 1992). Ethanol-dependent rats show increased dopamine release when intoxicated but reduced basal dopamine tone during withdrawal, which is increased by KOR antagonism (Diana et al., 1993; Lindholm et al., 2007). Consistent with this, KOR knockout mice showed elevated ethanol-evoked dopamine release in the NAcc (Zapata and Shippenberg, 2006).

The central nucleus of the amygdala (CeA) is a site where the dynorphin/KOR system likely contributes to ethanol dependence. KORs and dynorphin, the endogenous KOR agonist, are highly expressed in rat and mouse CeA (DePaoli et al., 1994; Slowe et al., 1999; Marchant et al., 2007), suggesting a functional role of the dynorphin/KOR system in this brain region. The CeA is a brain region critical in mediating anxiety- and stress-related behaviors (Tye et al., 2011) and is also involved in drug addiction, including drug reward and reinforcement and stress-induced reinstatement of drug dependence (Koob et al., 1998). Lesions of the CeA reduce voluntary alcohol consumption as well as anxiety in rats (Moller et al., 1997). $GABA_A$ receptor antagonists injected into the CeA significantly decrease ethanol consumption (Hyytiä and Koob, 1995; Foster et al., 2004). Because the dynorphin/KOR system is a key modulator of anxiety and fear conditioning (Bilkei-Gorzo et al., 2012), its dysregulation in CeA could contribute to ethanol dependence. Therefore, this study aims to examine the functional effects of the activation of KOR system in CeA, specifically its effects on inhibitory synaptic responses as well as interactions with acute ethanol.

The CeA is comprised of heterogeneous cell types with multiple physiological signatures. CeA projection neurons responsible for fear conditioning or anxiety-related behaviors are localized in the medial division, whereas these neurons are inhibited by lateral division neurons. Immunohistochemical studies show that dynorphin is highly localized in the lateral division of the rat CeA (Marchant et al., 2007). Therefore, in this study, we recorded from medial division neurons while local stimulation was applied in the lateral division.

Methods

Generation of Knockout Mice. The methods for generation of the knockout mice are as described previously (Simonin et al., 1998; Kovacs et al., 2005). In brief, gene inactivation was obtained by disruption of the first coding exon of the KOR gene in 129/SV embryonic stem cells. Germline transmission occurred from the breeding of chimeric males with C57BL/6Orl females. After mice were genotyped, those showing germline transmission were used as founder animals to produce the F1 animals used in these experiments. We used homozygous KOR KO and WT littermate mice (male, 120-180 days old) shipped from The Scripps Research Institute, La Jolla, CA to Duke University). The genetic background of these mice was a hybrid C57BL/6Orl X 129/SV strain. We housed mice two to four per cage in a temperature-controlled room in which the lights were on a 12-hr light/dark cycle with lights off at 6:00 PM, and animals were sacrificed for experiments between 9:00 and 11:00 AM. For pharmacological experiments, we also used wild type (WT) male C57BL/6 mice (60-90 days old, Charles River, Raleigh, NC).

In-Vitro Single-Cell Recordings. Following isoflurane anesthesia, we rapidly removed brains from the mice. Brains were immersed in ice-cold oxygenated (95% O_2-5% CO_2) artificial cerebrospinal fluid (ACSF), containing (in mM) 120 NaCl, 3.3 KCl, 1.23 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 0.9 $MgSO_4$, 10 glucose; for ACSF used only during the dissection, 2 $CaCl_2$ was replaced with 0.5 $CaCl_2$. We cut coronal slices (300 µm, between bregma -1.0 - ~1.9 mm, Paxinos and Franklin, 2004) using a Vibratome (Campden, model 752, Lafayette, IN) and incubated slices in ACSF continuously bubbled with 95% O_2-5% CO_2. After 30 minutes incubation at room temperature, we transferred slices singly to the recording chamber (volume 0.5 ml) in which oxygenated ACSF at 35 ºC was superfused over the submerged slice at approximately 3 ml/min. We viewed individual cells with an upright fixed-stage microscope (Zeiss Axioskop, Thornwood, NY) equipped with a water immersion objective (40X, 0.75 numerical aperture, NA), IR filtered light, differential interference contrast (DIC) optics, and a Hitachi CCD camera (Tokyo, Japan).

We made whole-cell patch recordings in the medial ventral division of the central amygdala. Patch pipettes were pulled from borosilicate glass capillary tubing (1.5 mm OD, 1.05 mm ID, World Precision Instruments, Sarasota, FL) using a Flaming-Brown horizontal microelectrode puller (model P-97, Sutter Instrument, Novato, CA). The pipettes (input resistance 2-5 MΩ) were filled with the following solution (in mM): Cs-methylsulfonate 65; CsCl 65; HEPES, 10; NaCl, 4; EGTA 0.2; Mg-ATP, 4; Tris-GTP, 0.3; Na_2creatine PO_4, 10 (pH 7.25; 285 mOsM). In some experiments with spontaneous synaptic responses, 140 mM CsCl was used instead of Cs-methylsulfonate 65 mM and CsCl 65 mM to maximize the driving force for $GABA_A$ receptor-mediated fast inhibitory postsynaptic currents (IPSCs). To study KOR effects on IPSCs, we voltage-clamped cells at -70 mV and isolated IPSCs using the NMDA and non-NMDA glutamate receptor blockers 6,7-dinitroquinoxaline-2,3-dion (DNQX, 20 μM) and D-2-amino-5-phosphonovalerate (APV, 50 μM), respectively, and the $GABA_B$ receptor blocker CGP 55845 (1 μM). To study evoked IPSCs, we placed a monopolar tungsten electrode (A-M system, Carlsborg, WA) in the lateral division of the CeA and delivered square wave current pulses (0.1 millisecond duration) every 20 seconds. For baseline responses we used IPSCs determined to be approximately 30-50% of maximal amplitude. Drugs (ethanol, U69593, norbinaltorphimine (nor-BNI)) were applied after establishing stable baseline responses. In some experiments we also examined “miniature” spontaneous IPSCs (mIPSCs) using tetrodotoxin (TTX; 1 µM) to block activity-dependent neurotransmission.

Data Acquisition and Analysis. We acquired and digitized responses at 10 KHz using an Axopatch 200B (Molecular Devices, Foster City, CA), filtered at 2 kHz (-3dB), and Clampex software (Molecular Devices, Foster City, CA). We made all recordings at 35 °C. Liquid junction potentials were not measured nor compensated. We monitored series resistance (10-30 MΩ) on-line throughout the experiment using pClamp (Molecular Devices, www.moleculardevices.com) and rejected cells if this resistance changed by > 20%. We did not use series resistance compensation.

For analysis of evoked IPSCs, we measured peak amplitudes off-line using Clampfit (Molecular Devices) on an IBM compatible computer, and compared the drug effects between groups using two-sample *t* test or two-way ANOVA when appropriate. For analysis of mIPSCs, we analyzed the frequencies and amplitudes using Mini Analysis (www.synaptosoft.com), and tested drug effects using the Komologrov-Smirnov (K-S) statistical method. We considered $p < 0.05$ as indicating statistical significance. We used Origin software (Origin Lab, Northampton, MA) for plotting figures and statistical analysis. Results in the text and figures are presented as the mean ± s.e.m.

Results

KOR activation reduces GABAergic synaptic transmission in CeA neurons.

Recent studies have reported that KOR agonists hyperpolarize a subpopulation of rat CeA neurons (Zhu and Pan, 2004; Chieng et al., 2006). However, effects of KOR activation on GABAergic synaptic transmission in the CeA have not been explored. To determine whether KOR activation modulates GABAergic synaptic transmission, we examined the effect of a selective KOR agonist, U69593, on IPSCs evoked by local stimulation within the CeA. Across all CeA neurons tested, U69593 (1 μM) significantly reduced the amplitude of evoked IPSCs by 18.1 ± 4.1%, from 674.0 ± 128.6 pA to 560.0 ± 115.6 pA (n = 10, Fig. 1Aa and Ac). However, 4 neurons showed no significant effect of the agonist. In responsive neurons, actions of U69593 were blocked by the specific KOR antagonist nor-BNI (Supplemental Figure 1). U69593 also significantly ($p < 0.01$) increased the paired-pulse ratio (PPR) of IPSCs at 100 msec inter-stimulus intervals from 0.94 to 1.1 (n = 10, Fig 1Ab and Ad). This suggests that the effect of U69593 is at least in part due to decreased GABA release, because changes in PPF are inversely related to transmitter release (Andreasen and Hablitz, 1994). We further examined the locus of KOR action on the IPSCs by measuring the effect of U69593 on mIPSCs after blocking action potentials with TTX (1 μM). Application of U69593 (1 μM) significantly ($p < 0.01$) decreased the frequency of mIPSCs in CeA neurons from 7.9 ± 1.5 Hz to 6.4 ± 1.3 Hz (n = 16, Fig. 1B) and significantly (k-s z = 1.72, $p < 0.01$) shifted the cumulative frequency distribution to longer inter-event intervals (Fig 1Bb), suggesting that U69593 reduces the vesicular release of GABA. U69593 did not significantly alter the amplitude of mIPSCs (means: control, 36.5 ± 4.9 pA; U69593, 35.9 ± 5.0, n = 16; Fig 1Bc).

To determine if there is tonic activation of KORs in CeA, we examined the effect of nor-BNI, a selective KOR antagonist, on evoked IPSCs (Fig 2A) in the CeA of WT mice. Across all neurons tested, nor-BNI (1 µM) significantly ($p < 0.05$) augmented the mean baseline IPSC amplitude by 14.1 ± 3.3 %, from 577.2 ± 60.3 pA to 651.0 ± 63.9 pA ($p < 0.01$, n=34; Fig 2). However, 17 (50%) of neurons tested showed no significant effect of the antagonist. These results suggest a constitutive activation of KORs or a tonic release of endogenous dynorphin in WT mice that affects at least a subgroup of CeA neurons.

Pharmacological block of KORs enhances ethanol-induced increases in GABAergic transmission in CeA.

We and others have previously shown that acute ethanol enhances GABA release at CeA synapses from rats and mice (Roberto et al., 2003; Nie et al., 2004; Kang-Park et al., 2007; Kang-Park et al., 2009). In addition, our previous physiological studies indicate that acute ethanol induces release of neuropeptides such as endogenous opioids and CRF in CeA (Nie et al., 2004; Lam et al., 2008) and that these peptides may further modulate GABA release (Nie et al., 2004; Kang-Park et al., 2007; Kang-Park et al., 2009; Roberto et al., 2010). We hypothesize that acute ethanol releases dynorphin in CeA that acts at presynaptic KORs by decreasing GABA release and partially ameliorating the overall effect of ethanol on such release. To test this idea, we first pretreated CeA slices with nor-BNI (1 µM) for 10 min and then added 40 mM ethanol (Fig 2B). Nor-BNI pretreatment significantly ($p < 0.05$) enhanced the effect of ethanol on the amplitude of evoked IPSCs. Ethanol alone increased mean IPSC amplitude by 14.1 ± 2.3 %, from

JPET Fast Forward. Published on April 15, 2013 as DOI: 10.1124/jpet.112.202903
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596.9 ± 53.1 pA to 679.4 ± 60.9 pA ($p < 0.01$, n =36) while in the presence of nor-BNI (1 μM) it increased the mean amplitude of IPSCs by 27.2 ± 3.9 %, from 565.8 ± 78.2 pA to 712.4 ± 97.2 pA ($p < 0.01$, n = 26). This result indirectly supports the suggestion that dynorphin released during acute ethanol exposure mitigates the effects of ethanol on GABA release.

Loss of KORs alters baseline GABAergic transmission and ethanol effects in CeA.

Based on behavioral studies of opioid receptor KO mice, KORs do not appear to mediate emotional responding under control conditions (Filliol et al., 2000). However, KORs mediate effects of cannabinoid receptor agonists in a manner opposite to that of mu opioid receptors (MORs) (Ghozland et al., 2002). Because a KOR antagonist augmented GABA responses in the CeA in the present study, we examined baseline GABA transmission in CeA of KOR KO and WT mice, first comparing a range of evoked IPSCs in response to five different stimulus intensities with 1x determined as the smallest stimulation that produced a discernible (Fig. 3A). Using two-way ANOVA, evoked IPSC amplitudes from KO (n =14) and WT (n = 21) mice were comparable ($F[1,33] = 0.10$; $p = 0.75$; Fig 3A), suggesting a modest contribution of KORs to tonic inhibition. Across all neurons tested, the effect of ethanol effect was significantly (p <0.01) greater in CeA of KOR KO mice compared to WT mice, increasing the evoked IPSC amplitudes by 14.2 ± 3.1% from 665.8 ± 86.2 pA to 764.2 ± 96.8 pA ($p < 0.01$, n = 19) in WT mice and by 33.5 ± 8.9% from 656.2 ± 105.0 pA to 841.5 ± 129.3 pA ($p < 0.01$, n = 13) in KOR KO mice. In addition, this ethanol effect was associated with a decrease in the paired-pulse ratio (PPR) (Fig 3B) in both groups, although CeA neurons of KOR KO mice showed a greater PPR decrease compared to WT mice. Thus, ethanol decreased the PPR of IPSCs (at 100 msec interstimulus intervals) significantly more in KOR KO mice (PPR = 0.79 ± 0.05, n = 13) than in WT mice (PPR = 0.91 ± 0.02, n = 19; $p < 0.02$), suggesting that the site of KOR action in modulating ethanol effects is at least in part presynaptic, by inhibition of GABA release.

To further examine the locus of the ethanol / KOR interaction, we studied mIPSCs in the presence of TTX (1 µM) in the CeA from KOR KO and WT mouse brain slices (Fig 4). There were no significant differences in the frequencies or amplitudes of the baseline mIPSCs between WT and KOR KO mice; the mean baseline frequencies were 2.0 ± 0.2 Hz and 2.2 ± 0.3 Hz for the WT mice (n = 14) and KOR KO mice (n = 16), respectively, while the mean baseline amplitudes were 22.7 ± 1.8 pA and 22.3 ± 1.1 pA, respectively. Ethanol (40 mM) increased the frequency of mIPSCs without changing their amplitudes in CeA neurons from both groups of mice; from 1.9 ± 0.2 Hz to 2.5 ± 0.3 Hz ($p < 0.01$, n = 11) in WT mice and from 2.2 ± 0.3 Hz to 3.4 ± 0.5 Hz ($p < 0.01$, n = 14) in KOR KO mice. However, the ethanol increase in mIPSC frequency in the CeA of KOR KO mice (56.8 ± 8.4 %; n = 14) was significantly ($p < 0.05$) greater than that in WT mice (35.0 ± 5.0 %, n = 11) (Fig 4B), further suggesting a presynaptic interaction of KORs and ethanol in regulating GABA release.

Discussion

In this study we explored the effects of activation of the KOR system in CeA on inhibitory GABAergic synaptic transmission and the interaction between the KOR opioidergic system and acute ethanol in regulating this transmission. First, we found that KOR activation decreased IPSCs in 6 of 10 neurons tested. This decrease appears to be mediated by presynaptic mechanisms. In addition, IPSCs appeared to be tonically inhibited by KOR activity in many neurons, as evidenced by the effect of the KOR antagonist alone. We also observed that removal of KOR activity through either genetic manipulation or pharmacological blockade enhanced the effectiveness of ethanol in increasing IPSCs in the CeA. This result suggests that acute ethanol actions in the CeA are regulated by KOR-mediated inhibition of GABAergic neurotransmission. Furthermore, this effect of KOR activation appears to be mediated by presynaptic mechanisms. Similar presynaptic actions of KOR activation have been recently demonstrated in the locus coeruleus (Kreibich et al., 2008) and bed nucleus of the stria terminalis (Li et al., 2012).

The KOR sensitivity was observed in approximately half of CeA neurons tested. We were unable to characterize this subpopulation of neurons at this time, as there was no electrophysiological signature specific to these neurons, nor was there a clear bimodal distribution of responses. However, these heterogeneous responses are consistent with the distribution of KORs and dynorphin in the CeA. Only a small fraction of CeA neurons express dynorphin (Marchant et al., 2007) or show direct effects of KOR agonists (Zhu and Pan, 2004). Although tonic KOR activity may be rather modest at baseline, this system may be activated during stress such as an ethanol dependent state or ethanol withdrawal (Walker and Koob, 2008; Sirohi et al., 2012; Berger et al., 2013). Further studies of the physiology of the KOR/dynorphin system in the ethanol dependent state appear warranted.

Modulation of GABAergic systems in the CeA was initially proposed as the primary mechanism underlying the motivational and reinforcing effects of ethanol (Hyytiä and Koob, 1995). Our previous work indicates that acute ethanol modulates GABAergic IPSCs in CeA indirectly through release of neuropeptides such as CRF and endogenous opioids; CRF increases GABA release (Nie et al., 2004; Roberto et al., 2010), whereas endogenous opioid peptides decrease GABA release (Kang-Park et al., 2007; Kang-Park et al., 2009). Thus, the overall effect of ethanol on GABAergic neurotransmission would be the net effect of these positive and negative modulators on GABA release.

The relationship between ethanol-induced modulation of the CeA GABAergic system and the motivational effect of ethanol is not entirely understood. The reinforcing effect of acute ethanol is blocked by inactivation of $GABA_A$ receptors in CeA, suggesting that an increase in GABA release may be a mechanism underlying reinforcing effects of ethanol in a rat model (Hyytiä and Koob, 1995). Similarly, Roberto and Siggins (Roberto and Siggins, 2006) reported that the opioid-like peptide nociceptin markedly blunted the rewarding effects of ethanol and also blocked acute ethanol-induced augmentation of the IPSCs in the rat CeA. In the same context, our results showing a KOR-mediated decrease in GABA release suggest one mechanism underlying the effect of KOR activity in inhibiting the motivational effects of acute ethanol. However, such a narrow interpretation raises several issues. First, the ethanol interaction with the GABAergic system is altered following chronic ethanol treatment, such that GABA agonists or KOR antagonists (that can augment GABAergic IPSCs) block the reinforcing effects of ethanol in dependent rats (Walker and Koob, 2008). Second, KOR KO mice (Kovacs et al., 2005), as well as dynorphin KO mice (Blednov et al., 2006), drink less ethanol than WT mice, although both KO mice should presumably exhibit disinhibition of GABAergic transmission in CeA (however, the study by Kovacs et al. (2005) also suggested KOR KO mice may have disrupted taste sensation). Third, activation of mu and delta opioid receptors also decreases GABAergic IPSCs in the mouse CeA (Kang-Park et al., 2007; Kang-Park et al., 2009), as with KOR activation. This contrasts with behavioral studies showing that KOR and MOR activation mediates opposing effects on reinforcing actions of tetrahydrocannabinol (Ghozland et al., 2002). In addition, while activation of mu and delta receptors is generally anxiolytic, KOR activation appears to be associated with dysphoria (Land et al., 2008) and frequently blocks behavioral effects of MOR activation (Pan, 1998).

The opposing behavioral effects of KOR and MOR activation could result from the different cellular localization of these receptors in CeA, despite a similar cellular mechanism. Such a model accounts for differing behavioral effects of oxytocin and vasopressin, despite identical physiological mechanisms at the cellular level in CeA (Huber et al., 2005). Previous work has indicated that discrete subpopulations of CeA neurons show direct sensitivity to either MOR or KOR activation (Zhu and Pan, 2004; Chieng et al., 2006). Localization of KORs or MORs on different CeA neurons may result in opposing functions in such a way that a decrease of GABA release onto the CeA output neurons could lead to *increased* excitation of these output neurons, whereas a decrease of GABA release onto the CeA GABAergic interneurons could lead to *decreased* excitation of CeA output neurons. However, under our current recording conditions, it is not possible to conclusively distinguish between projection neurons and interneurons in CeA based on electrophysiological markers.

Another possibility is that subpopulations of CeA neurons showing different MOR and KOR sensitivity could have distinct target projections. In the mouse ventral tegmental area, dopaminergic neurons projecting to nucleus accumbens are selectively sensitive to kappa receptor agonists, whereas neurons projecting to basolateral amygdala are sensitive to mu/delta receptor agonists (Ford et al., 2006; Ford et al., 2007); presynaptic GABAergic transmission was also differentially regulated (Ford et al., 2006). However, a similar study in rats comparing accumbens projections to basolateral amygdala and prefrontal cortex reported that the cortical projections, not the amygdala projections, were sensitive to KOR-mediated inhibition (Margolis et al., 2006). As the methods in these two studies were otherwise very similar we suggest that these seemingly contradictory findings result from species differences, though both studies support the notion of differential opioid peptide sensitivity based on target locations.

Our present results are consistent with the idea that increased ethanol-induced enhancement of presynaptic GABAergic function in CeA neurons is one of the cellular mechanisms underlying the enhanced anxiolytic effect of ethanol. The current finding indicates that KORs regulate presynaptic ethanol effects on GABAergic transmission in a manner similar to other opioid peptide systems (Kang-Park et al., 2007; Kang-Park et al., 2009). Given the divergent behavioral effects of activation of the various opioid peptide receptor subtypes, we believe that ethanol reinforcement is likely regulated by discrete neural circuits in the CeA with differing opioid sensitivities, reflecting in part the diverse role that opioid systems play in modulating voluntary ethanol intake (Roberts et al., 2000; Roberts et al., 2001; Kovacs et al., 2005). Our findings are consistent with the hypothesis that the anxiolytic effect of ethanol contributes to ethanol reinforcement and that presynaptic KORs in CeA may have a unique role modulating this effect. Therefore, we believe that further studies of KOR function on identified pathways projecting from CeA are warranted to establish the behavioral relevance of the interaction of ethanol with opioid peptide systems. Such studies may facilitate development of novel therapies for the treatment of alcohol use disorders (Walker et al., 2012).

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest.

Authorship Contributions

Participated in research design: Kang-Park, Kieffer, Roberts, Siggins, Moore

Conducted experiments: Kang-Park

Developed transgenic mice: Kieffer, Roberts

Performed data analysis: Kang-Park, Moore

Wrote or contributed to writing of the manuscript: Kang-Park, Kieffer, Siggins, Moore

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Footnotes

This study was supported by grants from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) Integrative Neuroscience Initiative on Alcoholism (INIA) grant [Grant UO1 AA013498] (GRS, SDM), [Grant R21 AA019553] (SDM); National Institute on Drug Abuse [Grant R01 DA03665] (GRS); and a Veterans Administration Merit Review [Grants NEUA-011-062 and 510BX001271-02] (SDM).

Figure Legends

Figure 1. U69593, a selective KOR agonist, reduces evoked IPSC amplitudes and mIPSC frequency in CeA from WT C57BL/6 mice. **A**: Time-course of the amplitudes of evoked IPSCs plotted over the course of a representative experiment (panel **Aa**) and averaged 6 experiments showing significant results (panel **Ac**); 1 µM U69593 reduces evoked IPSC amplitudes in a reversible manner. Representative traces of evoked IPSCs shown in panel **Ab** and the mean (± s.e.m.) results from all 10 neurons in panel **Ad, left**. U69593 also decreased the ratio of paired-pulse responses (PPR) at an interstimulus interval of 100 msec; mean (± s.e.m.) results in panel **Ad, right.** **B**: Effect of U69593 on mIPSCs. Representative traces in panel **Ba** and cumulative plots of frequency and amplitude in panel **Bb**; U69593 decreases the frequency of mIPSCs but does not change the mean amplitude. Mean (± s.e.m.) data from 16 neurons are shown in panels **Bc**. In this and subsequent figures; * represents $p < 0.05$; ** represents $p \leq 0.01$.

Figure 2. The effects of nor-BNI, a selective KOR antagonist, and ethanol on evoked IPSCs in CeA from WT C57BL/6 mice. **A**: Time-course of effect of nor-BNI (1 µM) and subsequent effect of ethanol on peak amplitudes of evoked IPSCs (panel **Aa**) and representative traces (panel **Ab; numbers correspond to the time-points indicated in Aa**). **B**: Ethanol effects on evoked IPSCs with and without nor-BNI treatment. Time courses of peak amplitudes of evoked IPSCs are plotted in panel **Ba,** with averaged data from neurons exposed to ethanol alone (n = 36) and with nor-BNI pre-treatment (n = 26) in panel **Bb**. Ethanol increases the amplitude of evoked IPSCs, and the magnitude of

enhancement is significantly greater in the presence of nor-BNI, suggesting that KOR-mediated inhibition partially regulates the potentiating effect of ethanol on IPSCs.

Figure 3. Comparison of ethanol effects on evoked IPSCs in CeA from KOR KO and WT mice. **A**: Baseline IPSCs from KOR KO and WT mice evoked in a CeA neuron by a range of five incrementally-increasing stimulus intensities, with 1x determined as the smallest stimulation that produced a discernible IPSC. Representative traces are shown in inset. Baseline evoked IPSCs were comparable in slices from KOR KO (n = 14) and WT mice (n = 21). **B**: Ethanol enhances evoked IPSCs, with representative traces in **Ba** and averaged results from experiments showing ethanol sensitivity (WT, n = 11; KOR KO mice, n = 9) in **Bb**. Ethanol (40 mM) significantly increased the peak amplitudes of IPSCs in both WT and KO mice, although the acute ethanol effect was significantly greater in CeA of KOR KO mice than WT mice. **Bc**: mean (± s.e.m.) ethanol effects on evoked IPSC amplitudes (left) and related PPRs (right) from WT (n = 19) and KOR KO mice (n = 13). Ethanol also significantly decreased the mean PPR from both groups, but showed a significantly greater decrease in CeA of KO mice, suggesting that the site of KOR action in modulating the ethanol effect is at least in part presynaptic by reducing release of GABA.

Figure 4. Ethanol increases mIPSC frequency in CeA neurons from both KOR KO and WT mice; mIPSCs recorded in the presence of 1 μM TTX. **A:** Upper panels show representative current traces; neuron from WT mouse in **Aa**, from KOR KO mouse in **Ab**, while lower panels present cumulative plots of mIPSC frequencies and amplitudes for

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each data. **B:** Mean (± s.e.m) results comparing ethanol effects on the frequency (**Ba**) and amplitude (**Bb**) of mIPSCs from WT (n = 11) and KOR KO mice (n = 14). Ethanol (40 mM) significantly increased the mean frequency (**Ba**) of mIPSCs, without significant change in the mean amplitude (**Bb**), in slices from both groups of mice, with a significantly greater ethanol effect in CeA neurons of KOR than WT mice, further suggesting that the KOR action in modulating ethanol effects is mediated at presynaptic sites.

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Figure 1

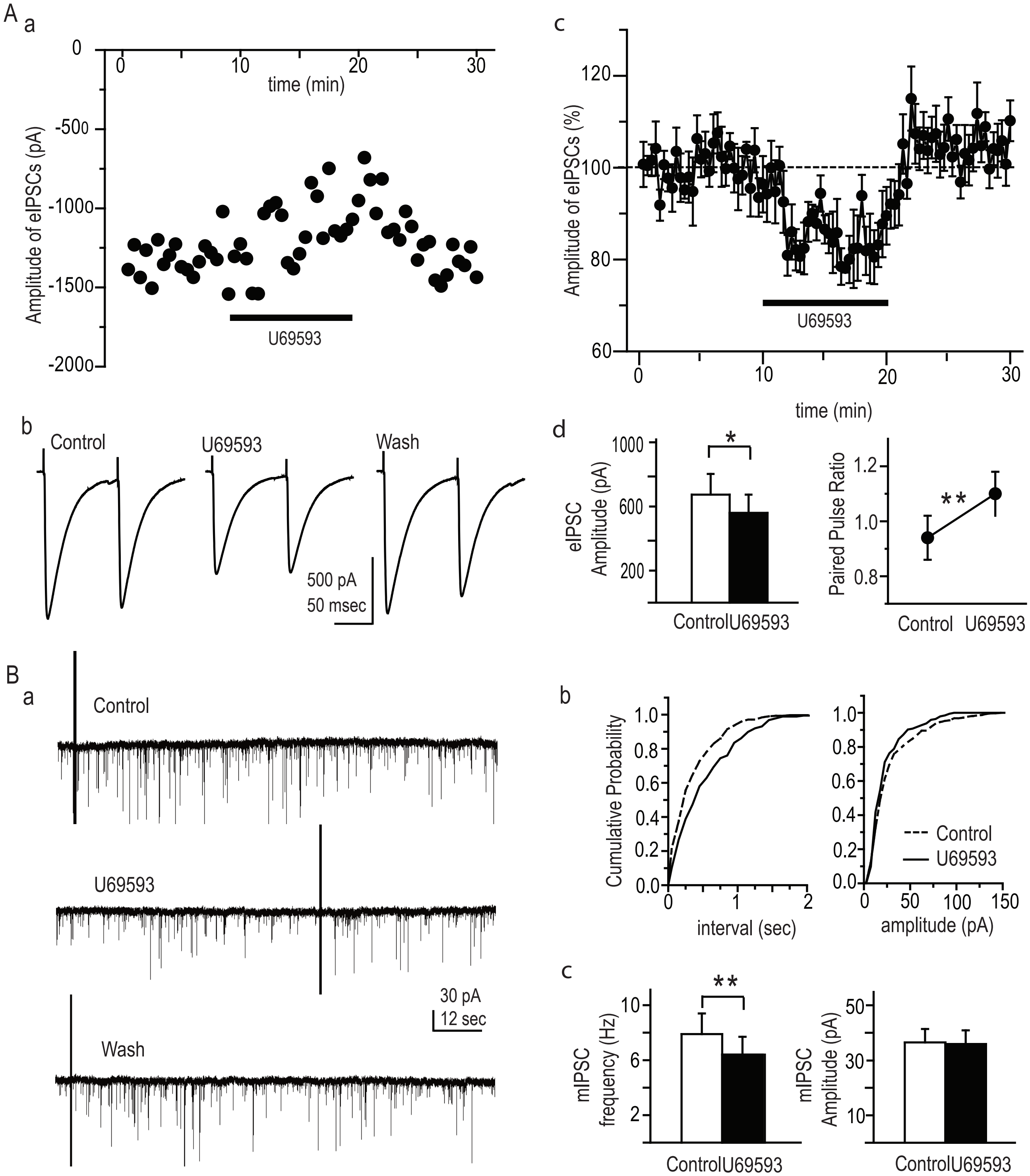

Figure 2

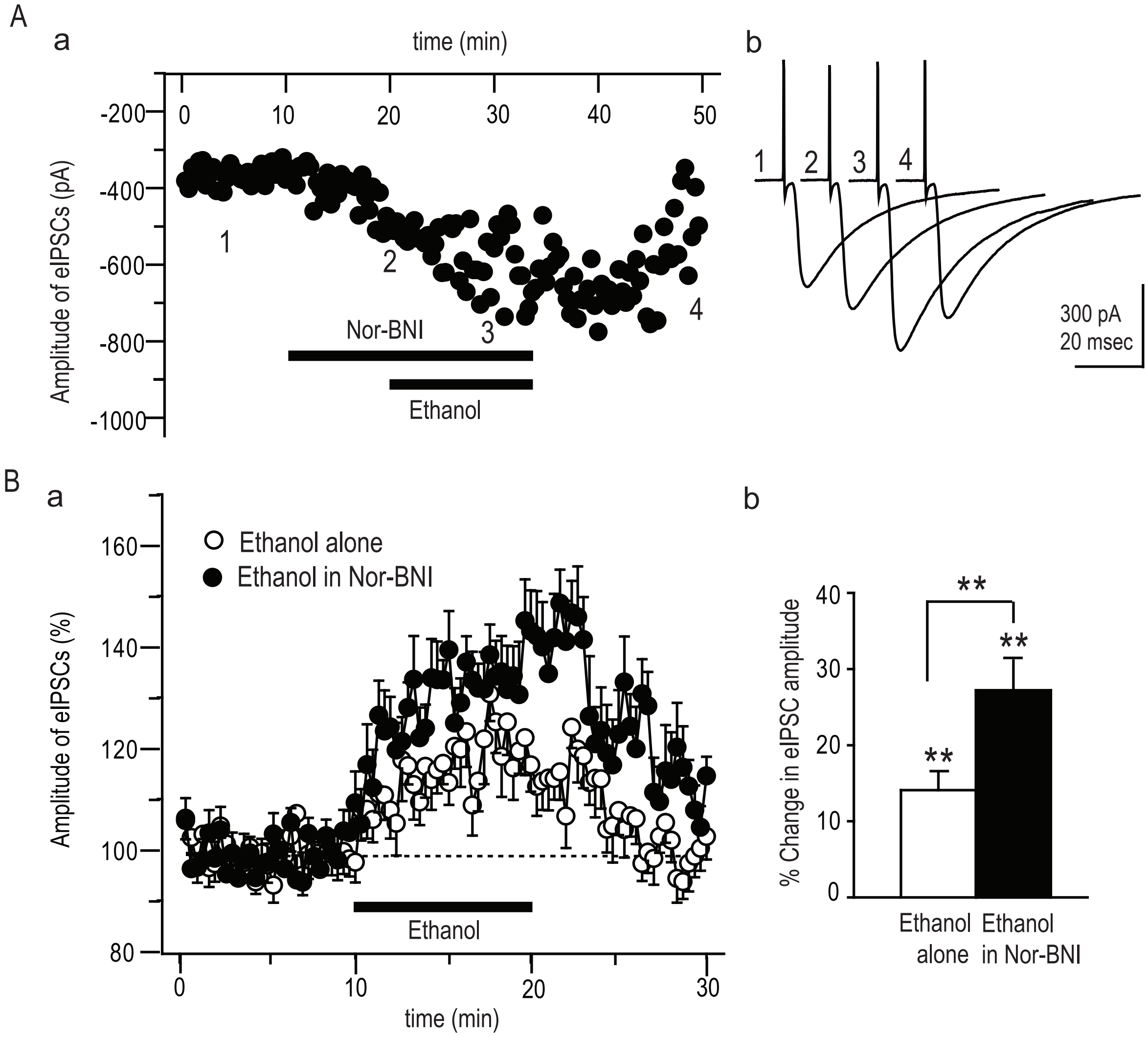
A
a
time (min)
0
10
20
30
40
50
-200
-400
-600
-800
-1000
Amplitude of eIPSCs (pA)
1
2
3
4
Nor-BNI
Ethanol
b
1
2
3
4
300 pA
20 msec
B
a
Ethanol alone
Ethanol in Nor-BNI
160
140
120
100
80
Amplitude of eIPSCs (%)
Ethanol
0
10
20
30
time (min)
b
40
30
20
10
0
% Change in eIPSC amplitude
**
**
**
Ethanol alone
Ethanol in Nor-BNI

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Figure 3

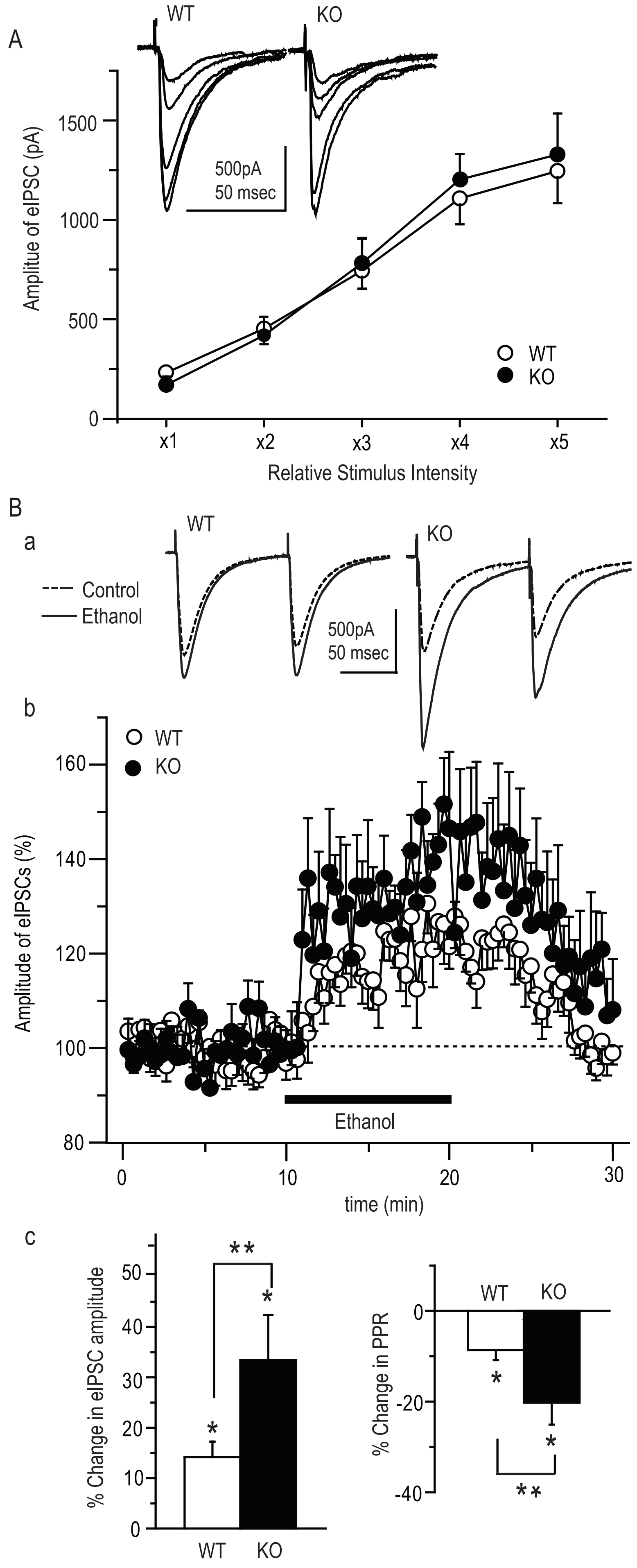

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Figure 4