κ -Opioid Receptors in the Central Amygdala Regulate Ethanol Actions at Presynaptic GABAergic Sites^S

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ABSTRACT

Human and animal studies indicate that κ -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 physiologic effects of KOR activation in the central amygdala (CeA) on GABAergic neurotransmission and its interaction with acute ethanol. A selective KOR agonist (U69593, 1 μ M) diminished evoked GABAergic inhibitory postsynaptic currents (IPSCs) by 18% ($n = 10$), whereas blockade of KORs with a selective antagonist (nor-binaltorphimine, 1 μ M) 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 pharmacologic blockade of KORs, from 14% ($n = 36$) to 27% ($n = 26$; $P < 0.01$), or by genetic deletion of KORs, from 14% in wild-type mice ($n = 19$) to 34% in KOR knockout 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 κ -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; Karpayak 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), whereas 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 nondependent rats (Walker and Koob, 2008; Walker et al., 2011). Mice lacking KORs drank half as much ethanol as either wild-type (WT) 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, 2009; Land et al., 2008).

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 on neuronal cell bodies, and they may act through two mechanisms: by inhibition of neurotransmission directly at terminal release sites (Svingos et al., 1999; Li et al.,

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; CeA, central nucleus of the amygdala; CGP 55845, 3-N-[1-(S)-(3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-*p*-benzyl-phosphinic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; IPSC, inhibitory postsynaptic current; KOR, κ -opioid receptor; mIPSC, miniature inhibitory postsynaptic current; MOR, μ -opioid receptor; NAcc, nucleus accumbens; nor-BNI, norbinaltorphimine; PPR, paired pulse ratio; TTX, tetrodotoxin; U69593, (+)-(5 α ,7 α ,8 β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide; WT, wild-type.

2012) as well as by 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 this region 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 comprises heterogeneous cell types with multiple physiologic 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.

Materials and 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 knockout and WT littermate mice (male, 120–180 days old) shipped from The Scripps Research Institute, La Jolla, California to Duke University, Durham, North Carolina. The genetic background of these mice was a hybrid C57BL/6Orl $\times$ 129/SV strain. We housed two to four mice per cage in a temperature-controlled room in which the lights were on a 12-hour light/dark cycle with

lights off at 6:00 PM, and animals were sacrificed for experiments between 9:00 and 11:00 AM. For pharmacologic experiments, we also used 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% O₂-5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM) 120 NaCl, 3.3 KCl, 1.23 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 0.9 MgSO₄, 10 glucose; for ACSF used only during the dissection, 2 CaCl₂ was replaced with 0.5 CaCl₂. We cut coronal slices (300 μ m, between bregma -1.0 to ~ 1.9 mm; Paxinos and Franklin, 2012) using a Vibratome (Campden, model 752, Lafayette, IN) and incubated slices in ACSF continuously bubbled with 95% O₂ and 5% CO₂. After 30 minutes of incubation at room temperature, we transferred slices singly to the recording chamber (volume 0.5 ml) in which oxygenated ACSF at 35°C 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 (40 $\times$, 0.75 numerical aperture), infrared filtered light, differential interference contrast optics, and a Hitachi charge coupled device 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 optical density, 1.05 mm internal diameter; 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 M Ω) 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; Na₂creatine PO₄, 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 *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate glutamate receptor blockers 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μ M) and D-2-amino-5-phosphonopentanoate (50 μ M), respectively, and the GABA_B receptor blocker CGP 55845 (3-*N*-(1-*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphonic acid) (1 μ M). To study evoked IPSCs, we placed a monopolar tungsten electrode (A-M Systems, Carlsborg, WA) in the lateral division of the CeA and delivered square wave current pulses (0.1 ms duration) every 20 seconds. For baseline responses, we used IPSCs determined to be approximately 30–50% of maximal amplitude. Drugs [ethanol, U69593 ((+)-(5 α ,7 α ,8 β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide), norbinaltorphimine (nor-BNI)] were applied after establishing stable baseline responses. In some experiments, we also examined “miniature” spontaneous IPSCs (mIPSCs) using tetrodotoxin (TTX; 1 μ M) 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 (-3 dB), and Clampex software (Molecular Devices). We made all recordings at 35°C. Liquid junction potentials were not measured or compensated. We monitored series resistance (10–30 M Ω) online throughout the experiment using pClamp (Molecular Devices) 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 offline using Clampfit (Molecular Devices) on an IBM-compatible computer and compared the drug effects between groups using two-sample *t* test or two-way analysis of variance when appropriate. For analysis of mIPSCs, we analyzed the frequencies and amplitudes using Mini Analysis (Synaptosoft, Decatur, GA) and tested drug effects using the Komolgorov-Smirnov statistical method. We considered $P < 0.05$ as indicating statistical significance. We used Origin software (Origin Laboratory, Northampton, MA) for plotting figures and statistical analysis. Results in the text and figures are presented as the mean $\pm$ 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 ($1 \mu\text{M}$) significantly reduced the amplitude of evoked IPSCs by $18.1\% \pm 4.1\%$, from

$674.0 \pm 128.6 \text{ pA}$ to $560.0 \pm 115.6 \text{ pA}$ ($n = 10$, Fig. 1, Aa and Ac). However, four neurons showed no significant effect of the agonist. In responsive neurons, actions of U69593 were blocked by the specific KOR antagonist nor-BNI (Supplemental Fig. 1). U69593 also significantly ($P < 0.01$) increased the paired-pulse ratio (PPR) of IPSCs at 100-millisecond interstimulus intervals from 0.94 to 1.1 ($n = 10$; Fig. 1, Ab 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 (Andreassen 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

Fig. 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 (eIPSCs) plotted over the course of a representative experiment (panel Aa) and averaged 6 experiments showing significant results (panel Ac); $1 \mu\text{M}$ U69593 reduces evoked IPSC amplitudes in a reversible manner. Representative traces of evoked IPSCs shown in panel Ab and the mean ($\pm$ 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 milliseconds; mean ($\pm$ 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 ($\pm$ S.E.M.) data from 16 neurons are shown in panel Bc. * $P < 0.05$; ** $P \leq 0.01$.

with TTX (1 μ M). Application of U69593 (1 μ M) 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 (Kolmogorov-Smirnov $z = 1.72$; $P < 0.01$) shifted the cumulative frequency distribution to longer interevent 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 μ M) significantly ($P < 0.05$) augmented the mean baseline IPSC amplitude by $14.1\% \pm 3.3\%$, 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.

Pharmacologic Block of KORs Enhances Ethanol-Induced Increases in GABAergic Transmission in CeA. We and others have previously shown that acute ethanol administration enhances GABA release at CeA synapses from rats and mice (Roberto et al., 2003; Nie et al., 2004; Kang-Park et al., 2007, 2009). In addition, our previous physiologic studies indicate that acute ethanol administration induces release of such neuropeptides as endogenous opioids and CRF in CeA (Nie et al., 2004; Lam et al., 2008), and these peptides may further modulate GABA release (Nie et al., 2004; Kang-Park et al., 2007, 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 μ M) for 10 minutes 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 $14.1\% \pm 2.3\%$, from 596.9 ± 53.1 pA to 679.4 ± 60.9 pA ($P < 0.01$, $n = 36$); in the presence of nor-BNI (1 μ M), it increased the mean amplitude of IPSCs by $27.2\% \pm 3.9\%$, 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. On the basis of results from behavioral studies of opioid receptor knockout 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 those of μ -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 knockout and WT mice. First, we compared a range of evoked IPSCs in response to five different stimulus intensities, with $1\times$ determined as the smallest stimulation that produced a discernible (Fig. 3A) response. Using two-way analysis of variance, evoked IPSC amplitudes from knockout ($n = 14$) and WT ($n = 21$) mice were similar ($F[1,33] = 0.10$; $P = 0.75$; Fig. 3A), suggesting

Fig. 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 μ M) and subsequent effect of ethanol on peak amplitudes of evoked IPSCs (eIPSCs; 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. $**P \leq 0.01$.

Fig. 3. Comparison of ethanol effects on evoked IPSCs (eIPSCs) in CeA from KOR knockout and WT mice. (A) Baseline IPSCs from KOR knockout (KO) and WT mice evoked in a CeA neuron by a range of five incrementally increasing stimulus intensities, with 1× determined as the smallest stimulation that produced a discernible IPSC. Representative traces are shown in inset. Baseline evoked IPSCs were similar in slices from KOR knockout ($n = 14$) 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 knockout mice, $n = 9$) in panel Bb. Ethanol (40 mM) significantly increased the peak amplitudes of IPSCs in both WT and knockout mice, although the acute ethanol effect was significantly greater in CeA of KOR knockout mice than

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 knockout mice compared with WT mice, increasing the evoked IPSC amplitudes by $14.2\% \pm 3.1\%$ from 665.8 ± 86.2 pA to 764.2 ± 96.8 pA ($P < 0.01$, $n = 19$) in WT mice and by $33.5 \pm 8.9\%$ from 656.2 ± 105.0 pA to 841.5 ± 129.3 pA ($P < 0.01$, $n = 13$) in KOR knockout mice. In addition, this ethanol effect was associated with a decrease in the PPR (Fig. 3B) in both groups, although CeA neurons of KOR knockout mice showed a greater PPR decrease compared with WT mice. Thus, ethanol decreased the PPR of IPSCs (at 100-millisecond interstimulus intervals) significantly more in KOR knockout 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 partially presynaptic, by inhibition of GABA release.

To further examine the locus of the ethanol/KOR interaction, we studied mIPSCs in the presence of TTX ($1 \mu\text{M}$) in the CeA from KOR knockout 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 knockout 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 knockout mice ($n = 16$), respectively, whereas 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 knockout mice. However, the ethanol increase in mIPSC frequency in the CeA of KOR knockout mice ($56.8 \pm 8.4\%$; $n = 14$) was significantly ($P < 0.05$) greater than that in WT mice ($35.0 \pm 5.0\%$, $n = 11$) (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 pharmacologic 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

WT mice. Panel Bc shows mean ($\pm$ S.E.M.) ethanol effects on evoked IPSC amplitudes (left) and related PPRs (right) from WT ($n = 19$) and KOR knockout mice ($n = 13$). Ethanol also significantly decreased the mean PPR from both groups but showed a significantly greater decrease in CeA of knockout mice, suggesting that the site of KOR action in modulating the ethanol effect is at least in part presynaptic by reducing release of GABA. * $P < 0.05$; ** $P \leq 0.01$.

Fig. 4. Ethanol increases mIPSC frequency in CeA neurons from both KOR knockout (KO) and WT mice; mIPSCs recorded in the presence of $1 \mu\text{M}$ TTX. (A) Upper panels show representative current traces; neuron from WT mouse in Aa, from KOR knockout mouse in Ab, whereas lower panels present cumulative plots of mIPSC frequencies and amplitudes for each data. (B) Mean ($\pm$ S.E.M.) results comparing ethanol effects on the frequency (Ba) and amplitude (Bb) of mIPSCs from WT ($n = 11$) and KOR knockout 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. * $P < 0.05$; ** $P \leq 0.01$.

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 electrophysiologic 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, 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 knockout mice (Kovacs et al., 2005), as well as dynorphin knockout mice (Blednov et al., 2006), drink less ethanol than WT mice, although both knockout mice should presumably exhibit disinhibition of GABAergic transmission in CeA (however, the 2005 study by Kovacs et al. also suggested KOR knockout mice may have disrupted taste sensation). Third, activation of μ - and Δ -opioid receptors also decreases GABAergic IPSCs in the mouse CeA (Kang-Park et al., 2007, 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, activation of μ and Δ receptors is generally anxiolytic, whereas 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 physiologic 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 electrophysiologic 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 κ -receptor agonists, whereas neurons projecting to basolateral amygdala are sensitive to μ/Δ receptor agonists (Ford et al., 2006, 2007); presynaptic GABAergic transmission was also differentially regulated (Ford et al., 2006). However, a similar study in rats comparing accumbens projections with 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, 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, 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).

Authorship Contributions

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

Conducted experiments: Kang-Park.

Contributed new reagents or analytic tools: 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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