Interaction of CRF and Kappa Opioid Systems on GABAergic Neurotransmission in the Mouse Central Amygdala

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ABSTRACT

The corticotropin-releasing factor (CRF) and kappa-opioid receptor (KOR) systems are both implicated in stress-related behaviors and drug dependence. Although previous studies suggest that antagonism of each system blocks aspects of experimental models of drug dependence, the possible interaction between these systems at the neuronal level has not been completely examined. We used an in vitro brain slice preparation to investigate the interaction of these two peptide systems on inhibitory neurotransmission in the central nucleus of the amygdala (CeA). Application of exogenous CRF increased the mean frequency of GABAergic miniature inhibitory postsynaptic currents (mIPSC) by 20.2%, suggesting an increase in presynaptic GABA release. Although the pharmacological blockade of KORs by norBNI alone did not significantly affect mIPSC

frequency, it significantly enhanced the effect of CRF (by 43.9%, $P = 0.02$). Similarly, the CRF effects in slices from KOR knockout (KO) mice (84.0% increase) were significantly greater than in wild-type (WT) mice (24.6%, $P = 0.01$), although there was no significant difference in baseline mIPSC frequency between slices from KOR KO and WT mice. The increase in CRF action in the presence of norBNI was abolished by a CRF-1 receptor antagonist but was unaffected by a CRF-2 receptor antagonist. We hypothesize that CRF facilitates the release of an endogenous ligand for KORs and that subsequent activation of KOR receptors modulates presynaptic effects of CRF in CeA. These results suggest that potential pharmacotherapies aimed at neurobehavioral and addictive disorders may need to involve both the KOR/dynorphin and the CRF systems in CeA.

Introduction

The brain stress system is activated during withdrawal states from various drugs of abuse, and avoiding drug withdrawal stress is known to provide strong motivation for continuing use of the drugs, leading to a drug-dependent state (Koob et al., 2014). Therefore, intervening within brain stress systems has been proposed as a strategic approach to decrease or block reinstatement of drug-seeking and/or drug-consuming behaviors. Such brain stress systems include corticotropin-releasing factor (CRF) and the dynorphin/kappa opioid receptor (KOR) neuropeptide systems. In support of this view, CRF-receptor antagonists block ethanol self-administration in ethanol-dependent rats (Funk et al., 2007) and cocaine self-administration (Boyson et al., 2011), whereas the block of KOR receptors decreases ethanol drinking in ethanol-dependent rats

(Walker and Koob, 2008; Walker et al., 2011) and also decreases heroin intake (Schlosburg et al., 2013).

Considering this apparent overlap in actions between CRF and dynorphin/KOR systems in regulating stress-related behavior, an interaction between these two systems has been proposed. It has been reported that CRF induces dynorphin release in the hypothalamus (Almeida et al., 1986), striatum (Sirinathsinghji et al., 1989), and spinal cord (Song and Takemori, 1992). Furthermore, the KOR antagonist norbinaltorphimine (norBNI) was shown to block CRF-induced anxiety-like behavior and CRF-induced conditioned place aversion, a measure of dysphoric effects (Land et al., 2008; Bruchas et al., 2009; Bruchas et al., 2010). However, KOR agonist-induced conditioned place aversion is still intact in animals lacking CRF1 receptors (Contarino and Papaleo, 2005). In contrast, Valdez et al. (2007) reported that selective CRF1-receptor antagonists blocked KOR agonist-induced reinstatement of cocaine seeking in primates. However, interactions between KOR and CRF systems may be reciprocal, as Wittmann et al. (2009) reported downregulation of CRF mRNA in select brain regions from dynorphin KO mice.

The central nucleus of the amygdala (CeA) is a brain locus mediating stress and anxiety-like behavior and is also

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; CRFR1, CRF type 1 receptor; CRFR2, CRF type 2 receptor; KO, knockout; KOR, kappa-opioid receptor; mIPSCs, miniature inhibitory postsynaptic currents; NBI, NBI-27914 (5-chloro-*N*-(cyclopropylmethyl)-2-methyl-*N*-propyl-*N'*-(2,4,6-trichlorophenyl)-4,6-pyrimidinediamine HCl); norBNI, norbinaltorphimine; WT, wildtype.

regarded as a critical region mediating drug dependence (Gilpin et al., 2014). The CeA contains both CRF and dynorphin, with colocalization reported in many neurons (Marchant et al., 2007), suggesting that it is a possible site for interaction of CRF and KOR systems. We previously observed that KOR activation decreases inhibitory neurotransmission in the CeA (Kang-Park et al., 2013), whereas CRF activation increases the inhibitory neurotransmission (Nie et al., 2004). Therefore, in the present study we have examined interactive effects of the CRF and KOR systems on CeA inhibitory GABAergic synaptic neurotransmission, and particularly on miniature inhibitory postsynaptic currents (mIPSCs), to characterize a possible interaction at the cellular and synaptic levels.

Methods

Animals. Female C57BL/6 mice (60–90 days old; Charles River Laboratories, Raleigh, NC), and homozygous KOR knockout (KO) mice and wild-type (WT) littermate mice (120–180 days old, shipped from the Scripps Research Institute, La Jolla, CA) were used for this study. We exclusively used female mice, as it has been reported that female animals showed a greater or selective sensitivity to KOR-mediated behaviors such as antinociception, motivation, mood-related disorders, and social interaction (Chang et al., 2000; Chakrabarti et al., 2010; Lawson et al., 2010; Liu et al., 2011; Robles et al., 2014; Russell et al., 2014). The genetic background of KOR KO and WT mice was a hybrid C57BL/6Orl $\times$ 129/SV strain. The methods for generation of the knockout mice were as described in detail previously (Simonin et al., 1998). We housed mice two to four 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. All mice from Scripps were quarantined in our facility for up to 6 weeks prior to use and all mice from Charles River Laboratories were allowed to acclimate in our facility for at least 1 week. Care was taken to minimize the discomfort and the number of animals used, and all procedures were approved by the Durham VA Medical Center and Duke University Medical Center institutional animal care and use committees and conducted in accordance with guidelines from the National Institutes of Health.

Brain Slice Preparations. Mice were anesthetized with isoflurane, and brains rapidly removed and immersed in ice-cold oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in millimolar concentrations) 125 NaCl, 3.3 KCl, 1.23 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 0.9 MgSO₄, 10 glucose; for ACSF used only during the dissection, we replaced 2 CaCl₂ with 0.5 CaCl₂. Coronal brain slices (300 μ m, between bregma $-1.0 \sim -1.9$ mm; Paxinos and Franklin, 2004) were cut using a Vibratome (Model 752; Campden Instruments Ltd., Loughborough, Leicestershire, England) and incubated in ACSF continuously bubbled with 95% O₂ and 5% CO₂ at 35°C for 30 minutes. After incubating in ACSF at room temperature for another 30 minutes, we transferred slices singly to the recording chamber (volume 0.5 ml), in which oxygenated ACSF 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).

Whole-Cell Patch Recordings. We made whole-cell patch recordings in the medial ventral division of the central amygdala. Recording pipettes (input resistance 2–5 M Ω) were pulled from borosilicate glass capillary tubing (1.5 mm o.d., 1.05 mm i.d.; World Precision Instruments, Sarasota, FL) using a Flaming-Brown horizontal microelectrode puller (Model P-97; Sutter Instrument, Novato, CA), and filled with the following solution (in millimolar concentrations): 140 CsCl, 10 HEPES, 4 NaCl, 0.2 EGTA, 4 Mg-ATP, 0.3 Tris-GTP, 10 Na₂Creatine PO₄ (pH 7.25; 285 mOsm). We voltage-clamped cells at -70 mV and applied isolated inhibitory postsynaptic currents

(IPSCs) using non-NMDA and NMDA glutamate-receptor blockers 6,7-dinitroquinoxaline-2,3-dione (20 μ M) and D-2-amino-5-phosphonovalerate (50 μ M), and the GABA_B-receptor blocker (CGP 55845, 1 μ M). At the end of the experiment, we blocked the IPSCs with bicuculline methiodide (20 μ M), to confirm mediation by GABA_A receptors (Kang-Park et al., 2013). We isolated miniature IPSCs (mIPSCs) by superfusing tetrodotoxin (1 μ M; Sigma-Aldrich, St. Louis, MO) throughout the recordings.

Drugs. Rat/human corticotropin-releasing factor (CRF; Beckman Coulter, Jersey City, NJ), nor-BNI (Sigma-Aldrich), 5-chloro-*N*-(cyclopropylmethyl)-2-methyl-*N*-propyl-*N'*-(2,4,6-trichlorophenyl)-4,6-pyrimidinediamine HCl (NBI-27914; Sigma-Aldrich), or astressin 2B (Sigma-Aldrich) were applied via bath perfusion.

Data Acquisition and Analysis. We acquired spontaneous synaptic current responses at 10 KHz using an Axopatch 200B (Molecular Devices, Foster City, CA), filtered at 2 kHz (-3 dB), and digitized by Clampex. All recordings were made at 33°C. We monitored series resistance (10–30 M Ω) online throughout the experiment using pClamp, and rejected cells if this resistance changed by more than 20%.

We analyzed the frequencies and amplitudes of mIPSCs using Minianalysis (www.synaptosoft.com) and tested drug effects using the Kolmogorov-Smirnov statistical method, paired sample *t* test, or two sample *t* tests when appropriate, with *P* < 0.05 considered 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

A total of 33 neurons were recorded in the central amygdala in brain slices from drug-naïve female mice (C57BL/6 from Charles River Laboratories) or KOR KO and their littermate WT female controls (a hybrid C57BL/6Orl $\times$ 129/SV background). To examine the interaction between CRF and KOR in the CeA, we measured the modulation of GABAergic mIPSCs by CRF after pharmacological blockade of KORs and compared it with the effect of CRF alone (control) in brain slices from drug-naïve C57 mice from Charles River Laboratories. CRF (100 nM) alone significantly increased the mean frequencies of mIPSCs by 20.2% (from 7.9 ± 0.8 Hz to 9.4 ± 0.9 Hz, *P* = 0.02; *n* = 5 neurons from four mice; Fig. 1). Pharmacological blockade of KORs with the KOR antagonist norBNI (1 μ M) alone had no significant effect on mean mIPSC frequency (baseline 7.81 ± 0.78 Hz versus norBNI 7.13 ± 1.25 Hz; *n* = 5). However, after the addition of norBNI, CRF further increased the frequency of mIPSCs (from 7.1 ± 1.2 Hz to 9.8 ± 1.4 Hz; *n* = 5; Fig. 1) in the same cells; the magnitude of increase (43.9%) in the presence of the KOR blocker was significantly greater than in the control (CRF alone) condition (*P* = 0.02; Fig. 1, B and C) in the same sets of cells. By contrast, CRF did not significantly change the amplitudes of mIPSCs either in the control condition (from 67.0 ± 17.0 pA to 60.2 ± 12.1 pA, *P* = 0.8; *n* = 5) or in the presence of KOR blockade (from 66.1 ± 12.3 pA to 65.3 ± 11.4 pA; *n* = 5; data not shown). These results suggest that an active KOR system dampens CRF effects on GABAergic IPSCs by a pre-synaptic mechanism.

Next, we examined the effect of deletion of KORs on CRF effects using KOR KO littermate control mice. There was no significant difference in baseline mean mIPSC frequency between the WT (8.01 ± 1.0 Hz; *n* = 10 neurons from 7 mice) and KOR KO mice (7.7 ± 1.6 Hz; *n* = 8 neurons from 4 mice). However, CRF (100 nM) enhanced the mean frequencies of mIPSCs significantly more (*P* = 0.01) in CeA neurons from KOR KO than in WT mice (Fig. 2, B and C). Thus CRF

Fig. 1. Pharmacological blockade of KORs increases the effect of CRF to enhance mIPSC frequencies in CeA neurons. Representative mIPSC traces are shown in (A); traces from control condition and KOR antagonist (norBNI) pretreated condition are shown in (A), a and b, respectively, and each panel shows traces before, during, and after bath application of CRF. In (B), the cumulative probability plot of interpulse intervals of mIPSCs was compared before and during CRF application, in the control condition (A) and norBNI-pretreated condition (B). The averaged percentage change in mIPSC frequencies are presented in (C), showing that percent change in mIPSC frequency during blockade of KORs with norBNI is significantly greater than in the control (CRF alone) condition.

increased the mean frequency of mIPSCs by $24.6 \pm 6.5\%$ (from 8.0 ± 1.0 Hz to 9.7 ± 1.2 Hz) in WT mice ($n = 10$), but increased the mean frequency of mIPSCs by $84.0 \pm 17.8\%$ (from 7.6 ± 1.6 Hz to 12.9 ± 2.3 Hz) in KOR KO mice ($n = 8$) (Fig. 2). By contrast, CRF did not significantly change the mean amplitudes of mIPSCs either in WT (from 64.9 ± 8.4 pA to 59.3 ± 6.1 pA) or in KOR KO mice (from 40.0 ± 5.2 pA to 42.0 ± 5.6 pA, data not shown). These results further support the suggestion that CRF activates the KOR system, which then reduces the CRF effect on IPSCs in CeA.

Of the two major CRF receptors that have been identified, blockade of the CRF type 1 receptor (CRFR1) is associated with decreased stress responsiveness and decreased ethanol intake (Funk et al., 2007; Lowery et al., 2010), whereas activation of the CRF type 2 receptor (CRFR2) is associated with decreased ethanol intake (Funk and Koob, 2007; Lowery et al., 2010). In the present experiments we examined the subtype of CRF receptors involved in the CRF-KOR interaction using female C57 mice from Charles River Laboratories. In the presence of the CRFR1 antagonist NBI-27914 (100 nM), CRF no longer significantly ($n = 5$ neurons from three mice, $P = 0.27$) affected the mean frequencies of mIPSCs in the control condition: Mean frequency increase was only 2.3% of control (from 9.0 ± 0.8 Hz to 9.21 ± 0.9 Hz; Fig. 3). Although mean mIPSC frequency with NBI-27914 pretreatment was somewhat higher than without pretreatment (Figs. 1 and 2), this difference was not statistically significant. In addition, CRF in the presence of NBI-27914 did not significantly affect the mean frequency of mIPSCs even in the norBNI pretreated condition ($n = 5$, $P = 0.52$): Mean frequency decreased by -0.6% from 9.1 ± 1.4 Hz to 9.1 ± 1.5 Hz. There was no significant difference in the CRF effect between the control

and norBNI-pretreated conditions (Fig. 3, B and C). Similarly, in the presence of NBI-27914, CRF did not significantly change the amplitudes of mIPSCs alone (from 45.7 ± 13.1 pA to 45.6 ± 13.3 pA) or with norBNI pretreatment (from 53.6 ± 16.2 pA to 46.4 ± 5.6 pA, (data not shown)). On the other hand, in the presence of the CRFR2 antagonist astressin 2B (100 nM), CRF still significantly increased the mean frequencies of mIPSCs in the control condition ($n = 5$ neurons from three mice, $P < 0.01$) by 29.7%: from 8.4 ± 2.4 Hz to 10.45 ± 2.6 Hz (Fig. 4). In addition, CRF also significantly increased the mean frequency of mIPSCs in the presence of norBNI ($n = 5$, $P < 0.01$), by 51.3% (from 7.8 ± 2.1 Hz to 11.2 ± 2.4 Hz). The effect of CRF on mean mIPSC frequency in the presence of astressin 2B was not statistically different from that in the absence of astressin 2B. As in the control condition, in the presence of astressin 2B the magnitude of the CRF effect was significantly greater with norBNI in the bath than with CRF alone ($P = 0.03$) (Fig. 4, B and C). By contrast, the mean amplitudes of mIPSCs were not significantly different with CRF treatment either in astressin 2B alone (from 61.0 ± 4.5 pA to 67.9 ± 11.1 pA) or in astressin 2B plus norBNI (from 56.2 ± 8.1 pA to 64.2 ± 9.5 pA). These results suggest that the enhanced release of GABA (and dynorphin, the endogenous KOR agonist; see below) by CRF is mediated by CRFR1 but not by CRFR2 receptors.

Discussion

In this study, we observed that pharmacological blockade of KORs by norBNI or genetic deletion of KORs enhanced the response to CRF in the CeA. Specifically, blockade or loss of KORs enhanced the effectiveness of CRF to increase

Fig. 2. The genetic deletion of KORs increases the effect of CRF to enhance mIPSC frequency in CeA neurons. Representative mIPSC traces are shown in (A); traces from WT mice and KOR KO mice are shown in (A), a and b, respectively, and each panel shows traces before, during, and after bath application of CRF. In (B), the cumulative probability plot of interpulse intervals of mIPSCs was compared before and during CRF application in slices from WT mice (A) and KOR KO mice (B). The averaged percentage changes in mIPSC frequencies are presented in (C), showing that percentage change in mIPSC frequency in neurons from KOR KO mice is significantly greater than that in WT mice.

presynaptic GABA release. Our data suggest that the KOR system in the CeA is activated by exogenous CRF, probably owing to release of the putative endogenous ligand dynorphin,

as has been previously reported in the amygdala and other brain regions (Chavkin et al., 1982; Sirinathsinghji et al., 1989; Song and Takemori, 1992; Bruchas et al., 2009; Lam and

Fig. 3. CRF R1 antagonism abolishes the CRF-KOR interaction. Pretreatment with the CRFR1 antagonist NBI-27914 not only blocked the CRF effect on mIPSC frequency in the control condition but also blocked the enhancement of the CRF effect in the presence of the KOR antagonist. The representative mIPSC traces are shown in (A); traces from the NBI-27914-alone-pretreated condition and both NBI-27914 and norBNI-pretreated condition are shown in (A), a and b, respectively, and each panel shows traces before, during, and after bath application of CRF. In (B), the cumulative probability plot of interpulse intervals of mIPSCs was compared before and during CRF application in the NBI-27914-pretreated condition (A) and NBI-27914 plus norBNI-pretreated condition (B). The averaged percentage changes in mIPSC frequencies in NBI-27914-pretreated condition (closed columns) are presented in (C) compared with control result (open columns).

Fig. 4. CRF R2 antagonism did not affect the CRF-KOR interaction. Pretreatment with the CRFR2 antagonist astressin 2B did not block the CRF effect on mIPSC frequency in the control condition, and did not block the enhancement of the CRF effect by KOR antagonism. Representative mIPSC traces are shown in (A); traces from the astressin 2B-alone-pretreated condition and both astressin 2B- and norBNI-pretreated condition are shown in (A), a and b, respectively, and each panel shows traces before, during, and after bath application of CRF. In (B), the cumulative probability plot of interpulse intervals of mIPSCs was compared before and during CRF application in the astressin 2B-pretreated condition (A) and astressin 2B plus norBNI-pretreated condition (B). The averaged percentage changes in mIPSC frequencies in NBI-27914 pretreated condition (closed columns) are presented in (C) compared with control results (open columns).

Gianoulakis, 2011). However, a microdialysis study of CRF effects in CeA suggested that dynorphin release was mediated by CRF2 rather than CRF1 receptors (Lam and Gianoulakis, 2011). The latter observation raises the possibility that CRF2 receptors mediate dynorphin release, whereas CRF1 receptors enhance GABA release. Our laboratory and others have previously shown that the CRF and KOR systems modulate GABAergic inhibitory neurotransmission in an opposite manner in the CeA: KOR activation decreases GABA neurotransmission (Kang-Park et al., 2013), whereas CRF activation increases this neurotransmission (Nie et al., 2004). If the KOR system is activated downstream from CRF receptors, KOR-mediated mechanisms may compensate for the CRF-mediated increase of the inhibitory synaptic neurotransmission in the control condition, whereas blockade of KORs would exaggerate the CRF-mediated increase of this inhibitory neurotransmission (Figs. 1 and 2). By contrast, if the CRF system is independent of the KOR system or acts downstream from KORs, exogenous CRF-evoked responses should not be different between control and KOR blockade conditions. In addition, we observed that the KOR-mediated CRF response is abolished by the CRFR1 antagonist NBI-27914 but not by the CRFR2 antagonist astressin 2B, suggesting that CRFR1-mediated mechanisms underlie this particular CRF-KOR interaction. However, the observation that activation of either CRF or kappa receptors produces anxiogenic effects (Land et al., 2008; Keen-Rhinehart et al., 2009) suggests that the CeA contains discrete circuitries mediating both anxiolytic and anxiogenic effects of neuropeptides and that these circuits are differentially sensitive to CRF and dynorphin. We propose this possibility to account for the behavioral similarities of CRF-R1 and kappa receptor activation in the context of

apparent opposite actions on terminal GABA release in the CeA.

In a previous study, we observed that ethanol activates the KOR system in the CeA in a manner similar to the exogenous CRF effect found in the present study, and ethanol enhancement of inhibitory synaptic transmission was greater in KOR KO mice than WT mice (Kang-Park et al., 2013). Because ethanol is known to release CRF in the CeA (Nie et al., 2004), and ethanol's effect in modulating inhibitory neurotransmission is mediated through the CRF system (Nie et al., 2004), we propose that ethanol activation of the KOR system is mediated by the sequential activation through the CRF system, although we are still testing this possibility. Further, the CRF system may be critical for KOR actions, as a CRF1-R antagonist can block KOR-mediated reinstatement of alcohol seeking (Funk et al., 2014) or cocaine seeking (Valdez et al., 2007). However, the suggestion that the KOR system is dependent on CRF may be overly simplistic, as mice lacking CRF1 receptors still exhibit KOR-dependent place aversion (Contarino and Papaleo, 2005). As noted above, it may be possible that the release of dynorphin, the putative endogenous KOR agonist, is mediated by CRF2 receptors (Lam and Gianoulakis, 2011), whereas release of GABA is mediated by CRF1 receptor activation (Nie et al., 2004; Roberto et al., 2010).

The functional significance of the CRF-KOR interaction remains to be determined. The characteristics of reciprocal CRF-KOR interactions could be compensatory or regulatory. Zhou et al. (2013) reported differential ethanol regulation of CRF and dynorphin mRNA levels: chronic ethanol decreased CRF mRNA but increased dynorphin mRNA. Therefore, the functional significance of these CRF-KOR interactions could result from different permutations of responses to their combination.

Currently, there are few studies on CRF-KOR interactions at the cellular level. Behavioral and molecular studies suggest a sequential connection between the CRF and KOR systems in the amygdala (Bruchas et al., 2009), and our study expands such a connection into the functional level in the CeA. This information could inform the development of more efficient therapeutic strategies for anxiety disorders and for drug and alcohol abuse. Thus, future pharmacotherapies may be improved by combining agents acting simultaneously on CRF and kappa opioid systems.

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Authorship Contributions

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

Conducted experiments: Kang-Park.

Contributed new reagents or analytic tools: Kieffer, Roberts.

Performed data analysis: Kang-Park.

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

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