Pro- and Anti-Nociceptive Effects of Corticotropin-Releasing Factor (CRF) in Central Amygdala Neurons Are Mediated Through Different Receptors

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Ji G, Neugebauer V. Pro- and anti-nociceptive effects of corticotropin-releasing factor (CRF) in central amygdala neurons are mediated through different receptors. J Neurophysiol 99: 1201–1212, 2008. First published January 2, 2008; doi:10.1152/jn.01148.2007. Corticotropin-releasing factor (CRF) is not only a stress hormone but also acts as a neuromodulator outside the hypothalamic-pituitary-adrenocortical axis, playing an important role in anxiety, depression, and pain modulation. The underlying mechanisms remain to be determined. A major site of extra-hypothalamic expression of CRF and its receptors is the amygdala, a key player in affect-related disorders such as anxiety. The latero-capsular division of the central nucleus of the amygdala (CeLC) is also important for pain modulation and pain affect. This study analyzed the effects of CRF on nociceptive processing in CeLC neurons and the contribution of CRF1 and CRF2 receptors and protein kinases A and C. Extracellular single-unit recordings were made from CeLC neurons in anesthetized adult rats. All neurons responded more strongly to noxious than innocuous mechanical stimulation of the knee. Evoked responses and background activity were measured before and during administration of CRF into the CeLC by microdialysis. CRF was administered alone or together with receptor antagonists or protein kinase inhibitors. CRF (0.01–1 μM; concentrations in microdialysis probe; 15 min) facilitated the evoked responses more strongly than background activity; a higher concentration (10 μM) had inhibitory effects. Facilitation by CRF (0.1 μM) was reversed by a selective CRF1 receptor antagonist (NB27914, 10 μM) but not a CRF2 receptor antagonist (astressin-2B, 100 μM) and by a protein kinase A (PKA) inhibitor (KT5720, 100 μM) but not a protein kinase C inhibitor (GF109203X, 100 μM). Inhibitory effects of CRF (10 μM) were reversed by astressin-2B. These data suggest that CRF has dual effects on amygdala neurons: CRF receptor-mediated PKA-dependent facilitation and CRF2 receptor-mediated inhibition.

INTRODUCTION

Corticotropin-releasing factor (CRF) not only mediates stress responses but also acts as a neuromodulator of synaptic transmission outside of the hypothalamic-pituitary-adrenocortical (HPA) axis. CRF plays an important role in emotional processes and psychiatric disorders such as anxiety and depression through a circuitry that includes the amygdala as a key element (Asan et al. 2005; Bale and Vale 2004; Dautzenberg and Hauger 2002; Gray 1993; Reul and Holsboer 2002; Takahashi 2001). The amygdala is a major site of extra-hypothalamic expression of CRF in cell bodies and terminals and contains CRF1 and, to a lesser extent, CRF2 receptors (Dautzenberg and Hauger 2002; Gray 1993; Reul and Holsboer 2002; Sanchez et al. 1999). CRF acts on both CRF1 and CRF2 receptors, but CRF1 receptors have emerged as drug targets for depression and anxiety disorders (Bale and Vale 2004; Charney 2003; Dautzenberg and Hauger 2002; Reul and Holsboer 2002; Tache and Bonaz 2007; Takahashi 2001). CRF1 receptor antagonists have been used successfully in humans to treat depression and anxiety (Kunzel et al. 2005; Zobel et al. 2000).

Accumulating evidence suggests that peripheral and central CRF is also an important pain modulator. Recent biochemical (Greenwood-Van Meerveld et al. 2006; Sinniger et al. 2004; Ulrich-Lai et al. 2006), behavioral (Lariviere and Melzack 2000; McNally and Akil 2002) and electrophysiological studies (Ji and Neugebauer 2007) point to the amygdala as an important site of CRF-mediated pain modulation. Long known as a key player in emotional-affective behavior (Maren 2005; Phelps and Ledoux 2005), the amygdala has emerged as an important brain center involved in the emotional-affective dimension of pain and pain modulation (Carrasquillo and Gereau 2007; Fields 2004; Gauriau and Bernard 2004; Heinricher and McGarvaughy 1999; Ikeda et al. 2007; Neugebauer 2006; Neugebauer et al. 2004; Pedersen et al. 2007; Rhudy and Meagher 2001). Pain not only carries a negative affective valence but is also intimately related to anxiety disorders and depression (Gallagher and Verma 2004; Rome and Rome 2000). The amygdala is believed to be an important substrate for the reciprocal relationship between pain and affective state (Ji et al. 2007; Meagher et al. 2001; Myers et al. 2005; Neugebauer et al. 2004; Rhudy and Meagher 2003). The implication of the amygdala and CRF in pain as well as anxiety sparked our interest in a detailed analysis of the CRF system in the amygdala.

The literature on pain-related CRF functions in the CNS, including the amygdala, is controversial. In the broader context of stress-induced analgesia, older studies observed anti-nociceptive effects of centrally (intracerebroventricularly, icv) administered CRF in phasic pain tests. The majority of studies, however, found no effect; one study reported hyperalgesic effects and others measured excitatory effects in several brain areas (reviewed by Lariviere and Melzack 2000). A recent study (Vit et al. 2006) showed anti-nociceptive effects of CRF (icv) on paw withdrawal thresholds and on the interphase of the formalin test, but CRF also increased pain-related vocalizations and the number of Fos immunopositive spinal neurons. A nonselective antagonist had opposite effects. The reason for the heterogeneity of CRF effects is not clear. It was pointed out that the effective dose range for CRF-induced analgesia was narrow and that lower doses of CRF (icv) increased behavioral

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activities, whereas higher doses had inhibitory effects (see Lariviere and Melzack 2000). This would be consistent with the higher affinity of CRF for CRF1 than CRF2 receptors and with divergent roles of these receptors (Arzt and Holsboer 2006; Blank et al. 2003; Dautzenberg and Hauger 2002).

An increasing number of studies now suggest that CRF1 receptors mediate pro-nociceptive effects of CRF, whereas CRF2 receptors serve anti-nociceptive functions (Martinez et al. 2004; Million et al. 2003, 2006; Nijsen et al. 2005; Tache and Bonaz 2007; Tache et al. 2004). CRF (icv) increased visceromotor responses indicating visceral hypersensitivity (Greenwood-Van Meerveld et al. 2005; Gue et al. 1997), which was blocked by a centrally administered nonselective CRF receptor antagonist (Gue et al. 1997) or by a systemically administered CRF1-selective antagonist (Greenwood-Van Meerveld et al. 2005). Microinjections of a nonselective CRF receptor antagonist into the amygdala reversed opiate withdrawal-induced hyperalgesia measured in the tail-flick test (McNally and Akil 2002). A CRF1-selective antagonist inhibited the increased visceromotor responses of rats with stereotaxic delivery of corticosterone to the amygdala (Myers et al. 2005). CRF1 receptor-deficient mice showed decreased anxiety and visceromotor responses (Trimble et al. 2007). CRF mRNA expression in the amygdala increased in models of colitis pain (Greenwood-Van Meerveld et al. 2006) and neuropathic pain (Urich-Lai et al. 2006). A recent study from our lab showed that administration of a selective CRF1 receptor antagonist into the amygdala inhibited central sensitization of amygdala neurons in a model of arthritic pain; a CRF2 receptor antagonist had no effect in this pain model (Ji and Neugebauer 2007).

Despite strong evidence for a role of the amygdala CRF system in pain modulation, it is not clear which CRF receptors and signaling mechanisms mediate the differential effects of CRF. In fact, the effect of CRF on nociceptive processing in individual amygdala neurons is not known. The amygdala consists of several nuclei. The lateral (LA), basolateral (BLA), and central nuclei (CeA) are particularly important for sensory processing (Neugebauer 2006; Phelps and Ledoux 2005; Price 2003). The LA receives polymodal sensory, including nociceptive, inputs from thalamic nuclei and cortical areas. Through associative processing in the LA-BLA circuitry, affective content is attached to sensory information and then transmitted to the CeA, a major output nucleus for amygdala functions (Maren 2005; Phelps and Ledoux 2005). The laterocapsular division of the CeA (CeLC) also receives nociceptive-specific information directly (not processed by thalamus or cortex) from the parabrachial area through the spino-parabrachio-amygdaloid pain pathway (Gauriau and Bernard 2004). Importantly, CRF containing neurons have been detected in the parabrachial area (Merchenthaler et al. 1982) and could serve as a source of CRF in the CeLC. The two principal types of CeLC neurons are nociceptive-specific (NS) neurons, which receive exclusively nociceptive input, and multireceptive (MR) neurons, which respond to innocuous and noxious stimuli and integrate nociceptive signals with affective information from the LA-BLA circuitry (Neugebauer 2006; Neugebauer et al. 2004). MR neurons, but not NS neurons, undergo central sensitization in the arthritis pain model (Neugebauer and Li 2003). Therefore the present study focused on MR neurons of the CeLC.

The present study shows that low concentrations of CRF facilitate nociceptive processing in CeLC neurons through CRF1 receptors and protein kinase A (PKA) activation. In contrast, higher concentrations of CRF have inhibitory effects mediated through CRF2 receptors, which is consistent with the emerging concept that CRF2 receptors serve to dampen or reverse CRF1-initiated responses (Tache and Bonaz 2007).

METHODS

Adult male Sprague Dawley rats (250–350 g) were housed in a temperature-controlled room and maintained on a 12-h day/night cycle. Water and food were available without restriction. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch and conform to the guidelines of the International Association for the Study of Pain and of the National Institutes of Health.

Animal preparation and anesthesia

On the day of the electrophysiological experiment, the animal was anesthetized with pentobarbital sodium (50 mg/kg ip). A cannula was inserted into the trachea for artificial respiration and to measure end-tidal CO2 levels. A catheter was placed in the jugular vein for continuous administration of anesthetic and for fluid support (3–4 ml·kg⁻¹·h⁻¹ iv lactated Ringer solution). Depth of anesthesia was assessed by testing the corneal blink, hindpaw withdrawal, and tail-pinch reflexes and by continuously monitoring the end-tidal CO2 levels (kept at 4.0 ± 0.2%; mean ± SE), heart rate, electrocardiogram (ECG) and breathing patterns. Core body temperature was maintained at 37°C by means of a homeothermic blanket system. Animals were mounted in a stereotaxic frame, paralyzed with pancuronium (induction: 0.3–0.5 mg iv; maintenance: 0.3 mg/hr iv) and artificially ventilated (3–3.5 ml; 55–65 stroke/min). Constant levels of anesthesia were maintained by continuous intravenous infusion of pentobarbital (15 mg·kg⁻¹·h⁻¹). A unilateral craniotomy was performed at the sutura frontoparietalis level for the recording of CeLC neurons and for the administration of drugs into the CeLC. The dura mater was opened and reflected; the pia mater was removed over the recording and drug administration sites to allow smooth insertion of the recording electrode and microdialysis probe, respectively.

Electrophysiological recording

As described previously (Han et al. 2005b; Ji and Neugebauer 2007; Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2002, 2003), extracellular recordings were made from single neurons in the CeLC with glass insulated carbon filament electrodes (4–6 MΩ) using the following stereotaxic coordinates (Paxinos and Watson 1998): 2.1–2.8 mm caudal to bregma; 3.8–4.5 mm lateral to midline; depth 7–9 mm. The recorded signals were amplified and displayed on analog and digital storage oscilloscopes. Signals were also fed into a window discriminator the output of which was processed by an interface (CED 1401 Plus) connected to a Pentium 4 PC. Spike2 software (CED, version 4) was used to create peristimulus rate histograms on-line and to store and analyze digital records of single-unit activity off-line. Spike size and configuration were continuously monitored on the storage oscilloscopes and with the use of Spike2 software.

Identification of amygdala neurons

An individual CeLC neuron was identified by its background activity and by its responses to brief mechanical stimuli applied to the knee with a calibrated forceps (see MECHANICAL STIMULI). While the recording electrode was slowly advanced through the CeLC, brief
search stimuli of innocuous intensity were applied every 30–50 μm (about once every 10 s). Innocuous stimuli are sufficient to identify MR neurons (see CLASSIFICATION AND RESPONSE THRESHOLDS). In this study, we included only MR neurons because they consistently and reliably become sensitized in the arthritis pain model (Han et al. 2005b; Ji and Neugebauer 2007; Li and Neugebauer 2004b; Neugebauer and Li 2003) and are believed to integrate nociceptive and affective information (Neugebauer 2006; Neugebauer et al. 2004). Configuration, shape, and height of the recorded action potentials (skips) were monitored and recorded continuously, using a window discriminator and Spike2 software for on- and off-line analysis. Skips were detected and recorded by the waveform signal that crossed a trigger level and matched a preset shape or template that was created for the individual neuron at the beginning of the recording period. Included in this study were only those neurons the spike configuration of which remained constant (matching the template) and could be clearly discriminated from activity in the background throughout the experiment, indicating that the activity of one and the same one neuron was measured.

**Experimental protocol**

In each experiment, one CeLC neuron was recorded before and during drug administration into the CeLC (see Drugs). Background activity was recorded for ≥10 min to calculate means ± SE and 95% confidence intervals (CI; GraphPad Prism 3.0). Before and during drug applications, intervals between the test stimuli were 5 min. Number of stimulations was kept at a minimum to avoid any “sensitization” that might be produced by repeated stimulation. Throughout the experiment we carefully monitored several physiological parameters (body temperature, heart rate, ECG, and endtidal CO2 levels) to ensure a stable recording situation (see Animal preparation and anesthesia).

**Drugs**

hrCRF (human, rat CRF) was purchased from Bachem California, Torrance, CA. 5-chloro-4-[(N-cyclopropyl)methyl-7-propylamino]-2-methyl-6-(2,4,6-trichlorophenyl) amino-pyridine (NBI 27914; CRF1 receptor antagonist) (Hoare et al. 2003; Ji and Neugebauer 2007; Pollandt et al. 2006) was purchased from Tocris Bioscience, Ellisville, MO. Cyclo(31–34) [t-Phe11, His12, CaMeLeu13,19, Nle17, Glu31, Lys34] Ac-Sauvagine (8–40) (astressin-2B; CRF2 receptor antagonist) (Hoare et al. 2005; Ji and Neugebauer 2007; Pollandt et al. 2006; Rivier et al. 2002) was purchased from Sigma, St. Louis, MO. (9R,10S,12S)-2,3,9,10,11,12-hexahydro-9-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-f:3′,2′,1′-kl]pyrrolo[3,4-ij]1,6benzodiazocine-10-carboxylic acid, hexyl ester (KT5720; PKA inhibitor) and 2-[1-(3-dimethylamino)propyl]indol-3-yl-3-(indol-3-yl) maleimide (GF10923x; PKC inhibitor) (see Bird et al. 2005) were purchased from Tocris Bioscience.

**Drug application by microdialysis**

Known concentrations of drugs were administered into the CeLC by microdialysis. Several hours before the start of the electrophysiological recordings a microdialysis probe (CMA11/Microdialysis; membrane diameter: 250 μm, membrane length: 1 mm) was lowered vertically into the CeLC and positioned stereotaxically anterior and ipsilateral to the recording electrode, using the following coordinates: 1.6 mm caudal to bregma; 4.0 mm lateral to midline; depth of tip 9.0 mm (Han and Neugebauer 2005; Han et al. 2005b; Ji and Neugebauer 2007; Li and Neugebauer 2004a,b, 2006). The distance between microdialysis probe and recording electrode was ~0.5 mm. In some experiments, a microdialysis probe was inserted into the striatum as a placement control, using the following stereotaxic coordinates 2.0 mm caudal to bregma; 4.5 mm lateral to midline; depth of tip 7.0 mm. Using PE-50 tubing, the microdialysis probe was connected to an infusion pump (Harvard) and perfused with artificial cerebrospinal fluid (ACSF) containing (in mM) 125.0 NaCl, 2.6 KCl, 2.5 NaH2PO4, 1.3 CaCl2, 0.9 MgCl2, 21.0 NaHCO3, and 3.5 glucose oxygenated and equilibrated to pH = 7.4. Before each drug application, ACSF was pumped through the fiber for ≥10 min to establish equilibrium in the tissue. Continued administration of ACSF did not change the neurons’ baseline activity (see Fig. 2). ACSF also served as a vehicle control.

Drugs were dissolved in ACSF on the day of the experiment at a concentration 100 times that predicted to be needed based on data from our previous studies (Ji and Neugebauer 2007; Ji et al. 2007; Liu et al. 2004) and data in the literature (Pollandt et al. 2006). Drug concentration in the tissue is ≥100 times lower than in the microdialysis probe due to the concentration gradient across the dialysis membrane and diffusion in the tissue (Han and Neugebauer 2005; Han et al. 2005b; Ji and Neugebauer 2007; Li and Neugebauer 2004a,b, 2006). Drugs were administered into the CeLC at a rate of 5 μl/min for 15 min. Different concentrations of CRF were administered in a cumulative fashion. When concentrations were tested individually in some experiments, no difference was found compared with the cu-
mulative concentration-response data. Background and evoked activity were measured every 5 min during drug application. The numbers given in this article refer to the drug concentrations in the microdialysis fiber.

Histology
At the end of each experiment the recording site in the CeLC was marked by injecting DC (250 μA for 3 min) through the carbon filament recording electrode. The brain was removed and submerged in 10% formalin and potassium ferrocyanide. Tissues were stored in 20% sucrose before they were frozen sectioned at 50 μm. Sections were stained with Neutral Red, mounted on gel-coated slide, and covered with a cover slip. The boundaries of the different amygdala nuclei were easily identified under the microscope. Lesion/recording sites were verified histologically and plotted on standard diagrams adapted from Paxinos and Watson (1998) (see Fig. 1).

Data analysis
Extracellularly recorded single-unit action potentials were analyzed off-line from peristimulus rate histograms using Spike2 software (CED, version 3). Responses to mechanical stimuli were measured and expressed as spikes per s (hertz). Background activity was subtracted from the total activity during the stimulus to obtain the “net” stimulus-evoked activity. The net firing rate throughout each stimulus was averaged to obtain the evoked response. Concentration-response relationships were measured for each neuron and then averaged across the sample of neurons. Sigmoid curves fitted to the cumulative concentration-response data by nonlinear regression using the formula

\[ y = A + \frac{(B - A)}{\left(1 + \left(\frac{C}{D}\right)^{X}\right)} \]

where

- \( A \) = bottom plateau,
- \( B \) = top plateau,
- \( C \) = log(EC50),
- \( D \) = slope coefficient,
- \( X \) = logarithm of concentration (Prism 3.0, GraphPad Software).

Concentration-response functions were analyzed statistically using a repeated-measures ANOVA followed by Dunnett’s multiple comparison test to compare the effect of individual drug concentrations to predrug control values (GraphPad Prism 3.0). The highest concentration of CRF, which was not tested in all neurons, was compared with predrug controls using a paired t-test. A repeated-measures ANOVA followed by Newman-Keuls multiple comparison test was used to determine the significance of effects of coapplications of CRF with antagonists or inhibitors compared with CRF alone and to predrug control values. All averaged values are given as the means ± SE. Statistical analysis was performed on the raw data (firing rate measured as spikes per second). Statistical significance was accepted at the level \( P < 0.05 \).

RESULTS
Sample of CeLC neurons
Extracellular single-unit recordings were made from 39 neurons in the laterocapsular division of the central nucleus of the amygdala (CeLC) in 19 anesthetized rats (recording sites are shown in Fig. 1). All neurons in this study were MR neurons, which responded significantly to innocuous but more strongly to noxious stimuli (see CLASSIFICATION AND RESPONSE THRESHOLDS). Individual examples are shown in Figs. 2, Figs. 4 Figs. 5 Figs. 6 Figs. 8 and Figs. 9. In agreement with our previous studies (Ji and Neugebauer 2007; Li and Neugebauer 2004b; Neugebauer and Li 2002, 2003), neurons with knee joint input typically had symmetrical receptive fields in the deep tissue of both hind limbs, including the area from the hip to the ankle; some neurons had additional receptive fields in the paw \((n = 11)\) and tail \((n = 9)\).
Concentration-dependent facilitatory and inhibitory effects of CRF

The responses of a CeLC neuron to innocuous (500 g/30 mm²) and noxious (2,000 g/30 mm²) stimulation of the knee and background activity remained constant during a control period of 45 min before drug administration (Fig. 2A). In the control period, ACSF was administered by microdialysis into the CeLC as a vehicle control (see METHODS). Administration of ascending concentrations of CRF (0.01, 0.1, and 1.0 µM, concentration in microdialysis fiber; 15 min each) into the CeLC increased the neuron’s evoked responses but not background activity. The neuron’s activity remained stable for a control period of 45 min before drug application (Fig. 2A). In the control period, ACSF was administered as a vehicle control before and during administration of ascending concentrations of CRF. Traces in B–E show recordings of the force (g/30 mm²) applied to the knee joint with a calibrated forceps (see METHODS).

Positive control material

A selective CRF1 receptor antagonist (NBI27914, NBI; 10 µM, concentration in microdialysis fiber) reversed the facilitatory effect of CRF (0.1 µM). Figure 4 shows an individual example. Administration of CRF into the CeLC increased the responses of this CeLC neuron to innocuous and noxious stimulation of the knee. NBI coapplied with CRF decreased the evoked activity (P < 0.05, paired t-test; Fig. 3, A and B) but not background activity (Fig. 3C). All averaged values are given as the means ± SE and are expressed as percent of predrug control values (set to 100%). Evoked activity was calculated by subtracting background activity preceding the stimulus from the total activity during stimulation.

CRF (0.1 and 1.0 µM) also increased the receptive field size (n = 7 neurons). New receptive fields appeared in the paw that was not part of the original receptive field in two neurons, trunk (abdomen, n = 7; thorax, n = 3), and forearm (n = 3). CRF1 receptor-mediated facilitation and CRF2 receptor-mediated inhibition

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neuron’s responses (Fig. 4A, time course; B–D, individual recordings of stimulus force and neuronal activity before and during drug applications).

A selective CRF2 receptor antagonist (astressin-2B, A-2B; 100 μM, concentration in microdialysis fiber) did not affect the facilitation by CRF (0.1 μM). An individual example is shown in Fig. 5. The evoked responses of this CeLC neuron increased during administration of CRF into the CeLC. The facilitation persisted during coapplication of A-2B (Fig. 5A, time course; B–D, individual recordings before and during drug applications).

The inhibitory effects of a higher concentration of CRF (10 μM) were reversed by a selective CRF2 receptor antagonist (A-2B; 100 μM). Figure 6 shows an individual example. CRF inhibited the neuron’s responses to innocuous and noxious stimulation of the knee. A-2B coapplied with CRF increased the neuron’s responses (Fig. 6A, time course; B–D, individual recordings before and during drug applications).

Figure 7 summarizes the effects of CRF1 and CRF2 antagonists on CRF-induced facilitation (Fig. 7, A and B) and inhibition (C). CRF (0.1 μM) increased the responses of CeLC neurons (n = 5) to innocuous and noxious stimulation of the knee significantly (P < 0.05, Newman-Keuls test after repeated-measures ANOVA; Fig. 7, A and B). Coapplication of a CRF1 receptor antagonist (NBI; 10 μM, n = 5) significantly reversed the CRF-induced facilitation (P < 0.05, Newman-Keuls post test; Fig. 7A). A CRF2 receptor antagonist (A-2B; 100 μM, n = 5) had no significant effect on the CRF-induced facilitation, i.e., the responses were still facilitated in the presence of the CRF2 receptor antagonist compared with predrug control values (P < 0.05, Newman-Keuls post test; Fig. 7B). A higher concentration of CRF (10 μM, n = 6) inhibited the evoked responses significantly (P < 0.05, Newman-Keuls post test). CRF-induced inhibition was reversed by A-2B (100 μM, n = 6) significantly (P < 0.05, Newman-Keuls post test).

These data suggest that CRF-induced facilitation of evoked responses is mediated through CRF1 receptors, whereas the inhibitory action of CRF involves CRF2 receptors.

**Protein kinase A (PKA), but not protein kinase C (PKC), mediates CRF-induced facilitation**

A selective PKA inhibitor (KT5720, KT; 100 μM, concentration in the microdialysis fiber; 15 min) inhibited the facilitatory effects of CRF (0.1 μM, 15 min). Figure 8 shows an individual example. Administration of CRF into the CeLC increased the responses of this CeLC neuron to innocuous and noxious stimulation of the knee. Coapplication of KT reversed the effects of CRF (Fig. 8A, time course; B–D, individual recordings of stimulus force and neuronal activity before and during drug applications).

A PKC inhibitor (GF109203x, GF; 100 μM, concentration in microdialysis fiber; 15 min) did not change the effects of CRF. Figure 9 shows recordings of an individual CeLC neuron. Administration of CRF (0.1 μM, concentration in microdialysis fiber; 15 min) increased the evoked responses. Co-application of GF did not block the effects of CRF (Fig. 9A, time course; B–D, individual recordings before and during drug applications).

Figure 10 summarizes the effects of PKA and PKC inhibitors on CRF-induced facilitation. CRF (0.1 μM, 15 min)
significantly reversed the CRF-induced facilitation (A). Intra-striatal administration of CRF alone (0.1 μM, concentration in microdialysis fiber; 15 min) inhibited the neuron’s responses to innocuous and noxious stimuli. Co-application of CRF (10 μM) and A-2B (100 μM, 15 min) reversed the inhibition. Each symbol shows the mean background or net evoked activity (see Fig. 2 and METHODS). B–D, peristimulus time histograms show action potentials (spikes) per second before (predrug control in ACSF, B) and during administration of CRF alone (C) and together with A-2B (D). Traces are recordings of the stimulus force (see METHODS).

increased the evoked responses of CeLC neurons (n = 6) significantly. Coapplication of KT (100 μM, n = 6; Fig. 10A) significantly reversed the CRF-induced facilitation (P < 0.05, Newman-Keuls test after repeated-measures ANOVA). GF (100 μM, n = 6) had no significant effect (Newman-Keuls post test). The data suggest that CRF-induced facilitation requires PKA but not PKC.

Placement controls

As a control for any drug effects due to diffusion from the microdialysis probe to other brain areas, microdialysis probes were stereotaxically inserted into the striatum (caudate-putamen) for drug application in a separate set of experiments (Fig. 11). The striatum was selected because it is located adjacent (dorsolateral) to the CeLC but does not form direct projections to the CeLC (Alheid et al. 1995). Thus drug application into this area should not produce any effect in the CeLC. We used the striatum as a placement control successfully in our previous studies (Han and Neugebauer 2005; Han et al. 2005b). Intrastriatal administration of CRF alone (0.1 μM, 15 min) and of CRF together with NBI (10 μM, 15 min) had no significant effect on the evoked and background activity of a CeLC neuron (Fig. 11A). The placement control data for the sample of neurons (n = 4) are summarized in Fig. 11B.

DISCUSSION

The key novelty of this study is that CRF has divergent effects on nociceptive transmission in amygdala neurons through concentration-dependent activation of different receptors. Low concentrations of CRF facilitate nociceptive processing through CRF1 receptors, whereas higher concentrations have inhibitory effects through CRF2 receptors. This result is significant because the literature has been controversial with regard to the role of the central CRF system in pain modulation. Strong evidence exists for pro- as well as anti-nociceptive effects (see INTRODUCTION).

Evidence for opposing but complimentary functions of CRF1 and CRF2 receptors is accumulating (Arzt and Holsboer 2006; Blank et al. 2003; Dautzenberg and Hauger 2002; Tache and Bonaz 2007). The anxiety-related literature suggests that CRF produces anxiogenic effects through CRF1 receptors, whereas CRF2 receptors serve to counterbalance these effects (Bale and Vale 2004; Charney 2003; Dautzenberg and Hauger 2002; Reul and Holsboer 2002; Tache and Bonaz 2007; Takahashi 2001). Our data support a similar scenario for pain mechanisms involving the CRF system in the amygdala.
previous study (Ji and Neugebauer 2007) showed that blockade of CRF1 receptors in the amygdala inhibited central sensitization of amygdala neurons in the arthritis pain model, suggesting the endogenous activation of CRF1 receptors has nociceptive effects. In contrast, a CRF2 receptor antagonist produced facilitation under normal conditions but had no effect in the arthritis pain model, suggesting inhibitory functions of endogenously activated CRF2 receptors under certain conditions.

It is not clear if the opposing functions of CRF1 and CRF2 receptors in pain and in other behavioral states and disorders involve different ligands or the same molecule (CRF). CRF can activate both receptor types but has a 10- to 40-fold higher affinity for CRF1 than CRF2 (Tache et al. 2004). The urocortin 2 and urocortin 3 members of the family of CRF-related peptides are selective CRF2 receptor ligands (Bale and Vale 2004; Dautzenberg and Hauger 2002; Martinez et al. 2004; Reyes et al. 2001; Tache and Bonaz 2007). The present study shows that low concentrations of CRF facilitate nociceptive processing through CRF1 receptors, whereas the anti-nociceptive effect of a higher concentration is mediated through CRF2 receptors. The mechanisms underlying the facilitatory and inhibitory effects remain to be determined. Because both receptors can couple to similar signal transduction pathways, we hypothesized previously (Ji and Neugebauer 2007) that CRF2 receptors could activate the well-documented powerful inhibitory circuits in the amygdala (Pure et al. 2004; Woodruff and Sah 2007), whereas CRF1 receptors regulate excitatory processes. Therefore the overall net effect of CRF receptor activation would be determined by the relative dominance of excitatory and inhibitory mechanisms. For example, the high concentration of CRF produced inhibition through CRF2 receptors in this study, although CRF1 receptors can be expected to be occupied by CRF at that concentration, suggesting that the activation of inhibitory processes through CRF2 receptors can override the facilitatory effects of CRF1 receptor activation.

CRF2-mediated inhibitory effects of CRF are consistent with the results of previous studies that showed anti-nociceptive effects of centrally administered CRF at a narrow dose range and/or higher concentrations (reviewed in Lariviére and Melzack 2000). It is interesting that urocortin 2, a CRF2 receptor ligand, had anti-nociceptive effects on visceral pain-related behavior (visceromotor responses) (Martinez et al. 2004; Million et al. 2006). A CRF2 receptor antagonist blocked the anti-nociceptive effects of intrathecally administered CRF on visceromotor responses (Nijsen et al. 2005). The results of another study (Vit et al. 2006) are more complicated. Centrally administered CRF increased the nociceptive threshold but also increased vocalizations in response to noxious stimuli; and CRF increased the number of activated (Fos positive) spinal neurons in the superficial and deep dorsal horn. These data suggest opposing effects of central CRF.

Evidence for CRF1 receptor-mediated pro-nociceptive effects of CRF comes from an increasing number of recent studies (Greenwood-Van Meerveld et al. 2005; Gue et al. 1997; Million et al. 2003; Myers et al. 2005; Nijsen et al. 2005; Tache et al. 2004; Trimble et al. 2007). Centrally administered CRF (i.ev) increased visceromotor responses (Greenwood-Van Meerveld et al. 2005; Gue et al. 1997). This effect was blocked by a systemically administered CRF1 receptor antagonist (Greenwood-Van Meerveld et al. 2005). Systemic administration of a selective CRF1 receptor antagonist that penetrated into the brain and occupied CRF1 receptors blocked stress-
induced visceral hyperalgesia (Million et al. 2003). Increased visceromotor responses following stereotactic delivery of corticosterone to the amygdala was also blocked by a CRF1 receptor antagonist (Myers et al. 2005). CRF1 receptor-deficient mice showed decreased visceromotor responses (Trimble et al. 2007). A recent study from our lab showed that administration of a selective CRF1 receptor antagonist into the amygdala inhibited central sensitization of amygdala neurons in a model of arthritic pain (Ji and Neugebauer 2007).

The differential effects of centrally administered CRF on pain behavior could be due to different sites of action in the brain, particularly after intracerebroventricular injection. However, the present study shows that within one brain area (amygdala) CRF produces facilitatory and inhibitory effects that depend on different receptors. The amygdala is a major site of extra-hypothalamic expression of CRF and its receptors in cell bodies and terminals (Dautzenberg and Hauger 2002; Gray 1993; Reul and Holsboer 2002; Sanchez et al. 1999). CRF has been shown before to increase and decrease neuronal excitability (Rainnie et al. 1992, 2004) and synaptic transmission (Liu et al. 2004; Pollandt et al. 2006) in amygdala neurons in vitro; but a clear picture has yet to emerge.

These opposing neuronal effects observed in the present study can explain pro- and anti-nociceptive effects of central CRF, assuming a positive correlation exists between amygdala activity and pain behavior. It is interesting that the earlier literature emphasized the role of the amygdala in stress- or environmentally induced analgesia, whereas in recent years, it has become increasingly clear that the amygdala can enhance or produce pain behaviors (see references and discussion in Neugebauer 2006, 2007; Neugebauer et al. 2004). Arthritis pain-related behavior was inhibited by decreasing activity of amygdala (CeLC) neurons with antagonists for group I metabotropic glutamate receptors mGlur1 and mGlur5 (Han and Neugebauer 2005; Li and Neugebauer 2004b; Neugebauer et al. 2003) and with calcitonin gene-related Cgrp1 receptor antagonists (Han et al. 2005b). ERK activation in the CeLC induced peripheral hypersensitivity in normal animals and inhibition of ERK activation reduced pain in the formalin pain model (Carrasquillo and Gereau 2007). A GABA$_A$ receptor agonist reduced mechanical hypersensitivity in a neuropathic pain model (Pedersen et al. 2007). Stimulation of the central amygdala (CeA) with corticosterone produced visceral hyper-sensitivity and increased responsiveness of viscerosensitive spinal neurons (Myers et al. 2005; Qin et al. 2003a,b).

Some technical aspects concerning drugs and drug administration need to be considered. This study used hrCRF rather than a ligand that is more selective for CRF1 receptors such as oCRF or stressin-1A (Rivier et al. 2007) because we wanted to determine the actions of the endogenous ligand, and CRF is present in the amygdala at particularly high levels (Dautzenberg and Hauger 2002; Gray 1993; Reul and Holsboer 2002; Sanchez et al. 1999). We also used antagonists selective for CRF1 and CRF2 (Arzt and Holsboer 2006; Hoare et al. 2003, 2005; Rivier et al. 2002) and selective PKA and PKC inhibitors (for references, see Bird et al. 2005; Han et al. 2005b). However, the drug dose administered by microdialysis is not known. On the other hand, microdialysis offers several advantages, including continued drug delivery and steady-state levels without a volume effect (Stiller et al. 2003). The concentration of the drug in the microdialysis fiber is known; based on our previous studies, drug concentration in the tissue is $\approx$100 times lower than in the microdialysis probe due to the concentration gradient across the dialysis membrane and diffusion in the tissue (Han and Neugebauer 2005; Rivier et al. 2002).
system in the amygdala may be a key link between pain and modulation and affective disorders suggests that the CRF system in the amygdala may be a key link between pain and affective states and disorders.

Another advantage of microdialysis is the ability to measure concentration-response functions. The concentration-response analysis in the present and our previous microdialysis studies (Ji and Neugebauer 2007; Ji et al. 2007) and the comparison with data from in vitro experiments in brain slices (see Liu et al. 2004; Pollandt et al. 2006) allowed us to estimate that the effective tissue concentrations of CRF and its receptor antagonists were in the nanomolar range, confirming that the drug concentration in the microdialysis fiber needed to be 100 times higher than the desired tissue concentration (see METHODS) (see also Han et al. 2005b; Ji and Neugebauer 2007; Ji et al. 2007).

Importantly, the differential effects of CRF1 and CRF2 receptor antagonists and PKA and PKC inhibitors in our present and previous studies (Bird et al. 2005; Han et al. 2005b; Ji and Neugebauer 2007) argue against nonselective drug effects at the concentrations used.

Placement control experiments, in which CRF and NB127914 were administered into the striatum (Fig. 11), suggest that the drugs did not spread beyond a distance of 1 mm around the tip of the microdialysis probe, which is consistent with our previous estimates (Li and Neugebauer 2004a,b). The distance between the tips of the microdialysis probes in the CeLC (effective drug administration site) and striatum (ineffective site) is ~2 mm. The striatum was selected because it is located adjacent (dorsolateral) to the CeLC but does not project directly to the CeLC (Alheid et al. 1995). Thus drug application into this area should not produce any effect in the CeLC. We used the striatum as a placement control successfully in our previous studies (Han and Neugebauer 2005; Han et al. 2005b). We also considered the BLA as a site for placement controls. However, the BLA projects heavily to the CeLC. Therefore drug administration into the BLA is not a useful control for drug diffusion because it could affect the responses of CeLC neurons through BLA-CeLC projections.

Another important control includes sufficiently long recording periods without drug application to rule out any systematic changes over time. Our data show that the neurons’ responses did not change during prolonged administration of ACSF (vehicle: Fig. 2). Constant activity was also measured during drug application into the striatum as a placement control (Fig. 11). These results are consistent with our previous studies (Ji and Neugebauer 2007; Li and Neugebauer 2004a, 2006) where stable responses were recorded during long (~2 h) control periods of ACSF administration into the amygdala before the induction of an experimental arthritis.

In summary, the present study shows that opposing effects of CRF on nociceptive processing in the amygdala are mediated through different receptors. The fact that the amygdala has emerged as an important brain center for pain modulation and pain affect and that CRF plays an important role in pain modulation and affective disorders suggests that the CRF system in the amygdala may be a key link between pain and affective states and disorders.

**References**


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