Differential Effects of CRF1 and CRF2 Receptor Antagonists on Pain-Related Sensitization of Neurons in the Central Nucleus of the Amygdala

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INTRODUCTION

Long known as a “stress hormone” in the hypothalamic–pituitary–adrenocortical (HPA) axis, corticotropin-releasing factor (CRF) is emerging as an important neuromodulator involved in emotional processes and psychiatric disorders such as anxiety and depression (Bale and Vale 2004; Reul and Holsboer 2002; Steckler and Holsboer 1999; Takahashi 2001). Pain, including arthritic pain, not only carries a negative affective state (Meagher et al. 2001; Rhudy and Meagher 2003). The amygdala also believed to be a key substrate of the reciprocal relationship between pain and affective state (Meagher et al. 2001; Rhudy and Meagher 2003).

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, the output nucleus for major amygdala functions (Maren 2005; Phelps and Ledoux 2005). The basolateral amygdala (CeA) 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). The CeA forms “downstream” connections with brain stem areas involved in descending pain modulation (Heinricher and McGaraughty 1999; Neugebauer 2006; Rizvi et al. 1991). Integration of unfiltered nociceptive input and affect-related information in the CeA is believed to generate the emotional response to pain (see Neugebauer 2006).

CeLC neurons develop central sensitization in vivo (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003) and synaptic plasticity in brain slices in vitro (Bird et al. 2005; Han et al. 2004, 2005b, 2006; Neugebauer et al. 2003) in the kaolin/carrageenan-arthritis pain model. Synaptic plasticity in the CeLC has also been shown in a model of chronic neuropathic pain (Ikeda et al. 2007). Reversing these changes by pharmacologic deactivation of the CeA with antagonists for glutamate receptors or calcitonin gene-related peptide (CGRP1) receptors decreases nociceptive and affective pain responses in arthritic animals (Han and Neugebauer 2005; Han et al. 2005b; Neugebauer 2006). Similarly, CeA lesion or deactivation inhibits negative affective behavior in models of pain.

Current understanding of pain-related effects of nonopioid neuropeptides such as CRF in the brain is limited, but recent biochemical (Greenwood-Van Meerveld et al. 2006; Sinniger et al. 2004; Ulrich-Lai et al. 2006) and behavioral (Cui et al. 2004; Lariviere and Melzack 2000; McNally and Akil 2002) studies point to the amygdala as a potentially important site of CRF action. The amygdala plays a key role in emotional–affective behavior (Maren 2005; Phelps and Ledoux 2005), including the emotional responses to pain (Fields 2004; Gauriau and Bernard 2002; Heinricher and McGaraughty 1999; Neugebauer 2006; Neugebauer et al. 2004; Rhudy and Meagher 2001). The amygdala is also believed to be a key substrate of the reciprocal relationship between pain and affective state (Meagher et al. 2001; Rhudy and Meagher 2003).

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visceral (Tanimoto et al. 2003) and neuropathic (Pedersen et al. 2007) pain.

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). Importantly, it is the MR neurons, but not NS neurons, that become sensitized to afferent inputs in the arthritis pain model, which is consistent with the concept that the CeLC attaches affective valence to a noxious event. Therefore the present study focused on MR neurons.

Mechanisms of pain-related plasticity in the CeLC are only beginning to emerge. Protein kinase A (PKA)–dependent increased N-methyl-D-aspartate (NMDA) receptor function appears to play an important role (Bird et al. 2005). CRF binds to G-protein–coupled CRF1 and CRF2 receptors, both of which activate the cyclic adenosine monophosphate (cAMP)–PKA signal transduction pathway directly, although coupling to other signaling molecules such as protein kinase C (PKC) and extracellular signal-related kinase (ERK) was also previously reported (Arzt and Holsboer 2006; Blank et al. 2003; Dautzenberg and Hauger 2002; Reul and Holsboer 2002; Steckler and Holsboer 1999). Importantly, despite similar effector mechanisms and a 70% sequence homology, there is evidence to suggest that these receptors mediate different, sometimes opposing functions (Bale and Vale 2004; Charney 2003; Reul and Holsboer 2002). CRF1 and CRF2 receptors show different pharmacological profiles. CRF has a higher affinity for CRF1 receptors, whereas the neuropeptides urocortin II and III are more selective for the CRF2 receptor. Selective CRF1 and CRF2 receptor antagonists are now available, including NB127914 (CRF1 receptor antagonist; Chen et al. 1996) and Astressin-2B (CRF2 receptor antagonist; Ricier et al. 2002), used in the present study.

The amygdala—and the CeA in particular—is a major site of extrahypothalamic expression of CRF in cell bodies and terminals, and contains CRF1 and, to a lesser extent, CRF2 receptors (Gray 1993; Reul and Holsboer 2002; Sanchez et al. 1999; Steckler and Holsboer 1999). Importantly, CRF-containing neurons are also found in the parabrachial area (Merchenenthaler et al. 1982), which provides nociceptive input to the CeA through the spino–parabrachio–amygdaloid pain pathway (Gauriau and Bernard 2004). The role of CRF1 and CRF2 receptors in pain processing in the amygdala is not known. The present electrophysiological study is the first to analyze the effects of selective CRF1 and CRF2 receptor antagonists on nociceptive processing in the amygdala (CeLC) under normal conditions and in a model of persistent pain.

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 (IASP) and of the National Institutes of Health.

Animal preparation and anesthesia

On the day of the electrophysiological experimental, the animal was anesthetized with pentobarbital sodium (50 mg/kg, administered intraperitoneally). 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 (see following text) and for fluid support [3–4 ml·kg\(^{-1}\)·h\(^{-1}\) lactated Ringer solution, administered intravenously (iv)]. Depth of anesthesia was assessed by testing the corneal blink, hindpaw withdrawal, and tail-pinch reflexes; by continuously monitoring the end-tidal CO2 levels (kept at 4.0 ± 0.2%), heart rate, and electrocardiogram (ECG) pattern; and by checking for abnormal breathing patterns. Core body temperature was maintained at 37°C by means of a homeothermic blanket system.

Animals were mounted in a stereotactic frame, paralyzed with pancuronium (induction: 0.3–0.5 mg, iv; maintenance: 0.3 mg/h, intravenous) and artificially ventilated (3–3.5 ml; 55–65 strokes/min). Constant levels of anesthesia were maintained by continuous iv infusion of pentobarbital (15 mg·kg\(^{-1}\)·h\(^{-1}\)). A unilateral craniotomy was performed at the suutra frontoparietalis level for the recording of CeLC neurons and for the administration of drugs into the CeLC contralateral to the knee joint in which the arthritis was induced. Our previous studies showed that multireceptive CeLC neurons with input from the contralateral knee become sensitized after induction of arthritis in that knee (Li and Neugebauer 2004a, 2006; Neugebauer and Li 2003). 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 and identification of amygdala neurons

As described previously (Li and Neugebauer 2004a, 2006; Neugebauer and Li 2002, 2003), long-term 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, whose output 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 (see individual examples in Figs. 2 and 5).

An individual CeLC neuron was identified by its background activity, if present, and by its response to brief mechanical stimuli applied to the knee with a calibrated forceps (see MECHANICAL STIMULI). Although the recording electrode was slowly advanced through the CeLC, brief (5-s) search stimuli of innocuous intensity were applied every 30–50 μm (about once every 10 s). Innocuous stimuli are sufficient to identify multireceptive (MR) neurons (see CLASSIFICATION OF CeLC NEURONS AND RESPONSE THRESHOLDS). In this study we included only MR neurons because they consistently and reliably become sensitized in the arthritis pain model (Li and Neugebauer 2004a, 2006; Neugebauer and Li 2003). After a neuron was identified, we optimized spike size, searched carefully for a receptive field in the knee joint(s), and determined size and threshold of its total receptive field in the deep tissue and skin.

Configuration, shape, and height of the recorded action potentials were monitored and recorded continuously, using a window discriminator and Spike2 software for on-line and off-line analyses. Spikes are detected and recorded by the waveform signal that crosses a
trigger level and matches a preset shape or template that is created for the individual neuron at the beginning of the recording period. Only those neurons were included in this study whose spike configuration 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 (see examples in Figs. 2 and 5).

Experimental protocol

RECEPTIVE FIELDS. Neurons were selected that had a receptive field in the knee. Size and thresholds of the receptive fields in deep tissue and skin were mapped using graded mechanical stimuli of innocuous and noxious intensities (see MECHANICAL STIMULI). Cutaneous input was distinguished from deep tissue input by selective stimulation of skin folds gently raised from the underlying deep tissue. Mechanical stimuli were considered to activate deep tissue (joints and muscles) if the stimulation of overlying skin evoked no or a clearly distinct response. The focus of this study was on the processing of nociceptive information from the deep tissue.

MECHANICAL STIMULI. Mechanical stimuli were applied to the deep tissue by means of a forceps equipped with a force transducer, whose calibrated output was amplified, digitized, and recorded on a Pentium PC for on- and off-line analyses. The hindlimbs, including knee and ankle, were readily accessible to compression of the medial and lateral aspects with the forceps. Stimulus–response relations were measured using brief (15-s) graded mechanical test stimuli of increasing intensities (100, 500, 1,000, 1,500, and 2,000 g/30 mm² at 15-s interstimulus intervals). Stimulus intensities of 100 and 500 g/30 mm² applied to the knee and other deep tissue are considered innocuous because they do not evoke hindlimb withdrawal reflexes in awake rats and are not felt to be painful when tested on the experimenters. An intensity of 1,000 g/30 mm² represents a firm but nonpainful stimulus that does not evoke a hindlimb withdrawal reflex. Pressure stimuli >1,500 g/30 mm² are noxious because they evoke hindlimb withdrawal reflexes and vocalizations in awake rats and are distinctly painful when applied to the experimenters (Han et al. 2005a; Neugebauer and Li 2002). If present, background activity before stimulation was subtracted from the total response during stimulation to calculate the net response evoked by a particular stimulus.

CLASSIFICATION OF CELC NEURONS AND RESPONSE THRESHOLDS. All neurons selected for this study were multireceptive (MR) neurons according to our classification of CeLC neurons with deep tissue input (Neugebauer and Li 2002, 2003). MR neurons were previously shown to develop increased responsiveness (“sensitization”) in the arthritis pain model (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003). Our classification is primarily based on the neurons’ responses to mechanical stimulation of the knee joint and other deep tissue. MR neurons respond consistently to innocuous stimuli (<500 g/30 mm²) but are more strongly activated by noxious stimuli (>1,500 g/30 mm²). Mechanical threshold was defined as the minimum stimulus intensity that evoked an excitatory response (spike frequency higher than the upper 95% confidence interval of background activity) or an inhibitory response (spike frequency less than the lower 95% confidence interval of background activity). A lowering of the threshold in the innocuous range correlates with allodynia. Increased responses to noxious stimuli reflect hyperalgesia.

EXPERIMENTAL PROTOCOL. In each experiment, one CeLC neuron was recorded before and for several hours after the induction of arthritis in one knee joint as in our previous studies using this pain model (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003). The following parameters were measured repeatedly before and after induction of arthritis and 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 (CIs; GraphPad Prism 3.0). Mechanical stimuli of innocuous and noxious intensities (see MECHANICAL STIMULI) were applied to knee joint and at least one other area of the receptive field (typically the ankle) to compare processing of input from arthritic (knee) and noninjured (ankle) tissue. During the development of arthritis, the test stimuli were repeated at regular intervals of about 30 min. Before and during drug applications, intervals between the test stimuli were 5–10 min. Number of stimulations was kept at a minimum to avoid any “sensitization” that might be produced by repeated stimulation. Sufficiently long control periods were included in each experiment (see Figs. 3 and 6) to establish the baseline responses before drug application and/or arthritis induction. Background activity, evoked responses, and receptive field size had to be stable for ≥2 h before the arthritis was induced. Throughout the experiment we carefully monitored several physiological parameters (body temperature, heart rate, ECG, end-tidal CO₂ levels) to ensure a stable recording situation (see Animal preparation and anesthesia).

Arthritis

Arthritis was induced as described in detail previously (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003). A kaolin suspension (4%, 100 μl) was slowly injected into the joint cavity through the patellar ligament with the use of a syringe and needle (1 ml, 25G, 5/8-in.). After repetitive flexions and extensions of the knee for 15 min, a carrageenan solution (2%, 100 μl) was injected into the knee joint cavity and the leg was flexed and extended for another 5 min. This treatment paradigm reliably leads to inflammation and swelling of the knee within 1–3 h and the inflammation persists for weeks (Min et al. 2001; Neugebauer and Li 2003).

Drugs

The following selective CRF receptor antagonists were used: S-chloro-4-[[N-(cyclopropyl)methyl-N-propylamino]-2-methyl-6-(2,4,6-trichlorophenyl)amino-pyridine (NBI27914; CRF1 receptor antagonist) (Chen et al. 1996) was purchased from Tocris Bioscience (Ellisville, MO). Cyclo(31–34) [s-Phe11, His12, CnMeLeu13,39, Nle17, Glu31, Lys34] Ac-Sauvagine(8–40) (Astressin-2B; CRF2 receptor antagonist) (Rivier et al. 2002) was a generous gift from Dr. Jean Rivier of The Salk Institute; additional amounts were purchased from Sigma, St. Louis, MO.

Drug application

Known concentrations of the antagonists were administered into the CeLC by microdialysis before and/or 5–6 h postinduction of arthritis. Changes of response behavior (“sensitization”) reach a maximum after 5–6 h postinduction of arthritis in the kaolin/carrageenan model (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003). Several hours before the start of the electrophysiological recordings a microdialysis probe (CMA11; CMA/Microdialysis; membrane diameter: 250 μm; membrane length: 1 mm) was lowered vertically into the CeA 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 (Li and Neugebauer 2004a,b, 2006). The distance between microdialysis probe and recording electrode was 0.5–1.0 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): NaCl 125.0, KCl 2.6, Na₂HPO₄ 2.5, CaCl₂ 1.3, MgCl₂ 0.9, NaHCO₃ 21.0, and glucose 3.5; oxygenated and equilibrated to pH = 7.4. Before each drug application, ACSF was pumped through the fiber for ≥1 h to establish equilibrium in the tissue.

Drugs were dissolved in ACSF on the day of the experiment at a concentration 100-fold that predicted to be needed based on data from our previous microdialysis and in vitro studies and data in the literature (Pollandt et al. 2006). NB127914, at a concentration of 100 5-chloro-4-[[N-(cyclopropyl)methyl-N-propylamino]-2-methyl-6-(2,4,6-trichlorophenyl)amino-pyridine (NBI27914; CRF1 receptor antagonist) (Chen et al. 1996) was purchased from Tocris Bioscience (Ellisville, MO). Cyclo(31–34) [s-Phe11, His12, CnMeLeu13,39, Nle17, Glu31, Lys34] Ac-Sauvagine(8–40) (Astressin-2B; CRF2 receptor antagonist) (Rivier et al. 2002) was a generous gift from Dr. Jean Rivier of The Salk Institute; additional amounts were purchased from Sigma, St. Louis, MO.
nM, and Astressin-2B were previously shown to be effective and selective in the amygdala (Pollandt et al. 2006). Drug concentration in the tissue is ≥100 times lower than in the microdialysis probe as a result of the concentration gradient across the dialysis membrane and diffusion in the tissue (Han et al. 2005b; Li and Neugebauer 2004a,b, 2006).

Drugs were administered into the CeLC at a rate of 5 μl/min for ≥15 min to establish equilibrium in the tissue. Different concentrations were administered in a cumulative fashion. When concentrations were tested individually in some experiments, no difference was found compared with the cumulative concentration–response data. Drug effects on background and evoked activity were measured every 5–10 min during drug application. The numbers given herein refer to the drug concentrations in the microdialysis fiber. ACSF served as a vehicle control.

### Histology

At the end of each experiment the recording site in the CeLC was marked by injecting DC (25 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 on 50 μm. Sections were stained with Neutral Red, mounted on gel-coated slides, and coverslipped. 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 second (Hertz). Background activity, if present, was subtracted from the total activity during the stimulus. Concentration–response relationships were measured for each neuron and then averaged across a sample of neurons. Sigmoid curves fitted to the cumulative concentration–response data by nonlinear regression using the formula $y = A + (B - A)/(1 + (10^c/10^x)^d)$, where $A$ is the bottom plateau, $B$ is the top plateau, $C$ = log ($IC_{50}$), and $D$ is the slope coefficient (Prism 3.0, GraphPad Software). Linear regression analysis with runs test was used to analyze whether the slope of the concentration–response curve was significantly different from zero (GraphPad Prism 3.0). Concentration–response functions under normal conditions and in arthritis were compared statistically using a two-way ANOVA followed by Bonferroni posttests (GraphPad Prism 3.0). A one-way ANOVA followed by Dunnett’s multiple comparison test was used to determine the significance of effects of individual drug concentrations compared with predrug control values. All averaged values are given as the means ± SE. Statistical significance was accepted at the level $P < 0.05$. 

### Results

**Sample of neurons and their sensitization in the arthritis pain model**

Extracellular single-unit recordings were made from 24 neurons in the laterocapsular division of the central nucleus of the amygdala (CeLC) in 20 anesthetized rats (recording sites are shown in Fig. 1). A CRF1 antagonist was tested in 12 neurons and a CRF2 antagonist in another 12 neurons. All neurons in this study were multireceptive (MR) neurons that responded significantly to innocuous but more strongly to noxious stimuli (see CLASSIFICATION OF CELC NEURONS AND RESPONS THRESHOLDS in METHODS). Individual examples are shown in Figs. 2 and 5. This study focused on MR neurons because they represent the class of CeLC neurons consistently becoming sensitized to afferent inputs in the arthritis pain model (Neugebauer 2006; Neugebauer et al. 2004).

Twelve MR neurons were recorded during the development of arthritis, whereas four MR neurons were recorded only in the arthritis state (5–6 h postinduction). CeLC neurons with MR-type behavior in the arthritis model are sensitized neurons because all MR neurons and only MR neurons become sensitized in this model (Neugebauer 2006; Neugebauer et al. 2004). In agreement with our previous studies (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003) all 12 MR neurons developed enhanced background activity and responses to stimulation of the knee and ankle after induction of the localized mono-arthritis in the knee (see examples in Figs. 2, 3, 5, and 6). Under normal conditions before arthritis induction, the receptive fields of these neurons included the knee and adjacent tissue in the thigh and lower leg of both hindlimbs; most neurons also had receptive fields in the ankle (11 of 12...
CRF1 receptor inhibition

The effects of a selective CRF1 receptor antagonist (NBI27914) were tested under normal conditions (no arthritis) in six neurons and 5–6 h postinduction of arthritis in nine neurons. Maximum changes of responsiveness occur after 5–6 h postinduction of arthritis when the sensitization process reaches a long-lasting plateau (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003). In three of these neurons, drug effects were assessed before and after induction of arthritis in the same neuron. In three neurons drugs were tested only before arthritis and in another six neurons only in the arthritic state (as a control for any pretreatment effect). Data obtained with these different experimental protocols were pooled because no difference in drug effects was found. The test concentration of NBI27914 (10 μM) administered by microdialysis was chosen based on data in the literature that showed 100 nM to be an effective and selective concentration (Chen et al. 1996; Pollandt et al. 2006). Concentration in the microdialysis fiber was adjusted to 100-fold the target concentration (see METHODS).

Figure 2 shows an individual example. In the control period before arthritis induction (Fig. 2A), this multireceptive (MR) CeLC neuron responded more strongly to noxious (2,000 g/30 mm²) than to innocuous (500 g/30 mm²) stimulation of the knee. Administration of NBI27914 (10 μM, concentration in the microdialysis probe; 15 min) into the CeLC had no apparent effect on the neuron’s responses (Fig. 2B). The activity of the same neuron was continuously recorded during development of the knee joint arthritis (see METHODS). Size, shape, and configuration of action potentials (“spikes”) were closely monitored to ensure that activity from only one and the same neuron was recorded (see insets in A–D).

The time course of arthritis- and drug-induced changes in one individual CeLC neuron is shown in Fig. 3. Administration neurons) and paw (nine of 12 neurons). In arthritis, the receptive fields increased to include the whole body. The enlargement of the receptive field is evidence for pain-related central sensitization as described in our previous studies (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003).

FIG. 2. Block of corticotrophin-releasing factor 1 (CRF1) receptors in the CeLC inhibits pain-related sensitization. Extracellular recordings of the responses of one CeLC neuron to brief (15-s) innocuous (500 g/30 mm²) and noxious (2,000 g/30 mm²) stimulation of the knee. A: in the control period before arthritis, the neuron responded more strongly to noxious than to innocuous stimuli. B: administration of a selective CRF1 receptor antagonist [5-chloro-4-[N-(cyclopropyl)methyl-N-propylamino]-2-methyl-6-(2,4,6-trichlorophenyl)amino-pyridine (NBI27914); 10 μM, concentration in the microdialysis probe; 15 min] into the CeLC had no apparent effect. C: neuron’s responses increased 5 h after arthritis induction. D: NBI27914 (10 μM; 15 min) inhibited the increased responses. Top traces: recordings of the stimulus intensity (force in g) that was applied to the knee joint with a calibrated forceps (see METHODS). Peristimulus time histograms (PSTHs) show number of action potentials (“spikes”) per second. Size, shape, and configuration of the spikes were closely monitored and matched to the preset template to ensure that activity from only one and the same neuron was recorded (see insets in A–D).

FIG. 3. Time course of arthritis- and drug-induced changes in one individual CeLC neuron. A: administration of NBI27914 (1, 10, and 100 μM; concentration in the microdialysis probe; 15 min) into the CeLC had no apparent effect on the neuron’s responses to innocuous (500 g/30 mm²) and noxious (2,000 g/30 mm²) stimulation of the knee joint arthritis. After induction of arthritis the responses of the neuron increased. Administration of NBI27914 5–6 h postinduction of arthritis reduced the enhanced responses in a concentration-dependent fashion. B: expanded time-scale of the effects of NBI27914 postinduction of arthritis (same data as in A). Each symbol in A and B shows the response (number of action potentials [spikes] counted per second) to one stimulus. Background activity preceding the stimulus was subtracted from the total activity during stimulation.
of NBI27914 (1, 10, and 100 μM; concentration in the microdialysis probe; 15 min) into the CeA had no apparent effect on the neuron’s responses to innocuous (500 g/30 mm²) and to noxious (2,000 g/30 mm²) stimulation of the knee before induction of the arthritis. Responses to stimulation of the arthritic knee increased after arthritis induction and reached a maximum at 5 h postinduction of arthritis as described before (Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003). There was also an increased responsiveness to stimulation of noninjured parts of the body such as ankle (data not shown), indicating central sensitization. NBI27914 concentration-dependently reduced the enhanced responses to stimulation of the arthritic knee. Background activity preceding the stimulus was subtracted from the total activity during stimulation.

Concentration–response data in Fig. 4 summarize the effects of NBI27914. Under control conditions, NBI27914 (1.0, 10, and 100 μM; concentrations in microdialysis probe; 15–20 min) had no significant effect on the responses of CeLC neurons (n = 6) to innocuous (Fig. 4A, 500 g/30 mm²) or noxious (Fig. 4B, 2,000 g/30 mm²) stimulation of the knee and on background activity (Fig. 4C). Linear regression analysis of the concentration–response data under normal conditions showed no significant deviation of the slope from zero [P > 0.05, F(1,2) = 9.402, innocuous; F(1,2) = 6.189, noxious; F(1,2) = 13.32, background; see METHODS]. In the arthritis pain state (5–6 h postinduction; n = 9), NBI27914 concentration-dependently inhibited the responses of CeLC neurons (Fig. 4, A, B, and C; P < 0.01, Dunn’s multiple comparison test comparing the effects of individual concentrations of NBI27914 to predrug control values). The changes of NBI27914 effects in arthritis compared with normal controls before arthritis were statistically significant [Fig. 4A, innocuous knee, P < 0.0001, F(1,37) = 79.05; Fig. 4B, noxious knee, P < 0.0001, F(1,37) = 87.79; Fig. 4C, background, P < 0.0001, F(1,37) = 21.79; two-way ANOVA; see METHODS].

Blockade of CRF1 receptors also reduced the receptive field size of CeLC neurons in arthritis (n = 9) but not under normal conditions (n = 6). The receptive fields of sensitized neurons covered the whole body and shrank in the presence of NBI27914 to include only the hindlimbs.

These data suggest that CRF1 receptors are endogenously activated in the arthritis pain model, but not under normal conditions, and contribute to pain-related increases of responsiveness in the amygdala (CeLC) in arthritis.

**CRF2 receptor inhibition**

The effects of a selective CRF2 receptor antagonist (Astrassin-2B) were tested under normal conditions (no arthritis) in seven neurons and 5–6 h postinduction of arthritis in five neurons. In three of these neurons, drug effects were assessed before and after induction of arthritis in the same neuron. In four neurons drugs were tested only before arthritis and in another two neurons only in the arthritic state (as a control for any pretreatment effect). Data obtained with these different experimental protocols were pooled because no difference in drug effects was found. The test concentration of Astrassin-2B (10 μM) administered by microdialysis was chosen based on data in the literature that showed 100 nM to be an effective and selective concentration (Pollandt et al. 2006; Rivier et al. 2002). Concentration in the microdialysis fiber was adjusted to 100-fold the target concentration (see METHODS).

Astrassin-2B facilitated the responses of CeLC neurons under normal conditions but had no significant effect in the arthritis pain model. Figure 5 shows an individual example. The neuron responded more strongly to noxious (2,000 g/30 mm²) than to innocuous (500 g/30 mm²) stimulation of the knee joint in the control period before arthritis induction (Fig. 5A) and was classified as MR neuron. Administration of Astrassin-2B (10 μM, concentration in the microdialysis probe; 15 min) into the CeLC enhanced the responses of the neuron to innocuous and noxious stimuli (Fig. 5B). The activity of the same neuron was continuously recorded during development of the knee joint arthritis (see METHODS). The neuron’s responses increased 5 h after arthritis induction (Fig. 5C). Administration of Astrassin-2B (10 μM, 15 min) had no effect on the responses in the arthritis pain state (Fig. 5D). Insets show that size, shape, and configuration of action potentials (“spikes”) remained constant, indicating that only one and the same neuron was recorded.

Figure 6 shows the time course of arthritis- and drug-induced changes in one individual CeLC neuron. Administration of Astrassin-2B into the CeLC concentration-dependently increased the neuron’s responses to innocuous (500 g/30 mm²) and noxious (2,000 g/30 mm²) stimulation of the knee under normal conditions. Responses of the neurons returned to baseline after washout of Astrassin-2B under normal conditions before the induction of arthritis. After induction of arthritis the responses of the same neuron increased. Astrassin-2B had no apparent effect on the enhanced responses 5–6 h postinduction of arthritis. Background activity preceding the stimulus was subtracted from the total activity during stimulation.

The effects of Astrassin-2B are summarized in Fig. 7. Under normal conditions (n = 8), Astrassin-2B concentration-dependently increased the responses of CeLC neurons to innocuous (Fig. 7A, 500 g/30 mm²) and noxious (Fig. 7B, 2,000 g/30 mm²) mechanical stimulation of the knee and on background activity (Fig. 7C). The facilitatory effects of Astrassin-2B on evoked activity, but not background activity, were statistically significantly (P < 0.05–0.01, Dunn’s multiple comparison test comparing the effects of individual concentrations to predrug control values). However, in the arthritis pain state (5–6 h postinduction; n = 7), Astrassin-2B (0.1, 1, 10, and 100 μM; concentrations in microdialysis probe; 15–20 min) had no effect. Linear regression analysis of the concentration–response data for Astrassin-2B in arthritis showed no significant deviation of the slope from zero [P > 0.05, F(1,3) = 1.401, innocuous; F(1,3) = 0.8304, noxious; F(1,3) = 7.903, background; see METHODS]. Changes of Astrassin-2B effects on evoked activity in arthritis compared with normal conditions were statistically significant [Fig. 7A, innocuous, P < 0.01, F(1,5) = 9.99; Fig. 7B, noxious, P < 0.001, F(1,5) = 38.70; two-way ANOVA; see METHODS]. There was no significant difference between Astrassin-2B effects on background activity under normal conditions and in arthritis (two-way ANOVA).

Blockade of CRF2 receptors also increased the receptive field size of CeLC neurons under normal conditions (n = 8) but not in arthritis (n = 7). In the presence of Astrassin-2B the receptive fields expanded to include hindlimbs and forelimbs in their entirety, thus partially mimicking the effects of arthritis.
on receptive field size (see Sample of neurons and their sensitization in the arthritis pain model).

The data suggest that CRF2 receptors are endogenously activated under normal conditions and contribute to an inhibitory tone under normal conditions, which is lost in the arthritis pain model.

FIG. 5. Block of CRF2 receptors has no effect in the arthritis pain model but facilitates responses under normal conditions. Extracellular recordings of the responses of one CeLC neuron to brief (15 s) innocuous (500 g/30 mm²) and noxious (2,000 g/30 mm²) stimulation of the knee. A: before arthritis, the neuron responded more strongly to noxious than innocuous stimuli [multireceptive (MR) type neuron]. B: administration of cyclo(31–34) [D-Phe11, His12, CoMeLeu13, Nle17, Glu31, Lys34] Ac-sauvagine(8–40) (Astressin-2B, 10 μM, concentration in the microdialysis probe; 15 min) into the CeLC enhanced the responses of the neuron. C: responses of the same neuron increased 5 h after arthritis induction. Administration of Astressin-2B (10 μM, 15 min) had no apparent effect on the increased responses in the arthritis pain state. Top traces: recordings of the stimulus intensity (force in g) that was applied to the knee joint with a calibrated forceps (see METHODS). PSTHs show number of action potentials (spikes) per second. Size, shape, and configuration of action potentials (“spikes”) remained constant throughout the experiment (see insets in A–D). E–H: traces show the spikes that matched the preset template (Spike2 software) and were counted in the PSTHs in A–D as the activity of the recorded neuron (see METHODS).

FIG. 4. Concentration-dependent inhibitory effects of a CRF1 receptor antagonist (NBI27914) in the arthritis pain model but not under normal conditions. Under control conditions (“normal”), NBI27914 had no significant effect on the responses of CeLC neurons (n = 6) to innocuous (A, 500 g/30 mm²) or noxious (B, 2,000 g/30 mm²) stimulation of the knee [P > 0.05, F(1,2) = 9.402, innocuous; F(1,2) = 6.189, noxious; linear regression analysis; see METHODS] and on background activity (C). In the arthritis pain state (5–6 h postinduction; n = 9), NBI27914 inhibited the evoked responses and background activity of CeLC neurons concentration-dependently (A, B, and C). Numbers refer to the concentrations in the microdialysis probe. Drugs were administered into the CeLC by microdialysis for 15–20 min. All averaged values are given as the means ± SE and are expressed as a percentage of predrug control values (set to 100%). Statistical analysis (2-way ANOVA; see METHODS) revealed significant changes of NBI27914 effects in arthritis compared with normal controls before arthritis [A, innocuous, P < 0.0001, F(1,37) = 79.05; B, noxious, P < 0.0001, F(1,37) = 87.79; C, background, P = 0.0001, F(1,37) = 21.79]. *P < 0.05, ***P < 0.001, Bonferroni posttests (following 2-way ANOVA) comparing normal vs. arthritis. Dunnett’s multiple comparison test (see METHODS) showed that the effects of NBI27914 in arthritis were significantly different from predrug control values where indicated (++P < 0.01).
expression of CRF and a key element of the extrahypothalamic circuits through which CRF contributes to anxiety-like behavior and affective disorders (Asan et al. 2005; Gray 1993; Reul and Holsboer 2002; Steckler and Holsboer 1999). 3) CRF1 receptors have emerged as drug targets for depression and anxiety disorders because CRF produces anxiety-like behaviors in preclinical studies through CRF1 receptors, whereas CRF2 receptors mediate anxiolytic effects (Bale and Vale 2004; Chalmers et al. 1996; Charney 2003; Dautzenberg and Hauger 2002; Reul and Holsboer 2002; Steckler and Holsboer 1999; Takahashi 2001). Importantly, a CRF1 receptor antagonist was previously used successfully in humans to treat major depression disorder, reducing depression and anxiety scores (Kunzel et al. 2005; Zobel et al. 2000). 4) The amygdala is emerging as a neuronal interface between pain and affective states and disorders such as anxiety (Fields 2004; Heinrich and McGarvaught 1999; Neugebauer 2006; Neugebauer et al. 2004; Pedersen et al. 2007; Rhudy and Meagher 2001). The CeLC integrates affect-related information from the lateral–basolateral amygdala (LA and BLA) with subcortical nociceptive inputs from the spino–parabrachio–amygdaloid pain pathway (Gauriau and Bernard 2002).

The presence of CRF-containing neurons in the parabrachial area (Merchanthaler et al. 1982) links the CRF system in the amygdala to the peridgergic spino–parabrachio–amygdaloid pain pathway and implicates CRF in the transmission of nociceptive information to the amygdala.

Our previous studies showed plastic changes in the CeLC in an arthritis pain model (Bird et al. 2005; Han et al. 2004, 2005b, 2006; Li and Neugebauer 2004a,b, 2006; Neugebauer and Li 2003; Neugebauer et al. 2003). Plasticity was measured as increased synaptic transmission in the nociceptive parabrachio–amygdaloid pathway, enhanced responsiveness to afferent input (sensitization), and increased neuronal excitability of CeLC neurons. These plastic changes were observed in so-called multireceptive (MR) neurons but not in nociceptive-specific (NS) neurons. MR neurons respond more strongly to noxious than innocuous stimuli, suggesting they are able to encode and distinguish nociceptive and nonnociceptive information. MR neurons are believed to be a site of convergence and integration of nociceptive and polymodal inputs in the CeA (Neugebauer 2006; Neugebauer et al. 2004).

Mechanisms of arthritis pain–related synaptic plasticity in the CeLC include the enhanced release of glutamate through presynaptic group I metabotropic glutamate receptors (mGlRs; particularly the mGlR1 subtype) (Li and Neugebauer 2004b; Neugebauer et al. 2003), which is controlled by inhibitory group II and group III mGlRs (Han et al. 2004, 2006; Li and Neugebauer 2006). Glutamate acts postsynaptically to activate NMDA receptors that become functional in the arthritis pain model through receptor phosphorylation by PKA but not PKC (Bird et al. 2005). Change in NMDA rather than α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function contributes to synaptic plasticity and sensitization in the CeLC (Bird et al. 2005; Li and Neugebauer 2004a). Endogenous release of CGRP from the parabrachio–amygdaloid tract activates postsynaptic CGRP1 receptors coupled to PKA, but not PKC, to increase NMDA, but not AMPA, receptor function (Han et al. 2005b). The present study shows that the endogenous activation of CRF1, but not CRF2, receptors also contributes to arthritis pain–related plasticity in the

**FIG. 6.** Time course of arthritis- and drug-induced changes in one individual CeLC neuron. **A** administration of Astressin-2B (0.1, 1, 10, and 100 μM; concentration in the microdialysis probe; 15 min) into the CeLC increased the neuron’s responses to innocuous (500 g/30 mm²) and noxious (2,000 g/30 mm²) stimulation of the knee under normal conditions. After induction of arthritis the responses of the neuron increased. Astressin-2B had no apparent effect 5–6 h postinduction of arthritis. **B** expanded timescale of the effects of Astressin-2B under normal conditions before the induction of arthritis. Each symbol in A and B shows the response (number of action potentials [spikes] counted per second) to one stimulus. Background activity preceding the stimulus was subtracted from the total activity during stimulation.

**DISCUSSION**

The main novel findings of this study are that CRF1 and CRF2 receptors mediate opposing effects in the amygdala (CeLC) and that they undergo differential changes in the arthritis pain model. Specifically, our data show that block of CRF1 receptors in the CeLC inhibits increased responsiveness of CeLC neurons in the arthritis pain model but has no effect under normal conditions. Conversely, block of CRF2 receptors in the CeLC facilitates the neurons’ responses under normal conditions but has no effect in the arthritis state. These data suggest that CRF1 receptors in the amygdala are endogenously activated in the arthritis pain model, but not under normal conditions, and contribute to pain-related increases of responsiveness in the amygdala. Endogenous activation of CRF2 receptors under normal conditions contributes to an inhibitory tone, which is lost in the arthritis pain model.

The rationale for the study of CRF receptor function in amygdala pain mechanisms is as follows. 1) Mechanisms of pain-related plasticity in the amygdala include the PKA-dependent phosphorylation of NMDA receptors (Bird et al. 2005) and CRF receptors are known to couple to the cAMP–PKA signal transduction pathway (Arzt and Holsboer 2006; Blank et al. 2003; Dautzenberg and Hauger 2002; Reul and Holsboer 2002; Steckler and Holsboer 1999). 2) The amygdala, particularly its central nucleus, is a major site of extrahypothalamic...
CeLC. It remains to be determined whether CRF1 receptor–mediated effects also involve PKA or other signal transduction mechanisms.

Antinociceptive effects of peripherally acting CRF and related peptides such as urocortin II are fairly well established (Lariviere and Melzack 2000; Martinez et al. 2004; Million et al. 2006; Mousa et al. 2003). They appear to be mediated through CRF2 and possibly CRF1 receptors and involve mainly beta-endorphins. The pain-modulatory role and mechanisms of centrally acting CRF and its receptors are controversial. Whereas early studies failed to detect any effect of centrally administered CRF, intracranial administration of CRF was more recently shown to produce analgesia with a very narrow dose range (Cui et al. 2004; Lariviere and Melzack 2000; Vit et al. 2006) but can also have hyperalgesic effects that are blocked by CRF1 receptor antagonists (Greenwood-Van Meerveld et al. 2005; Martinez et al. 2004). Interestingly, the antinociceptive effects of intracerebroventricular administration of CRF on thermal and mechanical withdrawal thresholds and on pain scores in the formalin test were accompanied by increased vocalizations (Vit et al. 2006), perhaps suggesting dual effects of centrally acting CRF.

Recent biochemical (Greenwood-Van Meerveld et al. 2006; Sinniger et al. 2004; Ulrich-Lai et al. 2006) and behavioral (Cui et al. 2004; Lariviere and Melzack 2000; McNally and Akil 2002; Myers et al. 2005) studies point to the amygdala as an important site for the pain-modulatory effects of CRF. Increased expression of CRF1, but not CRF2, receptor mRNA was detected in the amygdala in a model of somatovisceral pain induced by intraperitoneal acetic acid (Sinniger et al. 2004). CRF mRNA increased in the CeA in models of colitis pain (Greenwood-Van Meerveld et al. 2006) and chronic neuropathic pain (Ulrich-Lai et al. 2006). Intracerebroventricular or intra-CeA administration of a broad-spectrum CRF receptor antagonist (alpha-hCRF9-41) had antinociceptive effects on hyperalgesic behavior associated with opiate withdrawal (McNally and Akil 2002). Systemic administration of a CRF1 receptor antagonist nearly reversed colon hypersensitivity (visceromotor response) induced by stereotaxic delivery of corticosterone to the CeA (Myers et al. 2005). On the other hand, intra-CeA administration of a nonselcetive CRF receptor antagonist (alpha-hCRF9-41) produced hyperalgesic behavior (decreased mechanical and thermal withdrawal thresholds) and

**FIG. 7.** Concentration-dependent facilitatory effects of a CRF2 receptor antagonist (Astressin-2B) under normal conditions but not in the arthritis pain model. Under normal conditions (n = 8), Astressin-2B concentration-dependently increased the responses of CeLC neurons to innocuous (A, 500 g/30 mm²) and noxious (B, 2,000 g/30 mm²) mechanical stimulation of the knee but not background activity (C). Dunnett’s multiple comparison test (see METHODS) showed that the effects of Astressin-2B on evoked activity were significantly different from predrug control values where indicated (+P < 0.05, ++P < 0.01 after adjustment for multiple comparisons). In the arthritis pain state (5–6 h postinduction; n = 7), Astressin-2B had no significant effect [P > 0.05, F(1,15) = 1.401, innocuous; F(1,15) = 0.8304, noxious; F(1,15) = 7.903, background; linear regression analysis; see METHODS]. Drugs were administered into the CeLC by microdialysis for 15–20 min. All averaged values are given as the means ± SE and are expressed as a percentage of predrug control values (set to 100%). Statistical analysis (2-way ANOVA; see METHODS) showed significant changes of Astressin-2B effects on evoked activity in arthritis compared with normal conditions [A, innocuous, P < 0.01, F(1,5) = 9.99; B, noxious, P < 0.001, F(1,5) = 38.70; 2-way ANOVA; see METHODS]. Effects on background activity were not significant under either condition. *P < 0.05, ***P < 0.001, Bonferroni posttests (following 2-way ANOVA).
attenuated the antinociceptive effects of CRF administered into the CeA in normal animals (Cui et al. 2004).

The present study supports the concept of dual effects of CRF in the amygdala, which could be attributed to differential functions of CRF1 and CRF2 receptors. The facilitating effects of a CRF2 receptor antagonist under normal conditions would be consistent with inhibitory antinociceptive effects of CRF mediated through CRF2 receptors. CRF has a relatively low affinity for CRF2 receptors, which would explain the narrow dose range for the antinociceptive effects of CRF mentioned earlier (Lariviere and Melzack 2000). CRF2 receptors can also be activated by members of the urocortin peptide family, such as urocortin II and urocortin III, which are believed to be the endogenous ligands for CRF2 receptors (Bale and Vale 2004). They activate CRF2 receptor selectively and with a higher affinity than CRF. The amygdala is the major site of urocortin III expression outside the hypothalamus (Li et al. 2002). The inhibitory effect of a CRF1 antagonist in the arthritis pain model is consistent with facilitating pronociceptive effects of CRF. The differential effects of CRF1 and CRF2 receptor antagonists on pain-related processing in the amygdala are reminiscent of their reciprocal opposing influences on anxiety-like behaviors. CRF1 receptors mediate stimulatory anxiogenic effects of CRF, whereas CRF2 receptors have a dampening anxiolytic function (Bale and Vale 2004; Chalmers et al. 1996; Charney 2003; Dautzenberg and Hauger 2002; Reul and Holsboer 2002; Skelton et al. 2000; Steckler and Holsboer 1999; Takahashi 2001).

Evidence for dual effects of CRF also comes from neurophysiological studies of other brain areas and the spinal cord. CRF changed A-fiber stimulation-induced long-term depression to long-term potentiation (LTP) in the substantia gelatinosa of the spinal dorsal horn but inhibited LTP induction in the presence of inhibitory amino acid antagonists (Ikeda et al. 2003). CRF had predominantly excitatory actions on neurons in the hippocampus, cortex, locus coeruleus, and hypothalamic nuclei, and evoked epileptiform discharges in hippocampus and amygdala when administered intracerebroventricularly at higher doses (Siggins et al. 1985). CRF also induced LTP in the hippocampus (dentate gyrus; Wang et al. 1998) and in the CeA (Pollandt et al. 2006), but was critical for the induction of LTD in the cerebellum (Miyata et al. 1999). Further, prolonged (1-h) administration of CRF depressed LTP in the hippocampus (CA1) but higher concentrations of CRF were less effective (Rebaudo et al. 2001). Predominantly inhibitory actions of CRF on neuronal activity were observed in the lateral septum, thalamus, and hypothalamic paraventricular nucleus (Siggins 1985). CRF increased GABAergic transmission in the bed nucleus of the stria terminalis (Kash and Winder 2006). Inhibitory effects at low concentrations and excitatory effects at higher concentrations were measured in the dorsal raphe nucleus (Kirby et al. 2000).

Mechanisms underlying dual facilitatory and inhibitory effects of CRF remain to be determined. They may include the involvement of CRF1 versus CRF2 receptors, cellular locations (preversus postsynaptic), different circuits (glutamatergic vs. GABAergic), and different signal transduction mechanisms of the receptors (Arzt and Holsboer 2006; Blank et al. 2003; Dautzenberg and Hauger 2002; Reul and Holsboer 2002; Steckler and Holsboer 1999). Endogenous enhancement of CRF1 receptor activation and decrease or loss of CRF2 receptor activation seems to contribute to arthritis-induced hyperexcitability of amygdala neurons. Modulation of receptor activation in the arthritic state could be explained by changes in the levels of CRF and/or urocortin peptides in the amygdala, by changes in the levels of CRF1 and CRF2 receptor expression, by posttranslational modifications of these receptors, by changes in downstream effectors and targets, or by a combination of these mechanisms. Another possibility is that functional changes in the amygdala circuitry contribute to the differential changes of CRF1 and CRF2 receptor function. If CRF2 receptors activate well-documented inhibitory circuits in the amygdala (Par é et al. 2004) and CRF1 receptors regulate excitatory pathways, a shift of balance between excitatory and inhibitory transmission toward excitation in pain-related plasticity would result in the dominance of CRF1 over CRF2 receptor function. However, the underlying mechanisms remain to be determined.

In summary, the present study shows for the first time that CRF1 and CRF2 receptors in the amygdala mediate opposing effects on nociceptive processing and that they play different roles in normal transmission and pain-related sensitization. Agents that decrease CRF1 receptor activity in the amygdala would restore normal function and thus have beneficial effects in certain forms of pain.

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