Behavioral/Systems/Cognitive

Critical Role of Calcitonin Gene-Related Peptide 1 Receptors in the Amygdala in Synaptic Plasticity and Pain Behavior

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The role of neuropeptides in synaptic plasticity is less well understood than that of classical transmitters such as glutamate. Here we report the importance of the G-protein-coupled calcitonin gene-related peptide (CGRP1) receptor as a critical link between amygdala plasticity and pain behavior. A key player in emotionality and affective disorders, the amygdala has been implicated in the well-documented, but mechanistically unexplained, relationship between pain and affect. Our electrophysiological and pharmacological in vitro (patch-clamp recordings) and in vivo (extracellular single-unit recordings) data show that selective CGRP1 receptor antagonists (CGRP8-37 and BIBN4096BS) in the amygdala reverse arthritis pain-related plasticity through a protein kinase A (PKA)-dependent postsynaptic mechanism that involves NMDA receptors. CGRP1 receptor antagonists inhibited synaptic plasticity in the laterocapsular division of the central nucleus of the amygdala (CeLC) in brain slices from arthritic rats compared with normal controls. The effects were accompanied by decreased neuronal excitability and reduced amplitude, but not frequency, of miniature EPSCs; paired-pulse facilitation was unaffected. The antagonist effects were occluded by a PKA inhibitor. CGRP1 receptor blockade also directly inhibited NMDA-evoked, but not AMPA-evoked, membrane currents. Together, these data suggest a postsynaptic site of action. At the systems level, the antagonist reversed the sensitization of nociceptive CeLC neurons in anesthetized rats in the arthritis pain model. Importantly, CGRP1 receptor blockade in the CeLC inhibited spinal (hindlimb withdrawal reflexes) and supraspinal pain behavior of awake arthritic rats, including affective responses such as ultrasonic vocalizations. This study provides direct evidence for the critical dependence of pain behavior on CGRP1-mediated amygdala plasticity.

Key words: amygdala; synaptic plasticity; sensitization; pain; neuropeptide; vocalization

Introduction

The amygdala plays an important role in emotional learning and memory and affective disorders such as anxiety and depression (Davis, 1998; Davidson et al., 1999; LeDoux, 2000; Rodrigues et al., 2004) but has only recently been linked to pain processing (Neugebauer et al., 2004). The amygdala includes several anatomically and functionally distinct nuclei. The laterocapsular part of the central nucleus (CeLC) is now defined as the “nociceptive amygdala” because of its high content of neurons that process pain-related information (Bernard and Bandler, 1998; Neugebauer et al., 2004). The CeLC is the target of the spino-parabrachio-amgydaloid pain pathway (Bernard and Bandler, 1998) and also receives affect-related information from the lateral (LA) and basolateral (BLA) amygdala (Neugebauer et al., 2004). Associative learning and plasticity in the LA–BLA circuitry is critical for the emotional evaluation of sensory stimuli and affective states and disorders (LeDoux, 2000; Pare et al., 2004; Rodrigues et al., 2004; Walker and Davis, 2004). Therefore, the CeLC is well positioned to integrate pain-related information with affective content, contribute to the emotional response to pain, and serve as the neuronal interface through which affect modulates pain (Neugebauer et al., 2004).

The mechanisms and behavioral consequences of pain processing in the amygdala are only beginning to emerge. Our previous studies showed synaptic plasticity in the CeLC in the arthritis pain model (Neugebauer and Li, 2003; Neugebauer et al., 2003). This pain-related plasticity depends on presynaptic metabotropic glutamate receptors (Neugebauer et al., 2003; Li and Neugebauer, 2004a) and on postsynaptic NMDA receptor phosphorylation through protein kinase A (PKA) but not PKC (Li and Neugebauer, 2004b; Bird et al., 2005). Using an integrative approach that combines electrophysiology in vitro and in vivo and behavioral analysis, we show here that the calcitonin gene-related peptide 1 (CGRP1) receptor serves as the critical molecule that links these presynaptic and postsynaptic mechanisms and contributes to pain behavior organized at different levels of the pain neuraxis.

CGRP is a 37 amino acid peptide that activates adenyl cyclase and PKA through G-protein-coupled receptors, including the CGRP1 receptor for which selective antagonists are available (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997; Doods et al., 2000). Previous anatomical data and our present study suggest that CGRP is a molecular marker of the CeLC. The
CeLC is delineated by its abundance of CGRP-immunoreactive terminals of fibers from the external lateral parabrachial area (Kruger et al., 1988; Schwaber et al., 1988; Harrigan et al., 1994; de Lacle and Saper, 2000), which is part of the spino-parabrachio-amygdaloid pain pathway. These terminals innervate CeLC neurons that project to brainstem areas such as the periaqueductal gray (Schwaber et al., 1988; Harrigan et al., 1994), which is important for expression of behavior and descending pain modulation. The central nucleus of the amygdala (CeA) also represents one of the brain areas with the highest levels of CGRP and CGRP receptors (Skoftisch and Jacobowitz, 1985; Van Rossum et al., 1997; Oliver et al., 1998). Whereas the involvement of CGRP in peripheral and spinal pain mechanisms is well established (Galeazza et al., 1995; Neugebauer et al., 1996; Schaible, 1996; Ruda et al., 2000; Sun et al., 2004), less is known about its role in pain-related plasticity in the brain.

Materials and Methods

Arthritis pain model. The mono-arthritis was induced in the left knee joint of adult rats as described in detail previously (Neugebauer and Li, 2003; Neugebauer et al., 2003). A kaolin suspension (4%, 80–100 µl) was injected into the joint cavity through the patellar ligament with a syringe (1 ml, 25 ga, 5/8 inch). After repetitive flexions and extensions of the knee for 15 min, a carrageenan solution (2%, 80–100 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, reaches a maximum plateau at 5–6 h, and persists for days (Neugebauer and Li, 2003; Neugebauer et al., 2003). Electrophysiological and behavior measurements of arthritis pain-related changes were made at the 6 h time point.

In vitro electrophysiology: patch-clamp recording. Amygdala slice preparation. Brain slices containing the CeA were obtained from arthritic rats (1996; Ruda et al., 2000; Sun et al., 2004), less is known about its role in pain-related plasticity in the brain.
Cambridge Electronics Design) was used for on-line and off-line analysis of single-unit activity. An individual CeLC neuron was identified by the configuration, shape, and height of the recorded action potentials (spikes) in response to innocuous and noxious mechanical stimulation (compression) of the knee as described in detail previously (Neugebauer and Li, 2003; Li and Neugebauer, 2004a,b). Mechanical stimuli of innocuous (100 and 500 g/30 mm²) and noxious (1500 and 2000 g/30 mm²) intensity were applied to the knee and other parts of the receptive field (e.g., ankle) by means of a forceps equipped with a force transducer, whose calibrated output was amplified and displayed in grams on an liquid crystal display screen (Neugebauer and Li, 2003; Li and Neugebauer, 2004a,b). The output signal was also fed into the Cambridge Electronics Design interface and recorded on the computer for on-line and off-line analysis. Stimulus intensities of 100–500 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. Pressure stimuli >1500 g/30 mm² are nociceptive because they evoke hindlimb withdrawal reflexes in awake rats and are distinctly painful when applied to the experimenters (Neugebauer and Li, 2003; Li and Neugebauer, 2004a,b; Han and Neugebauer, 2005). Stimulus–response functions were obtained with the use of a wide range of innocuous and noxious stimuli (100–2000 g/30 mm²).

In this study, neurons that had a receptive field in the knee and responded more strongly to noxious than innocuous stimuli, because these so-called multireceptive (MR) neurons have been shown in our previous studies to become sensitized consistently in the arthritis pain model (Neugebauer and Li, 2003; Li and Neugebauer, 2004a,b). Size and threshold of the total receptive field, background activity, and responses to innocuous and noxious stimuli (see above) were recorded before and for 6–9 h after induction of arthritis in the knee. Innocuous and noxious stimuli (15 s duration each) were applied three times in a control period of at least 2 h before arthritis induction and then every hour after induction of arthritis.

**Drug application.** Known concentrations of CGRP1 receptor antagonists (CGRP₈₋₃₇ and BIBN4096BS; see above, Drugs) were administered into the CeLC by microdialysis 6 h after induction of arthritis as described in detail previously (Neugebauer and Li, 2003; Li and Neugebauer, 2004a,b). Several hours before the start of the electrophysiological recordings, a microdialysis probe (20 kDa cutoff, membrane length of 2 mm; CMA12; CMA/Microdialysis, North Chelmsford, MA) was inserted vertically into the CeLC and positioned stereotaxically 500 μm anterior to the recording electrode, using the following coordinates (Paxinos and Watson, 1998): 1.8 mm caudal to bregma; 4.0 mm lateral to midline; depth of tip, 9.0 mm. The microdialysis probe was connected with PE50 tubing to an infusion pump (Harvard Apparatus, Holliston, MA) and perfused with ACSF (see above, in vitro experiments) oxygenated and equilibrated to pH 7.4. ACSF was pumped through the fiber throughout the experiment to maintain stable conditions in the tissue. Drugs (see above) were dissolved in ACSF on the day of the experiment at a concentration 100 times that predicted to be needed based on data in the literature (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997) and our own in vitro data (this study) because of the concentration gradient across the dialysis membrane (Li and Neugebauer, 2004a,b; Han and Neugebauer, 2005). The numbers given in this article refer to the drug concentrations in the microdialysis fiber. ACSF administered alone served as a vehicle control. Behavior was measured at 15 min during continued drug administration and again at 30 min of washout with ACSF.

**Audible and ultrasonic vocalizations.** Vocalizations were recorded and analyzed as described in detail previously (Han and Neugebauer, 2005). The experimental setup [Han JS, Neugebauer V (2005) U.S. Patent Application 98006/28UTL, pending] included a custom-designed recording chamber, a condenser microphone (audible range, 20 Hz to 16 kHz) connected to a preamplifier and a ultrasound detector (25 ± 4 kHz), filter and amplifier (UltraVox four-channel system; Noldus Information Technology, Leesburg, VA), and data acquisition software (UltraVox 2.0; Noldus Information Technology), which automatically monitored the occurrence of vocalizations within user-defined frequencies (see above) and recorded the number and duration of digitized events (audible and ultrasonic vocalizations) on a computer (Dell Pentium 4) (Han and Neugebauer, 2005; Han et al., 2005). This computerized recording system was set to suppress nonrelevent audible sounds and to ignore ultrasound outside the defined frequency range (25 ± 4 kHz). Animals were placed in the recording chamber for acclimation 1 h before the vocalization measurements and for habituation (1 h on 2 d). The recording chamber ensured the stable positioning of the animal at a fixed distance from the sound detectors and allowed the reproducible stimulation of the knee joint. The chamber contained openings for the hindlimbs to allow the application of brief (15 s) innocuous (100 g/30 mm²) and noxious (2000 g/30 mm²) mechanical stimuli with a calibrated forceps (same as in the in vitro electrophysiology studies); it also had an opening for the head to permit drug administration into the amygdala through the microdialysis probe inserted into the implanted guide cannula. Rate and duration of vocalizations were recorded during application of the mechanical stimulus [vocalizations during stimulation (VDS), organized at the medullary level] and in the period immediately after stimulation [vocalization “afterdischarges” (VADs), generated in the limbic forebrain] (Borszcz and Leaton, 2003; Han and Neugebauer, 2005).

**Hindlimb withdrawal reflex.** The threshold of spinaly organized with-
Data analysis and statistics
All averaged values are given as the mean ± SEM. Statistical significance was accepted at the level p < 0.05. GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA) was used for all statistical analysis except when noted.

In vitro electrophysiology.
Input–output functions and concentration–response relationships were compared using a two-way ANOVA followed by post hoc tests when appropriate. Concentration–response curves were obtained by nonlinear regression analysis using the formula $y = A + (B - A)/(1 + (x/X_{0.5})^n)$, where A is the bottom plateau, B is the top plateau, C is log($IC_{50}$), and D is the slope coefficient. The paired t test was used to compare test EPSC amplitudes and PF excitation with the stimulus intensity and during drug application. Miniature EPSCs (mEPSCs) were analyzed for frequency and amplitude distributions using the MiniAnalysis program 5.3 (Synaptosoft, Decatur, GA). The Kolmogorov–Smirnov test was used for cumulative distribution analysis of mEPSC amplitude and frequency.

In vivo electrophysiology.
Extracellularly recorded single-unit activity (action potentials) was analyzed off-line from peristimulus rate histograms using Spike2 software (version 3; Cambridge Electronics Design). Evoked responses were expressed as spikes (action potentials) per second (Hertz) by subtracting from the total activity during the stimulus (15 s) any background activity (Hertz) in the 15 s preceding the stimulus. Responses before and during drug administration were compared using a paired t test. Concentration–response relationships were obtained and analyzed statistically as described above (see above, In vitro electrophysiology).

Audible and ultrasonic vocalizations.
Duration of audible and ultrasonic vocalizations was normalized to pre-arthritis (normal) conditions. The duration is defined as the arithmetic sum (total amount) of the durations of individual vocalization events that occur during (VDS) or after (VAD) a single stimulus. A paired t test was used to compare behavioral changes (vocalizations and withdrawal thresholds) in the same animal before and during drug administration.

Results
Endogenous activation of CGRP1 receptors is required for pain-related synaptic plasticity of CeLC neurons
Whole-cell voltage-clamp recordings of neurons in the CeLC were made in brain slices from untreated normal rats and from rats in which an arthritis pain state had been induced 6 h before (for details, see Neugebauer et al., 2003; Bird et al., 2005). CeLC neurons from arthritic rats showed significantly increased synaptic transmission (Fig. 1a,b), which indicates “synaptic plasticity” because it is preserved in the reduced slice preparation and maintained independently of peripheral or spinal pain mechanisms. Pain-related synaptic plasticity is evident from the increased synaptic strength measured as increased peak amplitudes of monosynaptic EPSCs at the nociceptive PB–CeLC synapse (Neugebauer et al., 2004). Individual current traces show that, compared

![Figure 1](image-url)

**Figure 1.** Pain-related synaptic plasticity in the amygdala depends in part on the activation of CGRP1 receptors. a, Peak amplitudes of monosynaptic EPSCs, a measure of synaptic strength, were larger in a CeLC neuron recorded in a brain slice from an arthritic rat (right) than in a control neuron from a normal rat (left). Individual traces show monosynaptic EPSCs (average of 8–10 EPSCs) evoked at the PB–CeLC synapse with increasing stimulus intensities. Calibration: 50 ms, 50 pA. b, Input–output functions were measured by increasing the stimulus intensity in 100 µA steps. CeLC neurons from arthritic animals (n = 19) showed significantly enhanced synaptic transmission compared with control neurons (n = 37) (p < 0.0001, F(1,220) = 60.29, two-way ANOVA followed by Bonferroni’s post hoc tests). c, A selective CGRP1 receptor antagonist (CGRP8–37, 1 µM) inhibited synaptic plasticity in a CeLC neuron from an arthritic animal (middle trace) but had little effect on basal synaptic transmission in a CeLC neuron from a normal animal (left trace). Likewise, the selective nonpeptide CGRP1 receptor antagonist (BIBN4096BS, 1 µM) inhibited synaptic plasticity in a CeLC neuron from an arthritic animal (right trace). Individual traces show monosynaptic EPSCs (average of 8–10 EPSCs) evoked at the PB–CeLC synapse with the stimulus intensity set to 70–80% of that required for generating maximum EPSC amplitude. Calibration: 50 µs, 50 pA. d, Averaged raw (current) data show that CGRP8–37 (1 µM) inhibited the increased EPSC amplitude in neurons (n = 17) from arthritic rats (right; p < 0.05, paired t test) but had no significant effects on the amplitude of EPSCs recorded in control neurons (n = 29) from normal rats (left). e, BIBN4096BS (1 µM) inhibited synaptic plasticity in a CeLC neuron from an arthritic animal (middle trace) but had little effect on basal synaptic transmission in a CeLC neuron from a normal animal (left trace). Individual traces show monosynaptic EPSCs (average of 8–10 EPSCs) evoked at the PB–CeLC synapse with the stimulus intensity set to 70–80% of that required for generating maximum EPSC amplitude. Calibration: 50 µs, 50 pA. f, Input–output function of the PB–CeA synapse (see b) significantly reduced by CGRP8–37 (1 µM) in CeLC neurons from arthritic rats (n = 12; p < 0.0001; F(1,120) = 76.32, two-way ANOVA followed by Bonferroni’s post hoc tests). Whole-cell voltage-clamp recordings were made from CeLC neurons held at −60 mV. g, % of control: *p < 0.05; **p < 0.01; ***p < 0.001.
with a control neuron (Fig. 1a, left), monosynaptic EPSCs were enhanced in a CeLC neuron recorded in a brain slice from an arthritic rat (6 h after induction) (Fig. 1a, right). Monosynaptic EPSCs of progressively larger amplitudes were evoked by electrical synaptic stimulation with increasing intensities, and input–output relationships were obtained by measuring EPSC peak amplitude (picoamperes) as a function of afferent fiber volley stimulus intensity (microamperes) for each neuron (Fig. 1b). The input–output function of the PB–CeLC synapse was significantly enhanced in a CeLC neuron recorded in a brain slice from an arthritic rat (6 h after induction) (Fig. 1). The inhibitory effects of CGRP1–37 were particularly pronounced at higher stimulus intensities (n = 12; p < 0.0001; F(1,242) = 76.32, two-way ANOVA followed by Bonferroni’s post hoc tests). These data suggest that enhanced activation of CGRP1 receptors by the endogenous ligand CGRP represents an important mechanism of pain-related synaptic plasticity in the amygdala.

**Figure 2.** CGRP1–37 inhibits neuronal excitability of CeLC neurons in the arthritis pain model but not under normal conditions. Action potentials were evoked in CeLC neurons by direct (through the patch electrode) intracellular injections of current pulses (250 ms) of increasing magnitude (50 pA steps) before and during CGRP1–37 administration. a, b, CGRP1–37 did not affect the action potential firing rate in CeLC neurons in slices from normal rats. c, d, However, the action potential firing rate was significantly decreased by CGRP1–37 in CeLC neurons from arthritic rats (n = 10; p < 0.0001; F(1,144) = 14.15, two-way ANOVA), suggesting a functional change of CGRP1 receptor activation that has postsynaptic effects in arthritis but not under normal conditions. For the measurement of action potential firing in current clamp, neurons were recorded at −60 mV. Calibration (in a, c): top traces, 100 ms, 25 mV; bottom traces, 100 ms, 150 pA. Symbols and error bars in b and d represent mean ± SE.

**Postsynaptic rather than presynaptic CGRP1 receptor activation in pain-related synaptic plasticity**

The major source of CGRP in the CeLC is the external lateral parabrachial area (Kruger et al., 1988;Schwabe et al., 1988; Harrigan et al., 1994; de Lacalle and Saper, 2000), which is part of the spino-parabrachio-amygdaloid pain pathway (Bernard and Bandler, 1998) and projects heavily to the CeLC. To assess whether CGRP acts on presynaptic or postsynaptic sites in the CeLC, we used a number of well established electrophysiological methods, including the analysis of neuronal excitability (Fig. 2), amplitude and frequency of spontaneous mEPSCs (Fig. 3a–c), and paired-pulse facilitation (Fig. 3d,e). These parameters were measured before and during application of CGRP1–37 in amygdala brain slices from normal and arthritic rats.

Action potentials were evoked in current-clamp mode by direct intracellular current injections of increasing magnitude through the patch electrode. Input–output functions of neuronal excitability were obtained by averaging the frequency of action potentials evoked at each current intensity. CGRP1–37 significantly decreased the input–output function of CeLC neurons from arthritic rats (Fig. 2c,d) (n = 10; p < 0.0001; F(1,144) = 14.15, two-way ANOVA) but had no significant effect in CeLC neurons from normal rats (Fig. 2a,b) (n = 14; p > 0.05; F(1,206) = 0.44, two-way ANOVA). Accordingly, the analysis of current–voltage relationships in voltage clamp showed that CGRP1–37 decreased the slope conductance in CeLC neurons from arthritic rats significantly (p < 0.01, paired t test; CGRP1–37, 4.88 ± 1.57 nS; predrug control, 5.80 ± 1.57 nS; n = 9). CGRP1–37 had no significant effect on the slope conductance of control neurons from arthritis in CeLC neurons from arthritic rats (Fig. 1e; p < 0.05, paired t test; n = 5; Fig. 1c, right, individual current traces). Synaptic responses were evoked by a stimulus intensity adjusted to 70–80% of that required for generating the maximum EPSC amplitude.

Cumulative concentration–response relationships show that CGRP1–37 inhibited synaptic plasticity in CeLC neurons (n = 29) from arthritic rats more efficaciously than basal synaptic transmission in control neurons (n = 17) from normal rats (Fig. 1f; p < 0.01; F(1,144) = 10.74, two-way ANOVA; Bonferroni’s post hoc tests indicate significant differences for individual concentrations). The IC50 did not change significantly in the arthritis pain model compared with normal controls (2.7 and 1.1 ns, respectively; see Materials and Methods). CGRP1–37 also changed the input–output function of the PB–CeLC synapse in the arthritis pain state to the level recorded under normal conditions (Fig. 1e). The inhibitory effects of CGRP1–37 were particularly pronounced at higher stimulus intensities (n = 12; p < 0.0001; F(1,242) = 76.32, two-way ANOVA followed by Bonferroni’s post hoc tests). These data suggest that enhanced activation of CGRP1 receptors by the endogenous ligand CGRP represents an important mechanism of pain-related synaptic plasticity in the amygdala.
normal animals ($p < 0.01$, paired $t$ test; CGRP$_{8-37}$ 4.45 ± 1.39 nS; predrug control, 4.69 ± 1.22 nS; $n = 14$). In agreement with our previous studies (Neugebauer et al., 2003; Bird et al., 2005), these data also show that the slope conductances of CeLC neurons in the arthritis pain model were increased compared with control neurons. Together, these results suggest that activation of CGRP1 receptors is involved in the pain-related increase of neuronal excitability in the amygdala.

The analysis of spontaneous mEPSCs in the presence of TTX is a well established electrophysiological approach to determine presynaptic versus postsynaptic mechanisms. Presynaptic changes at the transmitter release site affect mEPSC frequency, whereas changes at the postsynaptic membrane would alter mEPSC amplitude (quantal size) (Wyllie et al., 1994; Han et al., 2004). CGRP$_{8-37}$ decreased the amplitude, but not frequency, of mEPSCs recorded in TTX (1 μM) in slices from arthritic rats (Fig. 3). This postsynaptic effect is illustrated in the current traces recorded in an individual CeLC neuron (Fig. 3a). In the whole sample of neurons ($n = 4$), CGRP$_{8-37}$ caused a shift of the normalized cumulative mEPSC amplitude distribution toward smaller amplitudes ($p < 0.005$, Kolmogorov–Smirnov test) and decreased the mean mEPSC amplitude significantly ($p < 0.05$, paired $t$ test; $n = 4$) (Fig. 3b). CGRP$_{8-37}$ had no effect on the frequency of mEPSCs (see normalized cumulative interevent interval distribution and mean mEPSC frequency in Fig. 3c) ($p > 0.05$, paired $t$ test; $n = 4$).

Further arguing against a presynaptic site of action, CGRP$_{8-37}$ had no significant effect on PPF. PPF refers to the observation of a PKA inhibitor (KT5720, 1 μM) (Fig. 4), which has been shown before to block the NMDA-mediated component of synaptic transmission in CeLC neurons in the arthritis pain model (Bird et al., 2005). KT5720 was applied by superfusion of the slice (Fig. 4a–c) or directly into the cell through the patch pipette (Fig. 4d–f). In the presence of the PKA inhibitor, CGRP$_{8-37}$ produced no additional inhibition, suggesting that CGRP1 receptor activation requires PKA because the effects of CGRP$_{8-37}$ were occluded by the PKA inhibitor. Importantly, the fact that direct intracellular application of KT5720 also prevented the inhibitory effects of CGRP$_{8-37}$ strongly suggests a postsynaptic site of action. The magnitude of inhibition by KT5720 and CGRP$_{8-37}$ was comparable with that by an NMDA receptor antagonist (AP-5) reported previously (Bird et al., 2005). Because NMDA receptors contribute to pain-related synaptic plasticity but not normal transmission in the CeLC (Bird et al., 2005), these data suggest the selective involvement of PKA and CGRP1 receptors in NMDA-mediated synaptic plasticity.

![Figure 3.](image_url) mEPSC analysis and PPF suggest postsynaptic rather than presynaptic effects of CGRP$_{8-37}$. a, Original current traces of mEPSC recordings in an individual CeLC neuron in the presence of TTX (1 μM) showed that CGRP$_{8-37}$ (1 μM) reduced amplitude but not frequency of mEPSCs. Calibration: 1 s, 20 mV. The CeLC neuron was recorded in a slice from an arthritic rat. b, c, Normalized cumulative distribution analysis of mEPSC amplitude and frequency showed that CGRP$_{8-37}$ caused a significant shift toward smaller amplitudes (b) ($p < 0.005$; maximal difference in cumulative fraction, 0.175; Kolmogorov–Smirnov test) but had no effect on the interevent interval (frequency) distribution (c). d, CGRP$_{8-37}$ selectively decreased mean mEPSC amplitude ($p < 0.05$, paired $t$ test) but not mEPSC frequency (events per second) in the sample of neurons ($n = 4$; see bar histograms in d, e). PPF, a measure of presynaptic mechanisms, was not changed by CGRP$_{8-37}$. PPF was calculated as the ratio of the second and the first of two consecutive EPSCs evoked by two electrical stimuli of equal intensity at increasing interstimulus intervals. Peak EPSC amplitudes were measured as the difference between the current level before the stimulus artifact and the peak of the EPSC. d, Current traces (average of 8–10 EPSCs) recorded in an individual CeLC neuron illustrate that PPF evoked at a 50 ms interval was not affected by CGRP$_{8-37}$. e, CGRP$_{8-37}$ had no significant effect on PPF at various stimulus intervals in the whole sample of neurons ($n = 6$; $p > 0.05$, paired $t$ test), further arguing against a presynaptic action. Symbols and error bars represent mean ± SE. Neurons were recorded in voltage clamp at −60 mV.
CGRP8-37 significantly decreased the peak amplitude and area under the curve (total charge) of the NMDA receptor-mediated inward current (p < 0.05, paired t test; n = 5) (Fig. 5a, c). However, membrane currents evoked by exogenous AMPA (30 μM) were not affected by CGRP8-37 in terms of peak amplitude and area under the curve (p > 0.05, paired t test; n = 5) (Fig. 5b, d).

Together with our previous studies (Bird et al., 2005), these data show that CGRP1 receptors contribute to synaptic plasticity through the activation of PKA and PKA-dependent postsynaptic NMDA receptor function.

**Endogenous activation of CGRP1 receptors is required for pain-related sensitization of CeLC neurons**

Whereas the reduced brain slice preparation allows the definitive analysis of neuronal plasticity maintained in a particular brain area, the processing of pain-related information in identified nociceptive neurons and its behavioral consequence can only be studied at the systems level in the whole animal. To determine the...
significance of CGRP1 receptor activation, we therefore studied the effects of CGRP1 receptor block on electrophysiological responses in anesthetized animals and on behavioral responses in awake animals.

Extracellular single-unit recordings were made from CeLC neurons in anesthetized rats, and drugs were administered into the CeLC through microdialysis probes as described in detail previously (Neugebauer and Li, 2003; Li and Neugebauer, 2004a). All CeLC neurons included in this study responded more strongly to noxious (painful in the awake subject) than innocuous stimuli and were classified as MR neurons. MR neurons represent the class of amygdala neurons that consistently develop sensitization to afferent inputs in the arthritis pain model (Neugebauer and Li, 2003; Li and Neugebauer, 2004a). Responses to innocuous and noxious stimuli were recorded continuously in the same neuron before and after the induction of a localized mono-arthritis in the knee (Neugebauer et al., 2004). Figure 6a shows an individual example (histograms display the number of action potentials or spikes per second). The CeLC neuron responded more strongly to brief (15 s) noxious than innocuous stimulation (compression) of the knee joint under control conditions. In the arthritis pain state (6 h after induction), the responses of the same neuron increased not only to stimulation of the arthritic knee but also of non-injured tissue in the ankle, paw, and forelimb (data not shown). The increased responsiveness of neurons in the CNS to stimulation of non-injured tissues is generally accepted to indicate a state of “central sensitization” and is now also well documented for CeLC neurons (Neugebauer et al., 2004). Administration of CGRP8–37 (100 μM, concentration in microdialysis fiber; 20 min) into the CeLC next to the recorded neuron strongly reduced the enhanced responses in a reversible manner.

The inhibitory effects of CGRP1 receptor antagonists on pain-related sensitization in the amygdala (CeLC) are summarized in Figure 6b. CGRP8–37 (n = 7) and BIBN4096BS (n = 4) significantly (p < 0.01–0.05, paired t test) reduced the enhanced responses of CeLC neurons to stimulation of the arthritic knee with normally innocuous and noxious intensities (see Materials and Methods). Drug effects were reversible after washout for 20–30 min. Antagonists were administered by microdialysis into the CeLC (100 μM, concentration in microdialysis fiber; 20 min). Figure 6c shows the functional change of endogenously activated CGRP1 receptors in the CeLC in the arthritis pain model compared with normal conditions. Concentration–response relationships were obtained for the effects of CGRP8–37 on the responses of CeLC neurons to noxious stimulation of the arthritic knee and the non-injured ankle. CGRP8–37 was more efficacious in sensitized neurons 6 h after induction of arthritis (n = 7) than under normal conditions before arthritis (n = 7). The differences of drug effects between arthritis and normal conditions were statistically significant (knee, p < 0.001, F(1,25) = 26.57; ankle, p < 0.05, F(1,25) = 6.36; two-way ANOVA). The IC50 values were not significantly different (knee, 6.8 and 2.1 μM; ankle 4.3 and 11 μM, normal versus arthritis; concentrations in the microdialysis fiber). CGRP8–37 was administered by microdialysis for 20 min, and measurements were made at 15 min.

Recordings and drug administrations were made into the right CeLC contralateral to the arthritis because of the strong contralateral projection of the spino-parabrachio-amygdaloid pain pathway and our previous studies showing pain-related plasticity in the right (contralateral) CeLC (Neugebauer et al., 2004). All recording sites in the CeLC were confirmed histologically (see Fig. 8b). These data suggest that CGRP1 receptors in the

**Figure 6.** CGRP1 receptor antagonists inhibit nociceptive sensitization of CeLC neurons in anesthetized intact animals. a, Extracellular recordings of the responses of one multireceptive CeLC neuron to innocuous (100 g/30 mm2) and noxious (2000 g/30 mm2) mechanical stimulation of the knee joint before and 6 h after induction of the knee joint arthritis (see Materials and Methods). CGRP8–37 (100 μM) was administered into the CeLC by microdialysis for 20 min. Histograms show action potentials (spikes per second) (bin width, 1 s). Horizontal bars indicate duration of stimuli (15 s). b, Averaged raw data (spikes per second) for the sample of neurons tested with CGRP8–37 (100 μM; n = 7) and BIBN4096BS (100 μM; n = 4). Both antagonists significantly reduced the increased responses to normal levels (p < 0.01–0.05, paired t test). The inhibitory effects were reversible after washout for 20–30 min with ACSF in the microdialysis fiber. Bar histograms and error bars represent mean ± SE. *p < 0.05, **p < 0.01. Concentration–response relationships show that CGRP8–37 was more efficacious in sensitized neurons 6 h after induction of arthritis (n = 7) than under normal conditions before arthritis (n = 7). The differences of drug effects between arthritis and normal conditions were statistically significant (knee, p < 0.001, F(1,25) = 26.57; ankle, p < 0.05, F(1,25) = 6.36; two-way ANOVA). *p < 0.05; **p < 0.01; ***p < 0.001 (Bonferroni’s post hoc tests). Responses to brief (15 s) noxious stimulation of the knee or ankle during each concentration of CGRP8–37 were averaged and expressed as percentage of predrug (baseline) control (100%). CGRP8–37 was administered by microdialysis for 20 min, and measurements were made at 15–20 min. Concentrations shown indicate concentrations in the microdialysis fiber.
CeLC are activated endogenously in the arthritis pain state in the intact animal and are required for the pain-related sensitization of CeLC neurons.

Endogenous activation of CRGPR1 receptors in the amygdala is required for pain behavior organized at different levels of the pain neuraxis

To validate the significance of the CRGPR1 receptor activation observed in the electrophysiological studies, we analyzed the effect of CRGPR<sub>8-37</sub> on supraspinally (vocalizations) and spinally (hindlimb withdrawal reflexes) organized behavior in awake animals. Pain-related vocalizations in the audible and ultrasonic range were measured in the same animal before and after induction of arthritis and before and during administration of CRGPR<sub>8-37</sub> into the CeLC by microdialysis as described previously (Han and Neugebauer, 2005). Audible and ultrasonic vocalizations evoked by noxious stimuli represent nocifensive and affective responses, respectively (Han and Neugebauer, 2005). Vocalizations are further classified as VDSs, which are organized at the medullary brainstem level, and VADs, which outlast the actual stimulus and are organized in the limbic forebrain, particularly the amygdala (Borszcz and Leaton, 2003; Han and Neugebauer, 2005).

The duration of audible and ultrasonic vocalizations of the VAD and VDS types increased in the arthritis pain model (6 h after induction) (Fig. 7a,b). Administration of CRGPR<sub>8-37</sub> (100 μM, concentration in the microdialysis probe; 15–20 min) into the CeLC inhibited the duration of audible and ultrasonic VADs (Fig. 7a, right) and VDSs (Fig. 7b, right) evoked by noxious (2000 g/30 mm²) stimulation (15 s) of the arthritic knee (n = 9). The inhibitory effects of CRGPR<sub>8-37</sub> were significant (p < 0.01–0.05; paired t test) and reversible. In contrast, CRGPR<sub>8-37</sub> had no significant effect on the vocalizations of naive (non-arthritic) animals (n = 5) (Fig. 7a,b, left). Predrug vocalizations were measured before administration of ACSF through the microdialysis probe, thus serving as vehicle controls. Drugs were administered into the right CeLC contralateral to the arthritis because of the strong contralateral projection of the spino-parabrachio-amygdaloid pain pathway and our previous studies showing pain-related plasticity in the right CeLC (Neugebauer et al., 2004). All animals had guide cannulas for the microdialysis probes implanted on the day before the behavioral tests. The positions of the microdialysis probes in the CeLC were verified histologically (Fig. 8c).

The vocalization data suggest that chemical inactivation of the CeLC by CRGPR<sub>8-37</sub> inhibits pain responses organized in the brainstem (VDS) and limbic forebrain (VAD). Next we determined the contribution of CRGPR1 receptors in the CeLC to pain responses organized at the level of the spinal cord. Hindlimb withdrawal reflexes in response to stimulation (compression) of the knee were measured before and after induction of arthritis and before and during drug application (Fig. 7c). Mechanical stimuli of increasing intensity (steps of 50 g/30 mm²) were applied to the knee joint. Withdrawal threshold was defined as the minimum stimulus intensity that evoked a withdrawal reflex. Thresholds decreased after arthritis induction. Application of CRGPR<sub>8-37</sub> into the CeLC significantly increased the reduced thresholds in arthritic animals (right; n = 6; p < 0.05, paired t test) but had no effect in normal rats (left; n = 4; p > 0.05, paired t test) (Fig. 7c). Placebo control experiments showed that application of CRGPR<sub>8-37</sub> into the striatum (caudate-putamen dorsal lateral to CeLC) did not produce significant changes of audible and ultrasonic vocalizations in arthritic animals (n = 5; p > 0.05, paired t test).
As a control for any drug effects attributable 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 animals as placement controls (Fig. 7d). The striatum was selected because it is located adjacent (dorsolateral) to the CeLC but does not form direct projections to the CeLC (Neugebauer et al., 2004; Han and Neugebauer, 2005). Thus, drug application into this area should not have any effect on CeLC-mediated functions. This site, however, is sufficiently close to the CeLC to be useful as a control for drug diffusion. Administration of CGRP$_{8-37}$ (100 μM, 15 min; $n = 5$) (Fig. 7d) into the striatum had no significant effects on the audible and ultrasonic vocalizations and withdrawal reflexes (data not shown) in arthritic animals evoked by stimulation of the knee ($p > 0.05$, paired $t$ test). The positions of the microdialysis probes in the CeLC were verified histologically (Fig. 8d).

**Discussion**

The present study is the first to show that the endogenous activation of CGRP1 receptors in the nociceptive amygdala (CeLC) contributes critically to pain-related synaptic plasticity in the CeLC and consequently to pain behavior. Our integrative approach of *in vitro* and *in vivo* electrophysiology and behavioral analysis allowed us to determine the cellular mechanisms of CGRP1 receptor function and their significance at the systems level. The major findings are as follows. (1) Selective CGRP1 receptor antagonists (CGRP$_{8-37}$ and BIBN4096BS) inhibited synaptic plasticity and neuronal excitability in CeLC neurons *in vitro* in the arthritis pain model induced *in vivo*. (2) Analysis of spontaneous miniature EPSCs, PPF, and membrane effects indicated a postsynaptic rather than presynaptic mechanism. (3) The occlusion of CGRP$_{8-37}$ effects by a PKA inhibitor and the direct inhibition of NMDA, but not AMPA, receptor activation by CGRP$_{8-37}$ suggested that CGRP1 receptors couple to PKA activation and NMDA receptor function. (4) CGRP$_{8-37}$ and BIBN4096BS reversed the pain-related sensitization of nociceptive CeLC neurons recorded *in vivo*. (5) Chemical inactivation of the CeLC by CGRP$_{8-37}$ inhibited spinally (withdrawal reflexes) and supraspinally (vocalizations) organized pain behavior in awake animals.

The amygdala is well positioned to play an important role in the clinically important reciprocal relationship between pain and emotional-affective states (Rhudy and Meagher, 2001; Gallagher and Verma, 2004). The amygdala has long been known to be critically involved in mechanisms of fear, anxiety, and depression (Davis, 1998; Davidson et al., 1999; LeDoux, 2000; Rodrigues et al., 2004). More recent research has linked the amygdala also to the pain system (Neugebauer et al., 2004). The present study makes a major contribution toward a better understanding of the pain-affect relationship by providing valuable novel information about mechanisms of plasticity in the nociceptive amygdala and their behavioral consequences. Assuming that affective states and disorders can mimic pain-related plasticity in the amygdala, they would be able to gain access to the modulation of pain and pain behavior through the nociceptive amygdala. Although this hypothesis awaits proof, the present study sets the stage for mechanistic research into the pain-affect interaction.

Our previous studies showed plastic changes in the nociceptive amygdala in an arthritis pain model (Neugebauer and Li, 2003; Neugebauer et al., 2003; Li and Neugebauer, 2004a,b; Bird et al., 2005). Plasticity was measured as increased synaptic transmission in the nociceptive parabrachio-amygdaloid pathway, enhanced processing of nociceptive signals (sensitization), and increased neuronal excitability of CeLC neurons, which would result in increased output functions of the amygdala. Mechanisms and consequences of such plastic changes are only beginning to emerge (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Pain-related plasticity in the nociceptive amygdala (CeLC) depends on the activation and upregulation of presynaptic metabotropic glutamate receptors of the mGluR1 subtype (Neugebauer et al., 2003; Li and Neugebauer, 2004a), which can regulate the release of glutamate and other transmitters and modulators, including neuropeptides (Cartmell and Schoepp, 2000). Postsynaptically, normally “silent” NMDA receptors become functional through phosphorylation, but not upregulation, that depends on PKA but not PKC (Li and Neugebauer, 2004b; Bird et al., 2005). Which molecule, however, can account for PKA activation and thus serve as the missing link between presynaptic and postsynaptic sites?

CGRP is a prime candidate for several reasons. The exclusive source of CGRP in the amygdala is the lateral parabrachial area...
(Kruger et al., 1988; Schwaber et al., 1988; Harrigan et al., 1994; de Lacalle and Saper, 2000), which is part of the spino-parabrachio-amygdaloid pain pathway (Bernard and Bandler, 1998). CGRP-immunoreactive terminals target specifically the CeLC and innervate CeLC neurons that project to brainstem areas such as the periaqueductal gray (Schwaber et al., 1988; Harrigan et al., 1994; Xu et al., 2003). The central nucleus of the amygdala also contains particularly high levels of CGRP binding sites (Van Rossum et al., 1997; Oliver et al., 1998) but no CGRP mRNA-expressing or CGRP-immunoreactive neurons (Van Rossum et al., 1997). This mismatch suggests that the endogenous activation of CGRP receptors observed in the present study is attributable to release of CGRP from the spino-parabrachio-amygdaloid pain pathway but not from intrinsic circuits.

CGRP binds to G-protein-coupled receptors, which activate adenyl cyclase and cAMP-dependent PKA (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997). Pharmacologically, two classes of CGRP receptors have been proposed (CGRP1 and CGRP2 receptors), which have no significant affinity for calcitonin-like peptides (Poyner, 1996; Wimalawansa, 1996; Oliver et al., 1998; Hashbak et al., 2003). CGRP1 but not CGRP2 receptors have been cloned (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997) and are expressed at particularly high levels in the central nucleus of the amygdala (Oliver et al., 1998). Specific antagonists such as the C-terminal fragment CGRP8–37 and the nonpeptide compound BIBN4096BS are available for CGRP1 but not CGRP2 receptors (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997; Doods et al., 2000). CGRP1 receptors consist of three different proteins: the calcitonin receptor-like receptor (CRLR), receptor activity-modifying protein (RAMP1), and the receptor component protein (RCP) (Hashbak et al., 2003). RAMP1 defines the ligand-binding site, whereas RCP couples the receptor to signal transduction pathways (Hasbak et al., 2003). The low nanomolar affinities for CGRP8–37 measured in the present study are consistent with the binding to the CRLR component of the CGRP1 receptor (Oliver et al., 1998; Hasbak et al., 2003). The change in efficacy of CGRP8–37 can be the result of increased CGRP release as well as RCP-mediated enhanced coupling of the CGRP1 receptor to second messengers, including PKA activation.

The present study shows that CGRP1 serves as the critical molecule to link presynaptic and postsynaptic mechanisms of pain-related plasticity in the CeLC and contributes to pain behavior organized at different levels of the pain neuraxis. Patch-clamp experiments using CGRP8–37, PKA inhibition, and NMDA receptor activation suggest that CGRP1 receptor activation can modulate NMDA receptor function through PKA (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The synaptic component inhibited by block of PKA and/or CGRP1 receptors was comparable with that mediated by NMDA receptors in CeLC plasticity shown previously (Bird et al., 2005), suggesting the selective involvement of PKA and CGRP1 receptors in NMDA-mediated synaptic plasticity. This conclusion is also supported by the direct inhibitory effect of CGRP8–37 on NMDA-, but not AMPA-, evoked membrane currents (Fig. 5). Our data further suggest that CGRP1 receptor activation occurs at the postsynaptic rather than presynaptic site (Figs. 2, 3). CGRP8–37 inhibited action potential firing evoked by direct depolarizing current injections into the neuron (postsynaptic site). PPF, a measure of presynaptic changes, was not affected. Amplitude distribution (quantal size), but not frequency, of spontaneous miniature EPSCs was decreased by CGRP8–37. CGRP8–37 inhibited the membrane current evoked by direct application of NMDA.

Whereas the involvement of CGRP receptors in peripheral and spinal pain mechanisms is well documented (Galeazzi et al., 1995; Neugebauer et al., 1996; Schäible, 1996; Ruda et al., 2000; Sun et al., 2004), less is known about the role of CGRP receptors in the brain in models of prolonged or persistent pain states. One laboratory reported that administration of exogenous CGRP into periaqueductal gray (Yu et al., 2003), nucleus accumbens (Li et al., 2001), or amygdala (Xu et al., 2003) had antinoceptive behavioral effects in naive animals. Our electrophysiological data show that CGRP1 antagonists inhibit the processing of nociceptive signals in CeLC neurons in the arthritis model of persistent pain. Our behavioral studies further suggest that block of CGRP1 receptors in the CeLC inhibits spinally and supraspinally organized pain behavior, which is consistent with the inhibition of amygdala-mediated descending pain facilitation (Gebhart, 2004; Neugebauer et al., 2004; Vanegas and Schaible, 2004). Vocalizations have been used successfully previously to determine higher integrated pain behavior (Borszcz and Leaton, 2003; Han and Neugebauer, 2005; Han et al., 2005; Ko et al., 2005). Whereas our antagonist study suggests that endogenous activation of CGRP1 receptors in the amygdala produces pain behavior through descending facilitation, one behavioral study reported antinoceptive effects of exogenous CGRP administration into the central nucleus of the amygdala (Xu et al., 2003) (see above). However, these experiments were done in normal rats (not in a pain model). Additionally, drugs were administered into the left amygdala. In the present study, we targeted the right amygdala because our previous electrophysiological in vivo and in vitro studies showed pain-related plasticity in the right amygdala (Neugebauer and Li, 2003; Neugebauer et al., 2003) and our behavioral data indicated that the right amygdala is coupled to pain facilitation in the arthritis pain model (Han and Neugebauer, 2005). This is consistent with a strong contralateral projection of the spino-parabrachio-amygdaloid pain pathway (Bernard and Bandler, 1998; Neugebauer et al., 2004) (arthritis was induced in the left knee in this and our previous studies). It remains to be determined whether lateralization or differences between normal conditions and persistent pain can account for this difference.

In conclusion, the present study shows for the first time that the endogenous activation of CGRP1 receptors in the CeLC is critically involved in pain-related plasticity (in vivo and in vitro electrophysiology) and contributes to nociceptive and affective pain responses (audible and ultrasonic vocalizations, respectively). Therefore, CGRP1 receptors in the amygdala may be a novel therapeutic target for pain relief. Furthermore, they can play an important role in the pain–affect interaction if affective states and disorders indeed mimic pain-related plasticity in the amygdala and thus gain access to pain modulation. Our proposed model (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) will allow testing of this hypothesis.

References


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