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ROLE OF CGRP IN THE AMYGDALA IN PAIN-RELATED SYNAPTIC PLASTICITY AND BEHAVIOR

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ROLE OF CGRP IN THE AMYGDALA IN PAIN-RELATED SYNAPTIC PLASTICITY AND BEHAVIOR

by
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Dissertation
Presented to the Faculty of The University of Texas Graduate School of
Biomedical Sciences at Galveston
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy in Neuroscience

Approved by the Supervisory Committee

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December, 2005
Galveston, Texas

Key words: vocalizations, neuropeptides, arthritis

To my family

ACKNOWLEDGEMENTS

There are many people who helped and supported me in completing this dissertation. First of all, I would like to thank my mentor Dr. Neugebauer for all of his support throughout the Ph.D. study. I am very grateful that I have been able to have such a great relationship with my mentor. Working under his guidance has been a very pleasant and rewarding experience. I wish to offer special thanks and gratitude to all of the committee members for providing invaluable advice and guidance: Drs. William Willis, Karin High, Haring Nauta, Melvyn Soloff, and Robert Gereau.

I'm also indebted to many faculty members in the Neuroscience department for their help. I would like to thank Dr. Blankenship, Dr. Chung and Drs. Leonard for all of their support, encouragement and passion.

Finally, I wish to thank my wife, parents and colleagues who provided continued support as I traveled this journey.

ROLE OF CGRP IN THE AMYGDALA IN PAIN-RELATED SYNAPTIC PLASTICITY AND BEHAVIOR

Publication No._____

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Pain has a strong emotional component. In particular, arthritic pain is closely related to affective disorders such as anxiety and depression. A key player in the emotional evaluation of sensory stimuli, the amygdala has been suggested as a neural substrate of the reciprocal relationship between pain and affect. Nociceptive information reaches the central nucleus of the amygdala (CeA) through the spino-parabrachio-amygdaloid pain pathway and spino-amygdaloid connections. Highly processed polymodal information from thalamo-cortical areas is transmitted to the CeA through the lateral and basolateral amygdaloid nuclei. Previous studies have shown that CeA neurons undergo significant neuroplastic changes in a model of arthritic pain.

Importantly, high levels of calcitonin gene-related peptide (CGRP) are present in the CeA, most notably in the latero-capsular part (CeLC), which is now defined as the "nociceptive amygdala". The parabrachio-amygdaloid connection is essentially the exclusive source of CGRP in the CeA. The role of CGRP in pain processing has been studied mainly in peripheral tissues and in the spinal cord. However, information about CGRP function in the brain is still limited. Moreover, the role of neuropeptides in synaptic plasticity is less well understood than that of classical transmitters such as glutamate.

The present study determined the role of CGRP in the amygdala at the cellular and systems levels, using an integrative approach that combines patch-clamp recordings in brain slices in vitro and analysis of spinally (withdrawal reflexes) and supraspinally (vocalizations) organized behavior in awake animals.

A well-established preclinical animal model of arthritic pain induced by kaolin and carrageenan was used. Our results show that selective CGRP1 receptor antagonists ($CGRP_{8-37}$ and BIBN4096NS) in the CeLC reverse arthritis pain-related plasticity and behavior through a PKA-dependent postsynaptic mechanism that involves NMDA receptors. In addition, exogenous CGRP application in the CeLC in naïve animals mimics pain-related synaptic plasticity and behavior. Selective inhibitors of PKA and MEK (ERK1/2), but not PKC, inhibit pain-related synaptic plasticity and behavior in arthritic animals and CGRP-induced synaptic plasticity and behavior in naïve animals.

This work shows for the first time the importance of CGRP as a critical link between amygdala plasticity and pain behavior. The results provide direct evidence that CGRP receptor activation in the amygdala contributes to pain-related synaptic plasticity in the amygdala through a postsynaptic mechanism that involves PKA, ERK1/2 and NMDA receptors. This plasticity results in spinally and supraspinally organized pain behavior.

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LIST OF ABBREVIATIONS

ACSF…….	artificial cerebrospinal fluid
AMPA …….	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA …….	analysis of variance
BLA …….	basolateral nucleus of the amygdala
cAMP…….	cyclic adenosine monophosphate
CeA ……...	central nucleus of the amgydala
CeLC …….	latero-capsular division of the central nucleus of the amygdala
CHPG …….	carboxy-hydroxy-phenylglycine
CRLR …….	calcitonin receptor-like receptor
CGRP …….	calcitonin gene-related peptide
DHPG …….	dihydroxyphenylglycine
EPSC …….	excitatory postsynaptic current
ERK …….	extracellular signal-regulate kinase
fMRI …….	functional magnetic resonance imaging
I-V …….	current-voltage
LA …….	lateral nucleus of the amygdala
MEK …….	MAP/ERK kinase
mEPSC ……..	miniature excitatory postsynaptic current
mGluR …….	metabotropic glutamate receptor
MAP kinase …….	mitogen activated protein kinase
MR ……...	multi-receptive
NMDA …….	N-methyl-D-aspartate
NR …….	NMDA receptor
noSOM …….	non-responsive to somatic stimuli
PAG …….	periaqueductal gray matter
PB …….	parabrachial nucleus
PET …….	positron emission tomography

PKA ……. protein kinase A

PKC ……. protein kinase C

PPF ……. paired pulse facilitation

RCP ……. CGRP-receptor component protein

RVM …….rostro-ventral medulla

TTX ……..... tetrodotoxin

VAP ……. ventral amygdaloid pathway

VAD ……. vocalization afterdischarge

VDS ……. vocalization during stimulation

CHAPTER 1: INTRODUCTION

PAIN PROCESSING IN THE AMYGDALA

The amygdala plays a critical role in emotional learning and memory, affective state, and mood and anxiety disorders (Davis, 1998; Cahill, 1999; Gallagher and Schoenbaum, 1999; Maren, 1999; LeDoux, 2000; Cardinal et al., 2002; Davidson, 2002; Zald, 2003). Accumulating evidence suggests that the amygdala integrates nociceptive information with affective content, contributes to the emotional response to pain, and serves as a neuronal substrate for the reciprocal relationship between pain and affective states and disorders (Neugebauer et al., 2004). The amygdala includes anatomically and functionally distinct nuclei. The lateral, basolateral and central nuclei of the amygdala (LA, BLA and CeA, respectively) are of particular importance for the processing and evaluation of sensory information (**Fig. 1**). Polymodal sensory information reaches the amygdala from thalamus (midline and posterior areas) and cortex, including insular cortex, anterior cingulate cortex and association cortical areas (Shi and Davis, 1999; LeDoux, 2000; Stefanacci and Amaral, 2000; Price, 2003; Neugebauer et al., 2004; Pare et al., 2004). The LA serves as the major input region and initial site of sensory convergence in the amygdala. Associative learning and plasticity in the lateral-basolateral nucleus of the amygdala (LA-BLA) circuitry plays a key role in affective states and disorders such as fear and anxiety (Shi and Davis, 1999; LeDoux, 2000; Pare et al., 2004). This highly processed information with "affective" content is transmitted from the LA-BLA circuitry to the CeA, the output nucleus for major amygdala functions. The CeA receives inputs from other amygdala nuclei without forming reciprocal intra-amygdaloid connections.

The amygdala receives nociceptive-specific (i.e., strictly pain-related) information from the brainstem (parabrachial area) and spinal cord through the spino-parabrachio-amygdaloid pain pathway and spino-amygdaloid projections (Burstein and Potrebic, 1993; Wang et al., 1999; Gauriau and Bernard, 2002; 2004; Braz et al., 2005) (**Fig. 1**). The nociceptive inputs ultimately converge onto neurons in the latero-capsular part of the

CeA (CeLC), which is now defined as the "nociceptive amygdala" because of its high content of nociceptive neurons (Bourgeais et al., 2001; Neugebauer et al., 2004).

The CeA forms widespread connections with forebrain and brainstem areas to regulate autonomic, somatomotor, and other functions related to emotional behavior (**Fig. 2**). CeLC neurons project heavily to the substantia innominata dorsalis; midline and mediodorsal thalamic nuclei and paraventricular hypothalamus via the stria terminalis; and lateral hypothalamus and brainstem areas such as periaqueductal gray (PAG) and parabrachial area (PB) via the ventral amygdaloid pathway (VAP) (LeDoux, 2000; Bourgeais et al., 2001; Price, 2003; Neugebauer et al., 2004). The convergence of pain- and affect-related inputs onto the CeA and the widespread efferent projections of the CeA to brain areas involved in pain behavior and affective state, suggest an important role for this particular part of the amygdala in the emotional-affective pain response and pain modulation (Bernard et al., 1996; Millan, 1999; Heinricher and McGaraughty, 1999; Bourgeais et al., 2001; Rhudy and Meagher, 2001; Gauriau and Bernard, 2002; Neugebauer et al., 2004).

Evidence supporting a role of the amygdala in pain processing comes from imaging, behavioral, electrophysiological and pharmacological studies. Functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies using a variety of nociceptive stimuli have repeatedly detected pain-related activity changes in the amygdala in humans as well as experimental animals (Derbyshire et al., 1997; Becerra et al., 1999; Schneider et al., 2001; Bingel et al., 2002; Bornhovd et al., 2002; Paulson et al., 2002; Bonaz et al., 2002; Porro, 2003; Mayer et al., 2005). Lesions and pharmacological manipulations of the amygdala produced analgesic effects in behavioral tests in animals (Kang et al., 1998; Hebert et al., 1999) but also eliminated morphine induced analgesia (Manning and Mayer, 1995). Despite some discrepancies due to functional and structural complexities of the amygdala (activation *vs* deactivation in imaging studies and pronociceptive *vs* antinociceptive functions in behavioral studies), these studies have implicated the amygdala in pain processing and pain modulation.

More detailed information about pain processing in the amygdala has come from electrophysiological studies. The vast majority of CeLC neurons respond exclusively or predominantly to noxious stimulation of superficial and deep body tissue (Neugebauer and Li, 2002). CeLC neurons typically have large, often symmetrical bilateral receptive fields that can include the whole body, arguing against a sensory-discriminative role of the amygdala (Neugebauer et al., 2004). Several types of CeLC neurons have been identified that may reflect different contributions of the amygdala to pain (Neugebauer et al., 2004). Nociceptive-specific (NS) neurons are activated exclusively by noxious stimuli and preserve information directly related to pain. Multireceptive neurons (MR), which respond to innocuous but more strongly to noxious stimuli, integrate pain signals with other sensory and affect-related information; nonresponsive (noSOM) neurons, which do not have a somatic receptive field under normal conditions, may contribute specifically to persistent pain. MR neurons and noSOM neurons have been shown to exhibit substantial sensitization in a model of arthritic pain (Neugebauer and Li, 2002; Neugebauer and Li, 2003; Li and Neugebauer, 2004a; Li and Neugebauer, 2004b). These neurons could participate in the dual pronociceptive and antinociceptive functions of the amygdala by enhancing or decreasing output to pain modulation systems.

PAIN-RELATED PLASTICITY IN THE AMYGDALA

Neuronal plasticity may be defined as the ability of neurons to adapt their functions, electrophysiological properties, biochemical profile, or structure. Neuronal plasticity is essential to various nervous system functions. Plasticity also contributes to central sensitization, which refers to the increased neuronal responsiveness following prolonged peripheral noxious stimulation, tissue injury or nerve damage. Neuronal sensitization is a key mechanism as well as a consequence of persistent pain states. Pain-related central sensitization has been investigated mainly in spinal cord (Ji and Woolf, 2001; Willis, 2001; Ji et al., 2003; Salter, 2005). However, it has also been reported for higher levels of the pain neuraxis such as the rostroventral medulla (RVM), anterior

cingulate cortex and amygdala (Wei and Zhuo, 2001; Porreca et al., 2002; Neugebauer et al., 2003; Neugebauer et al., 2004).

The mechanisms and behavioral consequences of pain-related plasticity in the amygdala and the role of the amygdala in preclinical pain models are only beginning to emerge. Previous studies from our group showed central sensitization and synaptic plasticity in the CeLC in a model of arthritic pain (Neugebauer and Li, 2003; Neugebauer et al., 2003). In arthritic pain, synaptic transmission to the CeLC is potentiated at both the nociceptive PB-CeLC synapse and the polymodal BLA-CeLC synapse (Neugebauer et al., 2003). Also, action potential firing evoked by direct intracellular current injections increased, suggesting enhanced excitability of CeLC neurons in the arthritic pain model. The analysis of underlying cellular pain mechanisms in the amygdala may be crucial for a better understanding of the emotional aspects of pain. This pain-related plasticity depends on presynaptic metabotropic glutamate receptors (Neugebauer et al., 2003; Li and Neugebauer, 2004a) and postsynaptic N-methyl-D-aspartate (NMDA) receptor phosphorylation through PKA but not PKC (Li and Neugebauer, 2004b; Bird et al., 2005). The link between these pre- and postsynaptic mechanisms is not known.

CALCITONIN GENE RELATED PEPTIDE (CGRP) IN THE AMYGDALA

CGRP is a 37 amino acid peptide that binds to G-protein-coupled receptors, which activate adenylyl cyclase and cAMP-dependent PKA (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997). Pharmacologically two classes of CGRP receptors have been proposed, termed $CGRP_1$ and $CGRP_2$ receptors, respectively, which have no significant affinity for calcitonin-like peptides (Poyner, 1996; Wimalawansa, 1996; Oliver et al., 1998; Hasbak et al., 2003). CGRP1 but not CGRP2 receptors have been cloned (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997). Selective antagonists such as the C-terminal fragment $CGRP_{8-37}$ and the non-peptide compound BIBN4096BS are available for selectively blocking 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) (Hasbak et al., 2003). RAMP1 defines the ligand-binding site whereas RCP couples the receptor to signal transduction pathways (Hasbak et al., 2003).

Previous anatomical data showed that the CeA is one of the brain areas with the highest levels of CGRP and CGRP receptors (Skofitsch and Jacobowitz, 1985; Van Rossum et al., 1997; Oliver et al., 1998). The CeLC in particular is delineated by its abundance of CGRP-immunoreactive terminals of fibers from the external lateral parabrachial area (Schwaber et al., 1988; Kruger et al., 1988; Harrigan et al., 1994; de Lacalle 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 PAG (Schwaber et al., 1988; Harrigan et al., 1994), which is important for expression of behavior and descending pain modulation. Thus, CGRP can be considered as a molecular marker of the CeLC, the nociceptive amygdala.

The involvement of CGRP in peripheral and spinal pain mechanisms is well documented in various pain models including inflammatory pain (Sluka et al., 1992; Sluka and Westlund, 1993; Neugebauer et al., 1996; Schaible, 1996; Sun et al., 2003; Sun et al., 2004a; Sun et al., 2004b), visceral pain (Winston et al., 2005), neuropathic pain (Carlton and Coggeshall, 1996) and spinal cord injury (Christensen and Hulsebosch, 1997; Bennett et al., 2000). However, less is known about the role of CGRP in pain-related plasticity in the brain. Since PKA-dependent activation of postsynaptic NMDA receptors is known to contribute to enhanced synaptic transmission to CeLC neurons in our pain model, CGRP, which can activate the PKA pathway, is one of the prime candidates to link pre- and post-synaptic plasticity.

The overall purpose of this study was to define the role of CGRP and its mechanisms of action in the CeLC in the arthritis model of persistent pain, using a combination of systems (behavior) and cellular (electrophysiology) methods.

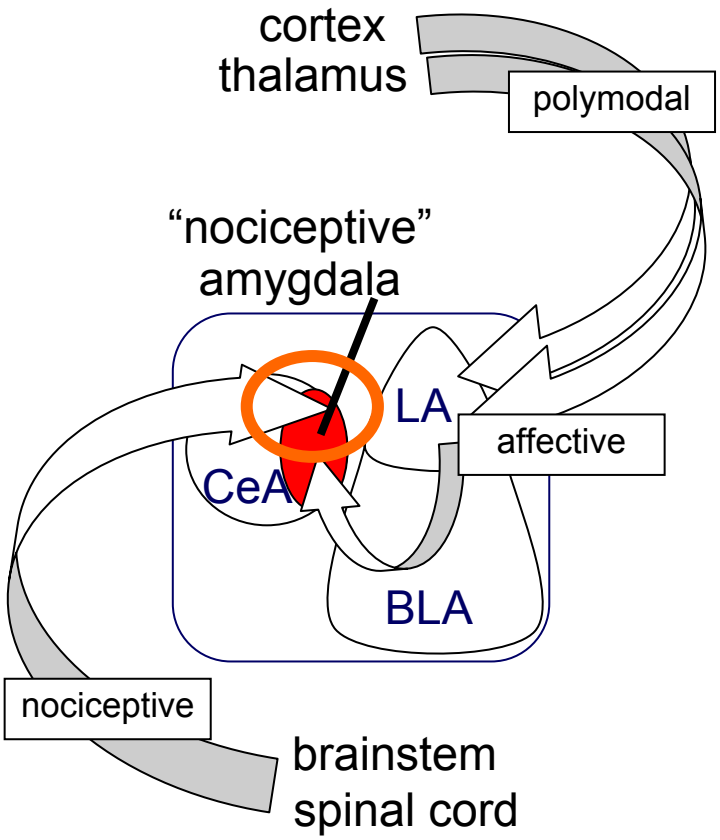

Figure 1

Major sensory inputs to the latero-capsular part of the central nucleus of the amygdala (CeLC).

CeLC neurons receive nociceptive-specific information from the spinal cord and brainstem through the spino-parabrachio-amygdaloid pain pathway and spino-amygdaloid projections. Highly processed polymodal, including nociceptive, information from thalamus (midline and posterior nuclei) and cortex reaches the lateral and basolateral amygdala (LA and BLA, respectively). The circle indicates the parabrachio-CeLC synapse that is the focus of the studies presented here (modified from Neugebauer et al., 2004).

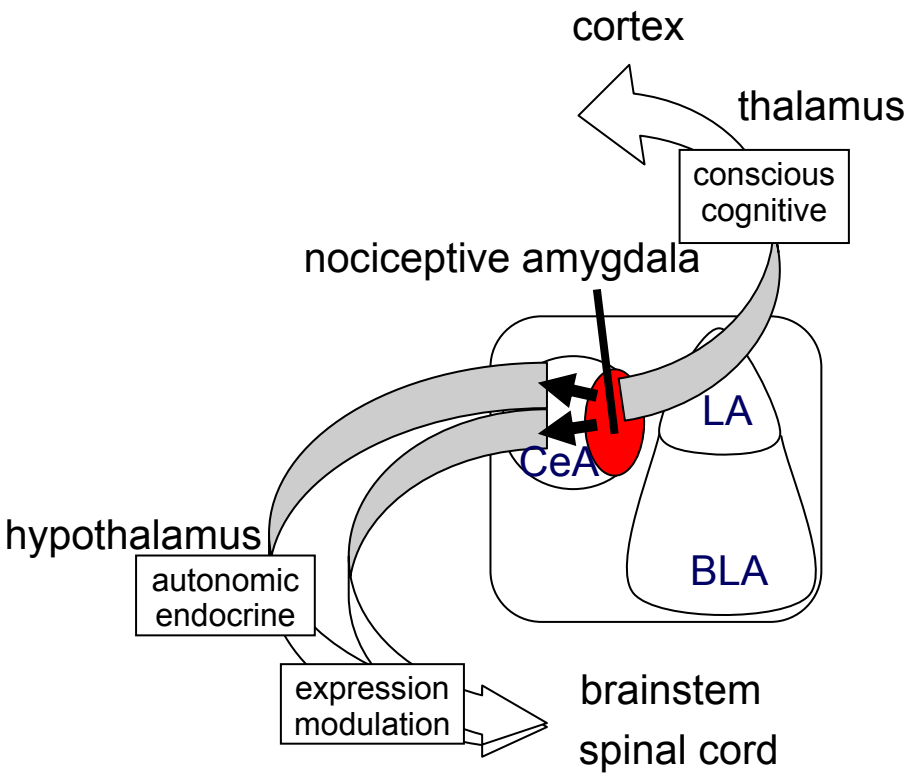

Figure 2

Major pain-related outputs from the CeLC.

The CeLC forms widespread direct and indirect connections with forebrain and brainstem areas. Projections to thalamus and cortical areas may be related to cognitive and conscious components of pain. Autonomic and neuroendocrine pain responses involve the hypothalamus. Emotional expression and modulation of pain behavior are regulated by the CeA through projections (mainly via the ventral amygdaloid pathway) to brainstem areas that are part of an endogenous pain-modulating system and dorsal horn of the spinal cord. The CeLC integrates polysensory and nociceptive-specific information, connecting emotional significance to painful stimuli. LA, lateral nucleus of the amygdala; BLA, basolateral nucleus of the amygdala (modified from Neugebauer et al., 2004).

CHAPTER 2: MATERIALS AND METHODS

ARTHRITIS PAIN MODEL

A mono-arthritis was induced in the left knee joint of adult rats as described in detail previously (Neugebauer et al., 1993; 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 G5/8). 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, which reaches a maximum plateau at 5-6 h, and persists for days (Neugebauer et al., 1993; Min et al., 2001; Neugebauer and Li, 2003; Neugebauer et al., 2003). Electrophysiological and behavioral measurements of arthritis pain-related changes were made at the 6 h time point, which is when the inflammation reached a maximum plateau.

IN VITRO ELECTROPHYSIOLOGY: WHOLE-CELL PATCH-CLAMP RECORDINGS

Amygdala slice preparation

Brain slices containing the central nucleus of the amygdala (CeA) were obtained from arthritic rats and normal rats (120g-250g; Sprague-Dawley) as described before (Neugebauer et al., 2003; Han et al., 2004; Bird et al., 2005). Rats were decapitated, the brains quickly dissected out and blocked in cold (4^0 C) ACSF. ACSF contained (in mM): NaCl 117, KCl 4.7, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, and glucose 11. ACSF was oxygenated and equilibrated to pH 7.4 with a mixture of 95% O_2/5% CO_2. Coronal brain slices (500 μm) were prepared using a Vibroslice (Camden Instruments, London, UK). After incubation in ACSF at room temperature (21^0 C) for at least 1 h, a single brain slice was transferred to the recording chamber and submerged in ACSF (31 ± 1^0 C), which superfused the slice at ~2 ml/min.

Whole-cell patch-clamp recordings

Whole-cell recordings using the ”blind” patch technique were obtained from neurons in the latero-capsular division of the CeA (CeLC) as described before (Neugebauer et al., 2003; Han et al., 2004; Bird et al., 2005). The different nuclei of the amygdala and the subdivisions of the CeA are easily discerned under the microscope. Patch electrodes (4-6 MΩ tip resistance) were made from borosilicate glass capillaries (1.5 mm and 1.12 mm, outer and inner diameter, respectively; Drummond, Broomall, PA) pulled on a Flaming-Brown micropipette puller (P-80/PC, Sutter Instrument Co., Novato, CA). The internal solution of the recording electrodes contained (in mM): 122 K-gluconate, 5 NaCl, 0.3 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 5 Na_2-ATP, 0.4 Na_3-GTP; pH was adjusted to 7.2-7.3 with KOH and the osmolarity to 280 mOsm/kg with sucrose. After tight (>2 GΩ) seals were formed and the whole-cell configuration was obtained, neurons were included in the sample if the resting membrane potential was more negative than -50 mV and action potentials overshooting 0 mV were evoked by direct cathodal stimulation. Voltage and current signals were low-pass filtered at 1 kHz with a dual 4-pole Bessel filter (Warner Instrument Corp., Hamden, CT), digitized at 5 kHz (Digidata 1322A interface, Axon Instruments, Foster City, CA), and stored on a computer (Dell Pentium 4). Data were also continuously recorded on an ink chart recorder (Gould 3400, Gould Instr., Valley View, OH). Current- and voltage-clamp (d-SEVC) recordings were made using an Axoclamp-2B amplifier (Axon Instruments) with a switching frequency of 5-6 kHz (30% duty cycle), gain of 3-8 nA/mV, and time constant of 20 ms. Phase shift and anti-alias filter were optimized. The headstage voltage was monitored continuously on a digital oscilloscope (Gould 400, Gould Instr.) to ensure precise performance of the amplifier. Voltage- and current data were analyzed with pCLAMP9 software (Axon Instruments).

Synaptic stimulation

A concentric bipolar stimulating electrode (Kopf Instr.; 22 kΩ resistance, Tujunga, CA) was positioned on the afferent fiber tract from the parabrachial area (PB, see Introduction and Bernard and Bandler, 1998; Neugebauer et al., 2004) under microscopic control as described previously (Neugebauer et al., 2003; Han et al., 2004; Bird et al., 2005) and illustrated in **Fig. 3**. Excitatory postsynaptic currents (EPSCs) were evoked in CeLC neurons by electrical stimulation (150 μs square-wave pulses; Grass S88 stimulator, Grass Instr.) at frequencies below 0.25 Hz to avoid facilitation or depression of the EPSCs. Input-output functions were obtained by increasing the stimulus intensity in 100 μA steps. For evaluation of a drug effect on synaptically evoked responses, the stimulus intensity was adjusted to 75-80% of the intensity required for orthodromic spike generation (Neugebauer et al., 2003; Han et al., 2004; Bird et al., 2005).

Paired pulse facilitation (PPF)

PPF is used to distinguish pre- vs. post-synaptic mechanisms in the CNS (see references in McKernan and Shinnick-Gallagher, 1997). Two orthodromic synaptic stimuli of equal intensity were applied at varying intervals and the resulting EPSCs were recorded. PPF refers to the phenomenon in which the amplitude of the second EPSC is usually larger than the initial EPSC if the interstimulus interval is sufficiently small (< 150-200 ms, see **Fig. 14**). In whole-cell voltage-clamp, peak amplitudes were measured as the difference between the current level before the stimulus artifact and the peak of the EPSC. PPF is calculated as [(EPSC2 – EPSC1)/EPSC1] x 100. If a drug increases neurotransmitter release, PPF is reduced while enhanced PPF would indicate decreased neurotransmitter release. Any alterations in PPF suggest a presynaptic site of action. PPF was tested before and during application of CGRP and CGRP receptor antagonists (see Drugs).

Miniature EPSC (mEPSC) analysis

The analysis of mEPSCs is another well established approach to distinguish between pre- and post-synaptic mechanisms. In the presence of tetrodotoxin (1 μM), mEPSCs were recorded before and during drug application. Amplitude and frequency of mEPSCs were compared and cumulative fractions were plotted using the MiniAnalysis program 5.3 (Synaptosoft, Fort Lee, NJ). Changes in the frequency of mEPSC suggest a presynaptic mechanism, while changes in the amplitude suggest a postsynaptic mechanism.

Drugs

CGRP and $CGRP_{8-37}$, a selective peptide CGRP1 receptor antagonist (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997), were purchased from Bachem, Torrance, CA. BIBN4096BS, a novel selective non-peptide CGRP1 receptor antagonist (Doods et al., 2000), was kindly supplied by Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany. KT5720, a potent and selective PKA inhibitor (Cabell and Audesirk, 1993; Bird et al., 2005); U0126, a MEK inhibitor; GF 109203X, a selective PKC inhibitor; N-methyl-D-aspartate (NMDA); and alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid; (AMPA); all purchased from Tocris Cookson Inc., Ellisville, MO.

Drugs were dissolved in ACSF on the day of the experiment and applied to the brain slice by gravity-driven superfusion in the ACSF. Solution flow into the recording chamber (1 ml volume) was controlled with a three-way stopcock. Drugs were applied for at least 15 min to establish equilibrium in the tissue.

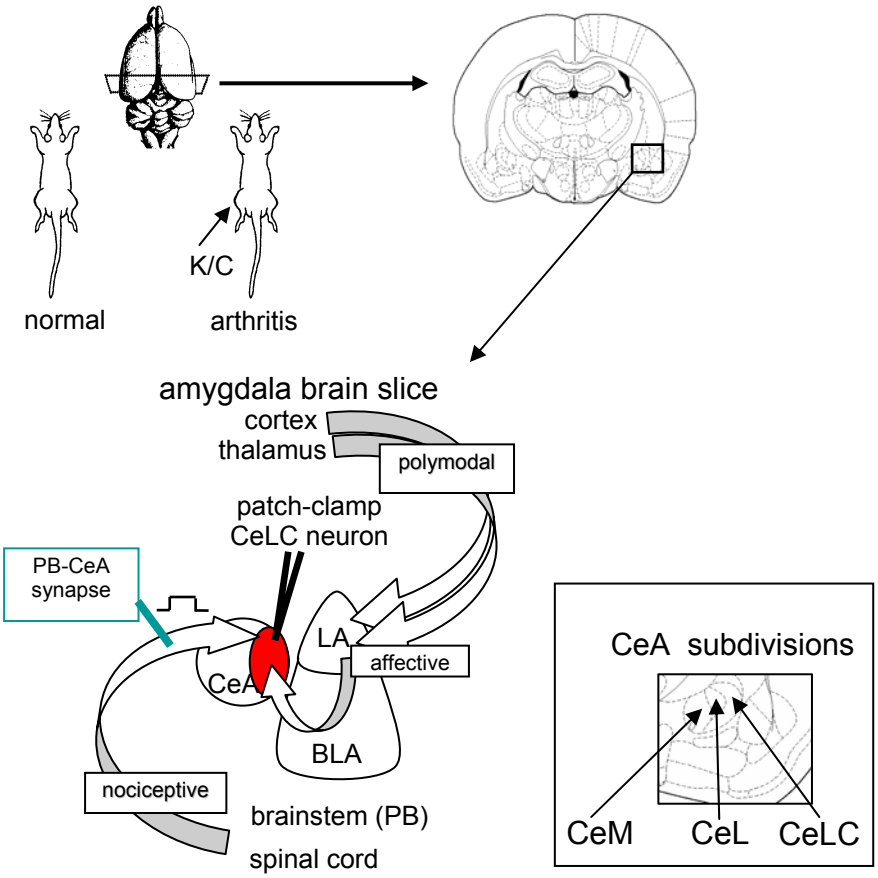

Figure 3

Experimental setup for whole-cell patch-clamp recordings.

Coronal brain slices containing the amygdala were obtained from normal (naïve) rats and from arthritic rats 6-8 h after injections of kaolin and carrageenan (K/C) into one knee joint. Under microscopic control, the patch-clamp electrode was positioned in the CeLC ("nociceptive amygdala"). Synaptic responses were evoked with a stimulation electrode positioned on the afferent fibers from the pontine parabrachial area (PB) providing nociceptive information to the CeLC (Neugebauer et al., 2004). CeM, CeL, CeLC: medial, lateral and latero-capsular divisions of the central nucleus of the amygdala.

BEHAVIOR: VOCALIZATIONS AND HINDLIMB WITHDRAWAL REFLEXES

Experimental protocol

On Day 1 a guide cannula for drug (and ACSF vehicle) application by microdialysis was stereotaxically inserted into the CeLC. Baseline (pre-arthritis) vocalizations and spinal withdrawal reflexes were measured in normal rats in the afternoon of Day 2. The behavioral tests were repeated in the afternoon of Day 3 in the same animals 6 h after arthritis induction in one knee (see above). Drugs were then administered into the CeLC through the microdialysis probe and behavior was measured at 15 min during the continued drug administration and again at 30 min of washout with ACSF. Drugs were also tested in a seperate set of naïve animals without arthritis.

Stereotaxic implantation of microdialysis guide cannula and drug administration

As described in detail before (Han and Neugebauer, 2005), rats were anaesthetized with pentobarbital sodium (50 mg/kg, i.p.) and a small unilateral craniotomy was performed at the sutura fronto-parietalis level. Using a stereotaxic apparatus (David Kopf Instr., Tujunga, CA), a guide cannula (CMA/12, CMA/Microdialysis Inc., North Chelmsford, MA) was inserted through a drill hole into the CeLC or striatum (as a placement control) using the following stereotaxic coordinates (Paxinos and Watson, 1998): CeLC, 2.0 mm caudal to bregma; 4.0 mm lateral to midline; depth 7.0 mm; striatum: 2.2 mm caudal to bregma; 4.5 mm lateral to midline; depth 5.0 mm. Once inserted, the microdialysis probe protruded beyond the tip of the guide cannula by 2 mm. The cannula was fixed to the skull with dental acrylic (PlasticsOne, Roanoke, VA). Antibiotic ointment was applied to the exposed tissue to prevent infection. On the day of the experiment, a microdialysis probe (CMA12; CMA/Microdialysis; 20 kD cut-off, membrane length 2 mm) was inserted through the guide cannula for administration of ACSF (same composition as in the in vitro electrophysiology studies) or drugs into the CeLC (or striatum) at a rate of 5 µl/min using an infusion pump (Harvard apparatus, Hollisten, MA).

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 and dilution in the tissue (Li and Neugebauer, 2004a; Li and Neugebauer, 2004b; Han and Neugebauer, 2005). The numbers given throughout the text 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 before (Han et al., 2005; Han and Neugebauer, 2005). The experimental setup (**Fig. 4**, Provisional Patent 98006/28PRV) included a custom designed recording chamber; a condenser microphone (audible range: 20 Hz-16 kHz) connected to a preamplifier and an ultrasound detector (25 ± 4 kHz); filter and amplifier (UltraVox 4-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). The number and duration of digitized events (audible and ultrasonic vocalizations) was recorded on a computer (see **Fig. 5** for original recordings, Dell, Pentium 4) (Han et al., 2005; Han and Neugebauer, 2005). The computerized recording system was set to suppress non-relevant audible sounds and to ignore ultrasounds 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 days). 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 hind limbs to allow the application of brief (15 s) noxious (2000 g/30 mm^2) mechanical stimuli with a calibrated forceps equipped with a force transducer, whose calibrated

output was amplified and displayed in grams on an LCD screen (Neugebauer and Li, 2003; Li and Neugebauer, 2004a; Li and Neugebauer, 2004b; Han et al., 2005). Stimulus intensities >1500 g/30 mm^2 are noxious (Neugebauer and Li, 2003; Li and Neugebauer, 2004a). The chamber also had an opening for the head to permit drug administration into the amygdala through the microdialysis probe inserted into the implanted guide cannula. Duration of vocalizations was recorded during application of the mechanical stimulus (VDS) and in the period immediately after stimulation (VAD) (**Fig. 5**). Selective lesion studies showed that VDS is mainly organized at the brain stem level, while VAD is organized at the limbic system including the amygdala (Borszcz et al., 1994; Borszcz and Leaton, 2003).

Hindlimb withdrawal reflexes

The threshold of spinal reflexes in response to stimulation of the knee was determined subsequently to the vocalization measurements. Mechanical stimuli of increasing intensity (steps of 50 g/30 mm^2) were applied to the knee joint as in the vocalization experiments by means of a calibrated forceps equipped with a force transducer. Withdrawal threshold was defined as the minimum stimulus intensity that evoked a withdrawal reflex.

Histology

The positions of the microdialysis probe were confirmed histologically as described before (Li and Neugebauer, 2004a; Li and Neugebauer, 2004b; Han and Neugebauer, 2005) (**Figs. 22, 30 and 37**). The brain was removed and submerged in 10% formalin and potassium ferrocyanide. Tissues were stored in 20% sucrose before they were frozen sectioned at 50 μm. Sections were stained with Neutral Red, mounted on gel-coated slides and cover-slipped. The boundaries of the different amygdalar nuclei were easily identified under the microscope.

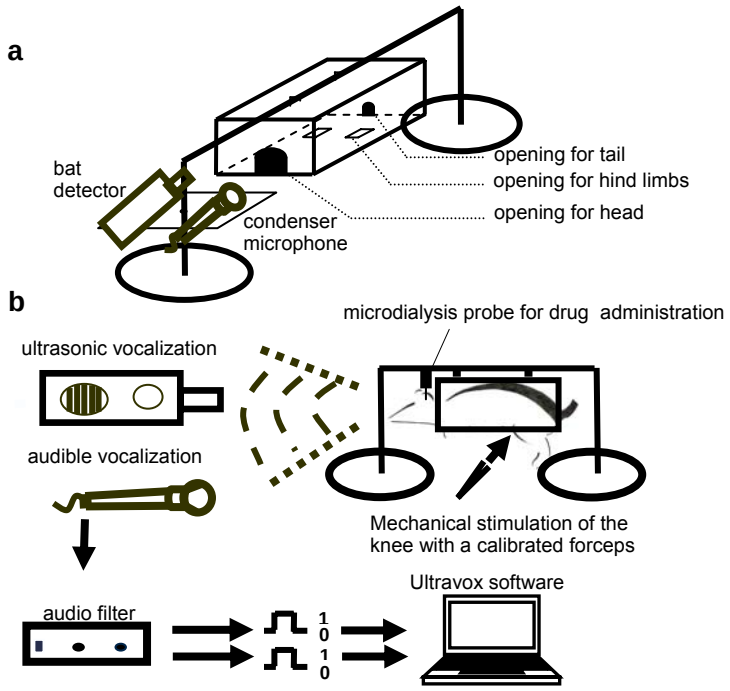

Figure 4

Experimental setup for the recording of vocalizations.

(a) Diagram of the suspended recording chamber for rats. The customized plexiglas box is adjustable for the size of the animal and contains openings for head, tail and hind limbs. The animal is positioned in the box at a fixed distance (6 cm) from the recording devices. **(b)** Audible and ultrasonic vocalizations are recorded simultaneously with a condenser microphone (20 Hz – 16 kHz) and a bat detector (25 ± 4 kHz), respectively. The audio signals are then fed into separate channels of an audio filter. Duration and rate of vocalizations are analyzed on a personal computer using a software analysis program (Ultravox 2.0, Noldus Technology). Noxious mechanical stimuli (2000 g/ 30 mm^2, 15s) are applied to the knee joint using a calibrated forceps equipped with a force transducer. The recording chamber allows free access to the hindlimbs for the application of test stimuli (from Han et al., 2005).

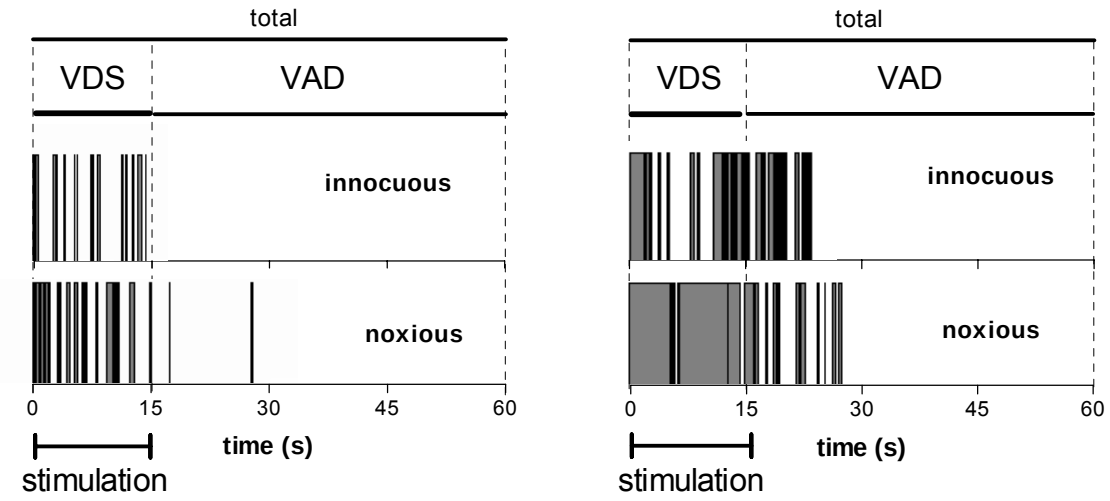

Figure 5

Original recordings of ultrasonic vocalizations.

Vocalizations were evoked by innocuous (500 g/ 30 mm^2) and noxious (2000g/ 30 mm^2) stimulation of the knee joint in a rat before **(a)** and after induction of arthritis **(b)**. The bars indicate the duration of individual vocalization events. Arthritis was induced by intraarticular injections of kaolin and carrageenan. Mechanical stimuli were applied for 15 s; duration of the recording period was 1 min. Vocalizations during and after stimulation (VDS and VAD, respectively) were analyzed separately (from Han et al 2005).

DATA ANALYSIS AND STATISTICS

All averaged values are given as the mean ± SE. Statistical significance was accepted at the level $P < 0.05$. GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses except where noted.

In vitro electrophysiology

Input–output functions and concentration–response relationships were compared using a two-way analysis of variance (ANOVA) followed by Bonferroni post-tests where appropriate. Concentration–response curves were obtained by non-linear regression analysis using the formula $y=A+(B-A)/[1+(10^{C}/10^{X})^{D}]$, where A is the bottom plateau, B top plateau, $C=\log(EC_{50}$ or $IC_{50})$, and D is the slope coefficient. The paired t-test was used to compare test EPSC amplitudes and PPF evoked by one stimulus intensity before and during drug application in the same neuron. mEPSCs were analyzed for frequency and amplitude distributions using the MiniAnalysis program 5.3 (Synaptosoft Inc., Fort Lee, NJ). The Kolmogorov-Smirnov test was used for cumulative distribution analysis of mEPSC amplitude and frequency.

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) and 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.

CHAPTER 3: CGRP1 RECEPTOR ANTAGONISTS INHIBIT PAIN-RELATED PLASTICITY AND BEHAVIOR

SYNAPTIC PLASTICITY AND ENHANCED EXCITABILITY OF CELC NEURONS IN A MODEL OF ARTHRITIC PAIN

Whole-cell voltage-clamp recordings of neurons in the latero-capsular division of the central nucleus of the amygdala (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; Han et al., 2004; Bird et al., 2005). CeLC neurons from arthritic rats showed significantly increased synaptic transmission (**Fig. 6**), which indicates "synaptic plasticity" because it is preserved in the reduced slice preparation and thus 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 excitatory postsynaptic currents (EPSCs) at the nociceptive PB-CeLC synapse (see Neugebauer et al., 2004) (see individual current traces in **Fig. 6a**).

Monosynaptic EPSCs were enhanced in a CeLC neuron recorded in a brain slice from an arthritic rat (6 h postinduction) compared to a control neuron. 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 (pA) as a function of afferent fiber volley stimulus intensity (μA) for each neuron (**Fig. 6b**). The input-output function of the parabrachial (PB)-CeLC synapse was significantly increased in CeLC neurons from arthritic rats (n = 19) compared to control neurons from normal rats (n = 37) as evidenced by the steeper slope and upward shift at higher stimulus intensities (**Fig. 6b**, $P < 0.0001$, $F_{1,593} = 60.29$, two-way ANOVA followed by Bonferroni post-tests). The sites of synaptic stimulation and patch-clamp recording in the amygdala brain slice are schematically illustrated in **Fig. 3**.

Compared to neurons from normal rats, CeLC neurons from arthritic rats showed higher frequency of action potentials generated by direct depolarizing current pulse

injected into the cell via the recording electrode in current-clamp mode (see individual examples in **Fig. 7a**). Analysis of the input-output functions showed that the action potential firing rate was significantly ($P < 0.001$, $F_{1,768176} = 40.20$, two-way ANOVA) increased in CeLC neurons (n = 44) from arthritic rats compared to neurons (n = 54) from normal rats (**Fig. 7b**), suggesting increased neuronal excitability and output function.

These data show enhanced information processing in CeLC neurons, which is maintained at least in part independently of continuous nociceptive input from peripheral and spinal sites as synaptic plasticity and increased excitability are measured in the reduced brain slice preparation in vitro. The consequence of sensitization, synaptic plasticity and increased excitability is the enhancement of both the input to and the output from the CeLC in the arthritis model of persistent pain (Neugebauer et al., 2004).

ENDOGENOUS ACTIVATION OF CGRP1 RECEPTORS IS REQUIRED FOR PAIN-RELATED SYNAPTIC PLASTICITY IN THE CELC

Next we addressed the role of CGRP1 receptor activation in pain-related synaptic plasticity compared to normal synaptic transmission. A selective CGRP1 receptor antagonist ($CGRP_{8-37}$; 1 μM) inhibited synaptic plasticity in CeLC neurons in slices from arthritic rats but had little effect on basal synaptic transmission in CeLC neurons from normal rats (**Fig. 8a, b**). $CGRP_{8-37}$ inhibited the EPSC peak amplitude in the arthritis pain model significantly ($P < 0.05$, paired t-test, n = 17) and restored the level of synaptic transmission to normal, but had no significant effect on synaptic transmission in control neurons from normal animals (**Figs. 8a, b,** left, n = 29). To ensure the selectivity of CGRP1 receptor blockade, we also tested a selective non-peptide CGRP1 antagonist (BIBN4096BS, 1 μM). BIBN4096BS inhibited synaptic plasticity in CeLC neurons from arthritic rats (**Fig. 8a, c**, $P < 0.05$, paired t-test, n = 5). Synaptic responses were evoked by a stimulus intensity adjusted to 70-80% of that required for generating the maximum EPSC amplitude.

$CGRP_{8-37}$ also changed the input-output function of the PB-CeLC synapse in the arthritis pain state to the level recorded under normal conditions (**Fig. 9**). The inhibitory

effects of $CGRP_{8-37}$ were particularly pronounced at higher stimulus intensities (n = 12, P < 0.0001, $F_{1,242}$ = 76.32, two-way ANOVA followed by Bonferroni post-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. Cumulative concentration-response relationships show that $CGRP_{8-37}$ 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. 10**, P < 0.01, $F_{1,220}$ = 10.74, two-way ANOVA). The IC_{50} did not change significantly in the arthritis pain model compared to normal controls (2.7 nM and 1.1 nM, respectively; see Methods).

POST- RATHER THAN PRE-SYNAPTIC CGRP1 RECEPTOR ACTIVATION IN PAIN-RELATED SYNAPTIC PLASTICITY IN THE CELC

The major source of CGRP in the CeLC is the external lateral parabrachial area (Schwaber et al., 1988; Kruger et al., 1988; Harrigan et al., 1994; de Lacalle and Saper, 2000). This projection to the CeLC is part of the spino-parabrachio-amygdaloid pain pathway (Bernard and Bandler, 1998). To assess whether CGRP acts at pre- or post-synaptic sites in the CeLC we used a number of well-established electrophysiological methods, including the analysis of neuronal excitability (**Fig. 11**), slope conductance (**Fig. 12**), amplitude and frequency of spontaneous miniature EPSCs (mEPSCs) (**Fig. 13**), and paired pulse facilitation (**Fig. 14**). These parameters were measured before and during application of $CGRP_{8-37}$ in amygdala brain slices from normal and arthritic rats.

Action potentials were evoked in current-clamp mode by direct intracellular depolarizing 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. $CGRP_{8-37}$ significantly decreased the input-output function of CeLC neurons from arthritic rats (**Fig. 11c,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. 11a,b**, n = 14, P > 0.05, $F_{1,206}$ = 0.44, two-way ANOVA).

The analysis of current-voltage (I-V) relationships in voltage-clamp showed that $CGRP_{8-37}$ decreased the slope conductance in CeLC neurons from arthritic rats significantly (**Fig. 12**, $P < 0.01$, paired t-test; $n = 9$) but had no significant effect on the slope conductance of control neurons from normal animals ($P > 0.05$, paired t-test; $n = 14$). 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 are increased compared to control neurons. Taken together, these results suggest that the endogenous activation of CGRP1 receptors in the CeLC is involved in pain-related increased neuronal excitability and produces direct membrane effects.

The analysis of spontaneous mEPSCs in the presence of TTX is a well established electrophysiological approach to determine pre- versus post-synaptic 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. 13**). This postsynaptic effect is illustrated in the current traces recorded in an individual CeLC neuron (**Fig. 13a**). In the whole sample of neurons ($n = 4$), $CGRP_{8-37}$ caused a shift of the normalized cumulative mEPSC amplitude distribution towards smaller amplitudes ($P < 0.005$, Kolmogorov-Smirnov test) and decreased the mean mEPSC amplitude significantly ($P < 0.05$, paired t-test, $n = 4$; **Fig. 13b**). $CGRP_{8-37}$ had no effect on the frequency of mEPSCs ($P > 0.05$, paired t-test, $n = 4$; see normalized cumulative inter-event interval distribution and mean mEPSC frequency, **Fig. 13c**).

Further arguing against a presynaptic site of action, $CGRP_{8-37}$ had no significant effect on paired pulse facilitation (PPF; **Fig. 14,** $n = 6$, $P > 0.05$, paired t-test). PPF refers to the observation that the amplitude of the second of two consecutively evoked EPSCs is larger than the initial EPSC if the interstimulus interval is sufficiently small. Any changes in PPF suggest a presynaptic site of action (see McKernan and Shinnick-Gallagher, 1997; Neugebauer et al., 2003; Han et al., 2004; Bird et al., 2005). Thus, if a drug (e.g., $CGRP_{8-37}$) decreases transmitter release, PPF is enhanced. $CGRP_{8-37}$ had no significant effect on

PPF recorded in CeLC neurons. PPF was calculated as the ratio of the second and the first of two consecutive EPSCs evoked by electrical stimuli of equal intensity.

The analysis of PPF, mEPSCs and neuronal excitability suggests that endogenous CGRP1 receptor activation occurs at a post- rather than pre-synaptic site in CeLC neurons. These data also show an increase of CGRP1 receptor function in the arthritic pain model.

CGRP1 RECEPTORS ACT THROUGH PKA TO MODULATE NMDA RECEPTOR FUNCTION

Our previous studies showed that postsynaptic NMDA receptor phosphorylation through a PKA dependent mechanism plays a critical role in pain-related synaptic plasticity in the CeLC (Bird et al., 2005). Since CGRP1 receptors are known to couple to the activation of PKA (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997), we tested the hypothesis that endogenous activation of CGRP1 receptors modulates PKA and NMDA receptor function.

Pain-related synaptic plasticity was reduced in the presence of a selective PKA inhibitor (KT5720, 1 μM; **Fig. 15**), 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. 15a-c**) or directly into the cell through the patch pipette (**Fig. 15d-f**). In the presence of the PKA inhibitor $CGRP_{8-37}$ produced no further inhibition, suggesting that CGRP1 receptor function requires PKA activation. Importantly, the fact that direct intracellular application of KT5720 occluded 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 to that by an NMDA receptor antagonist (AP5) reported previously (Bird et al., 2005). Since 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.

Next we determined the effects of $CGRP_{8-37}$ on NMDA- and AMPA-mediated membrane currents, because NMDA, but not non-NMDA, receptor function depends on PKA activation in the CeLC in the arthritis pain model (Bird et al., 2005). Membrane currents were evoked by exogenous NMDA (1 mM) (**Fig. 16a**) and AMPA (**Fig. 16b**) applied to the recording chamber using the microdrop application technique (Bird et al., 2005). $CGRP_{8-37}$ 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. 16a, c**). However, membrane currents evoked by exogenous AMPA (30 μM) were not affected by $CGRP_{8-37}$ in terms of peak amplitude and area under the curve ($P > 0.05$, paired t-test, $n = 5$; **Fig. 16b, 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 IN THE AMYGDALA IS REQUIRED FOR PAIN BEHAVIOR ORGANIZED AT DIFFERENT LEVELS OF THE PAIN NEURAXIS

To validate the significance of CGRP1 receptor activation observed in the electrophysiological studies, we analyzed the effect of $CGRP_{8-37}$ 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 $CGRP_{8-37}$ 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 (see Han and Neugebauer, 2005). Vocalizations are further classified as vocalizations during stimulation (VDS), which are organized at the medullary brainstem level, and vocalization afterdischarges (VAD), 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 postinduction, **Fig 17;** see also **Fig. 5**). Administration of $CGRP_{8-37}$ (100 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC inhibited the duration of audible and ultrasonic VAD (**Fig. 18b**) and VDS (**Fig. 19b**) evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the arthritic knee (n = 9). The inhibitory effects of $CGRP_{8-37}$ were significant ($P < 0.05$-0.01; paired t-test) and largely reversible. In contrast, $CGRP_{8-37}$ had no significant effect on the vocalizations of naive (non-arthritic) animals (n = 4, **Fig. 18a, 19a**). Predrug vocalizations were measured during 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 (see discussion in 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 (see **Fig. 22**).

The vocalization data suggest that $CGRP_{8-37}$ inhibits pain responses organized in the brainstem (VDS) and limbic forebrain (VAD). Next we determined the contribution of CGRP1 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. 20**). Mechanical stimuli of increasing intensity (steps of 50 g/30 mm^2) were applied to the knee joint. Withdrawal threshold was defined as the minimum stimulus intensity that evoked a withdrawal reflex (Han and Neugebauer, 2005). Administration of $CGRP_{8-37}$ (100 μM, concentration in the microdialysis probe; 15 min) into the CeLC had no significant effect on the withdrawal thresholds measured in naïve (non-arthritic) animals (n = 4; **Fig. 20a**). After arthritis induction, hindlimb withdrawal thresholds decreased, suggesting increased pain sensitivity (**Fig. 20b**). Administration of $CGRP_{8-37}$ (100 μM; 15 min) into the CeLC partially reversed the effects of arthritis by

increasing the hindlimb withdrawal thresholds significantly ($n = 6$, $P < 0.05$, paired t-test). These data suggest an important role of CGRP1 receptor activation in the amygdala in the descending modulation of spinal nociceptive processing.

As placement controls for any drug effects due to diffusion from the microdialysis probe to other brain areas, microdialysis probes were stereotaxically inserted into the striatum (caudate-putamen) for drug application in a separate set of animals (**Fig. 21**). 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). 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; $n = 5$) into the striatum had no significant effects on audible and ultrasonic vocalizations (**Fig. 21**) or withdrawal reflexes (not shown) evoked by stimulation of the arthritic knee ($P > 0.05$, paired t-test). The location of the microdialysis probes in the CeLC was verified histologically (see **Fig. 22**).

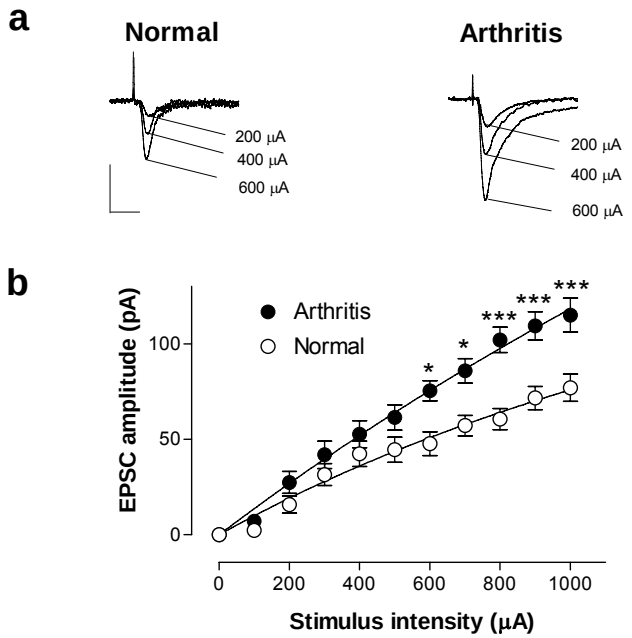

Figure 6

Pain-related synaptic plasticity in CeLC neurons.

(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 (scale bars, 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 to control neurons (n = 37) ($P < 0.0001$, $F_{1,593} = 60.29$, two-way ANOVA followed by Bonferroni post-tests).

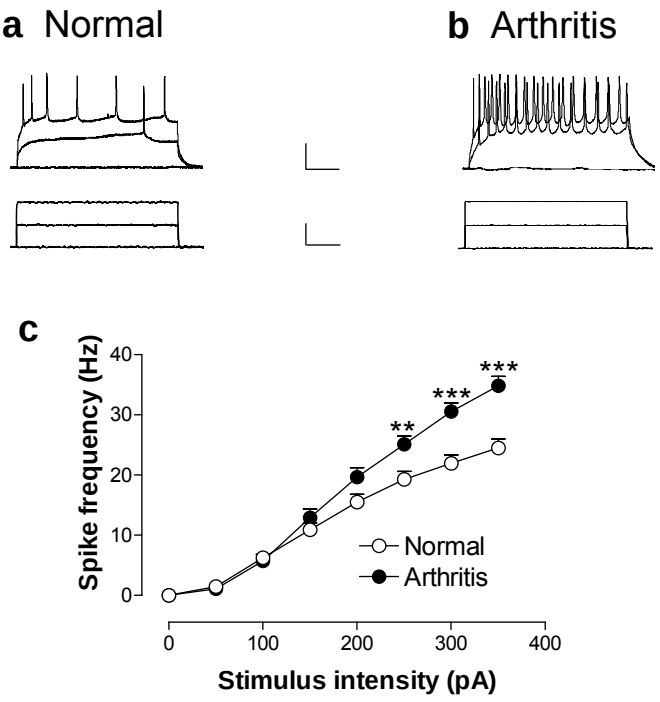

Figure 7

Pain-related increased neuronal excitability of CeLC neurons.

(a,b) Current-clamp recordings of action potentials (top traces) generated by direct current pulses of increasing magnitude (50 pA steps, 500 ms duration; bottom traces in **a** and **b**) injected via the recording electrode directly into a CeLC neuron from a normal rat **(a)** and a CeLC neuron from an arthritic rat 6 h postinduction of arthritis **(b)**. Scale bars in **a** and **b** are 100 ms/25 mV (upper traces) and 100 ms/150 pA (lower traces). **(c)** Action potential firing rate in CeLC neurons in the arthritis pain model (n = 44) was significantly ($P < 0.001$, $F_{1,768} = 40.20$, two-way ANOVA followed by Bonferroni post-tests) increased compared to control CeLC neurons (n = 54). ** $P < 0.01$, *** $P < 0.001$.

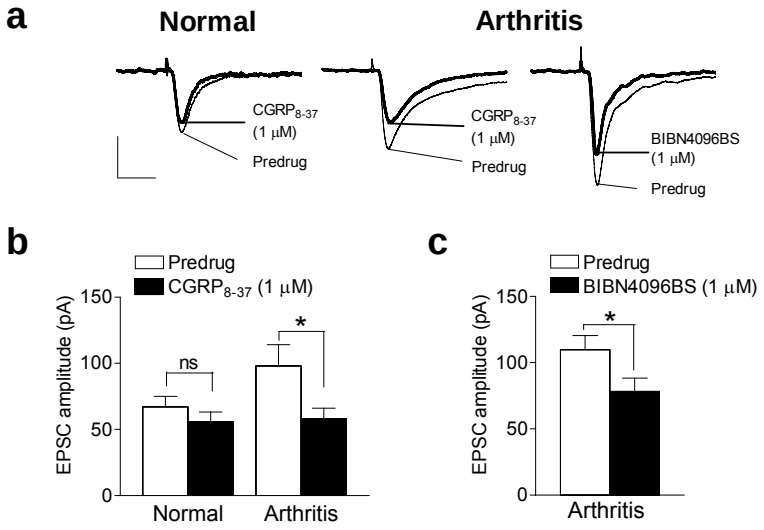

Figure 8

Enhanced endogenous activation of CGRP1 receptors in the CeLC in the arthritic pain model.

(a) A selective CGRP1 receptor antagonist ($CGRP_{8-37}$, 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 traces). Likewise, the selective non-peptide CGRP1 receptor antagonist (BIBN4096BS, 1 μM) inhibited synaptic plasticity in a CeLC neuron from an arthritic animal (right traces). 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 (scale bars, 50 ms/50 pA). **(b)** Averaged raw (current) data show that $CGRP_{8-37}$ (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). **(c)** BIBN4096BS (1μM) also decreased the EPSC amplitude (averaged raw data) significantly ($P < 0.05$, paired t-test, n = 5).

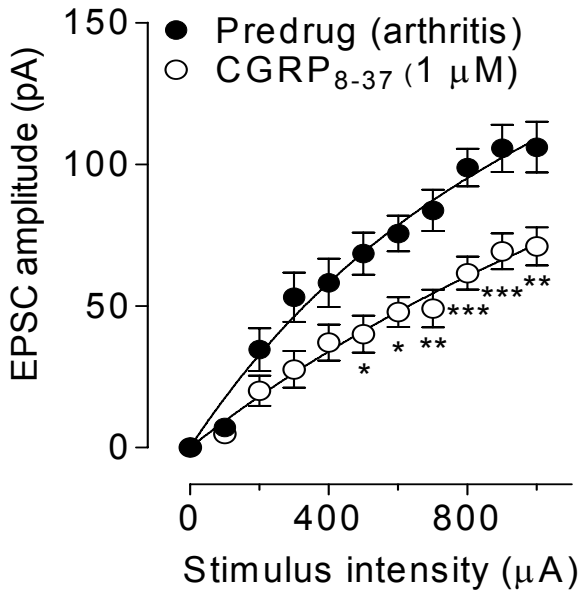

Figure 9

Inhibitory effect of $CGRP_{8-37}$ on the input-output function of the PB-CeLC synapse. Input-output functions were measured by increasing the stimulus intensity in 100 μA steps. $CGRP_{8-37}$ (1 μM) inhibited the input-output function in CeLC neurons from arthritic rats significantly ($n = 12$, $P < 0.0001$, $F_{1,242} = 76.32$, two-way ANOVA followed by Bonferroni post-tests). Whole-cell voltage-clamp recordings were made from CeLC neurons held at -60 mV. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

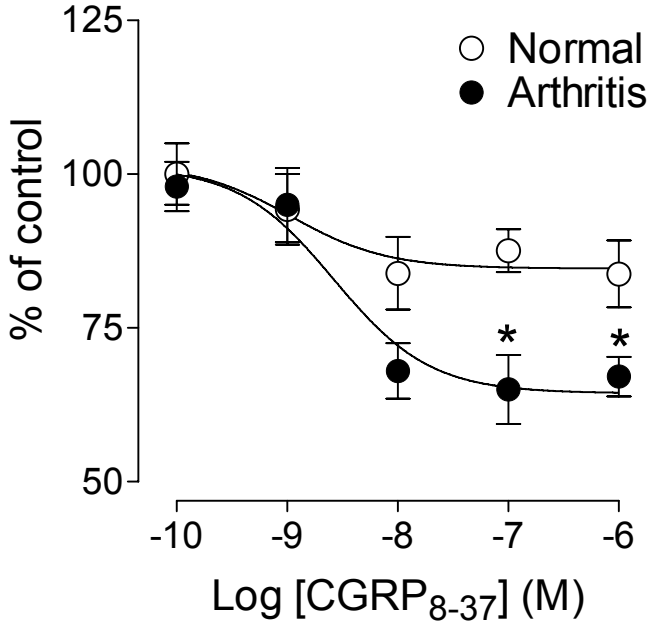

Figure 10

Enhanced inhibitory effect of $CGRP_{8-37}$ in CeLC neurons from arthritic rats.

Concentration-response relationships show that $CGRP_{8-37}$ was more efficacious in neurons from arthritic rats (n = 17) than in control neurons (n = 29) from normal rats ($P < 0.01$, $F_{1,220} = 10.74$, two-way ANOVA; Bonferroni post-tests indicate significant differences for individual concentrations). Peak amplitudes of monosynaptic EPSCs during each concentration of $CGRP_{8-37}$ were averaged and expressed as percent of predrug (baseline) control (100 %). $CGRP_{8-37}$ was applied for at least 15 min and measurements were made at 12 min. Whole-cell voltage-clamp recordings were made from CeLC neurons held at -60 mV. * $P < 0.05$.

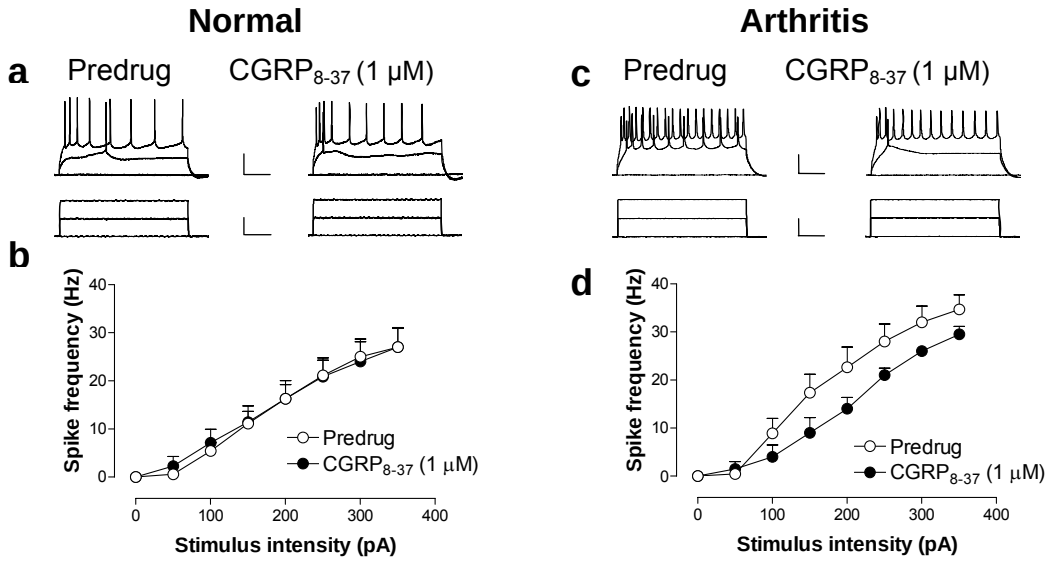

Figure 11

$CGRP_{8-37}$ 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 depolarizing current pulses (500 ms) of increasing magnitude (50 pA steps) before and during $CGRP_{8-37}$ administration. **(a,b)** $CGRP_{8-37}$ did not affect the action potential firing rate in CeLC neurons in slices from normal rats ($P > 0.05$, two-way ANOVA; n=14). **(c,d)** However, the action potential firing rate was significantly decreased by $CGRP_{8-37}$ in CeLC neurons from arthritic rats ($P < 0.0001$, $F_{1,144} = 14.15$, two-way ANOVA, n = 10), suggesting a functional change of CGRP1 receptor activation that has postsynaptic effects in arthritis but not under normal conditions. The same neurons as in **Fig. 7** were tested. For the measurement of action potential firing in current clamp, neurons were recorded at -60 mV. Scale bars in **a** and **c** are 100 ms/25 mV (upper traces) and 100 ms/150 pA (lower traces). Symbols and error bars in **b** and **d** represent mean ± SE.

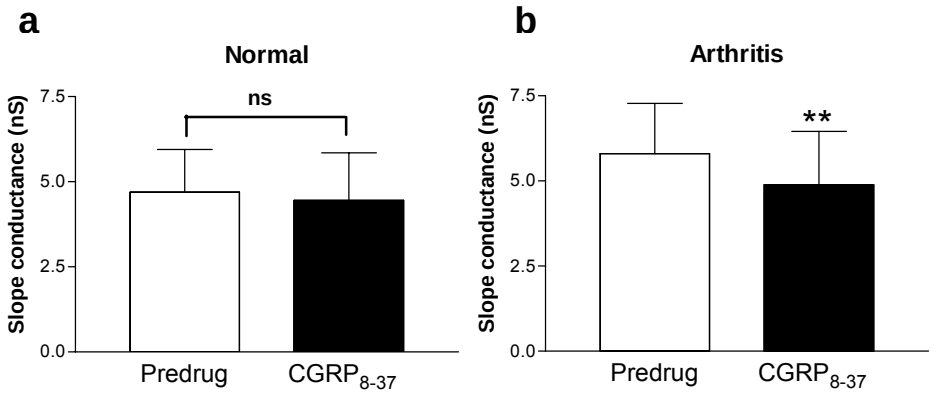

Figure 12

$CGRP_{8-37}$ decreases slope conductance of CeLC neurons in the arthritic pain model but not under normal conditions.

Slope conductance (in nS) was calculated from the linear portion of current-voltage (I-V) relationships in the presence and absence of $CGRP_{8-37}$. I-V relationships were obtained by plotting steady-state current changes against the amplitudes of transient voltage steps (500 ms) injected via the recording electrode in 10 mV steps (-130 mV to -30mV). Slope conductance was significantly decreased by $CGRP_{8-37}$ in neurons (n = 9) from arthritic rats **(b)** but not in control neurons (n = 14) from normal rats **(a)**. This finding further suggests a direct postsynaptic membrane effect of $CGRP_{8-37}$ in neurons from arthritic rats. ** $P < 0.01$ (paired t-test).

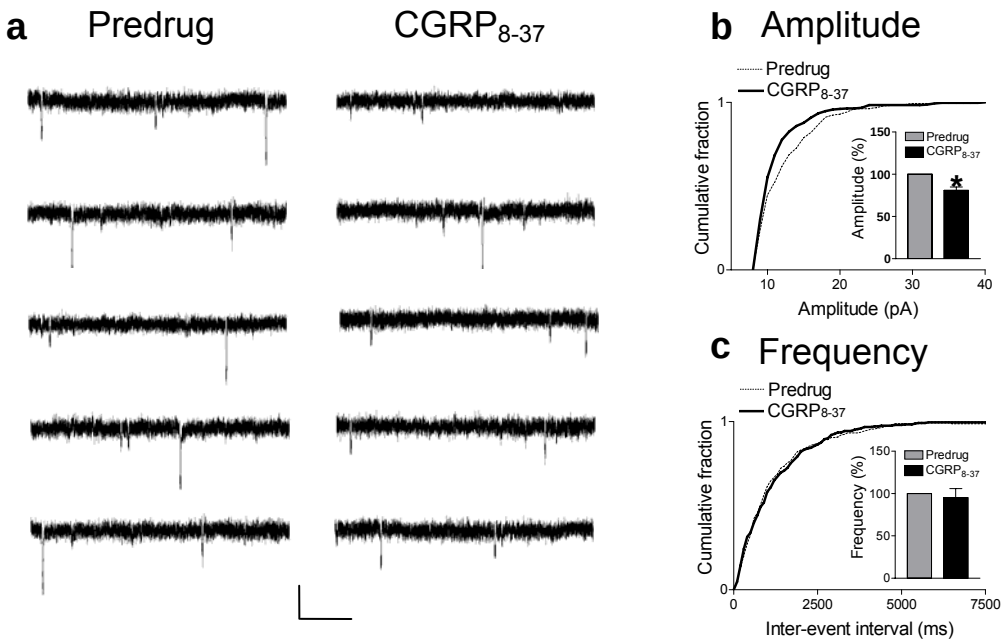

Figure 13

Miniature EPSC (mEPSC) analysis suggests post- rather than pre-synaptic effects of $CGRP_{8-37}$.

(a) Original current traces of mEPSC recordings in an individual CeLC neuron in the presence of TTX (1 μM) show that $CGRP_{8-37}$ (1 μM) reduced amplitude but not frequency of mEPSCs, (scale bars, 1 s/20 pA). The CeLC neuron was recorded in a slice from an arthritic rat. **(b,c)** Normalized cumulative distribution analysis of mEPSC amplitude and frequency show 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)**. $CGRP_{8-37}$ selectively decreased mean mEPSC amplitude ($P < 0.05$, paired t-test) but not mEPSC frequency (events/s) in the sample of neurons (n = 4; see bar histograms in **b, c**). *$P < 0.05$ (paired t-test).

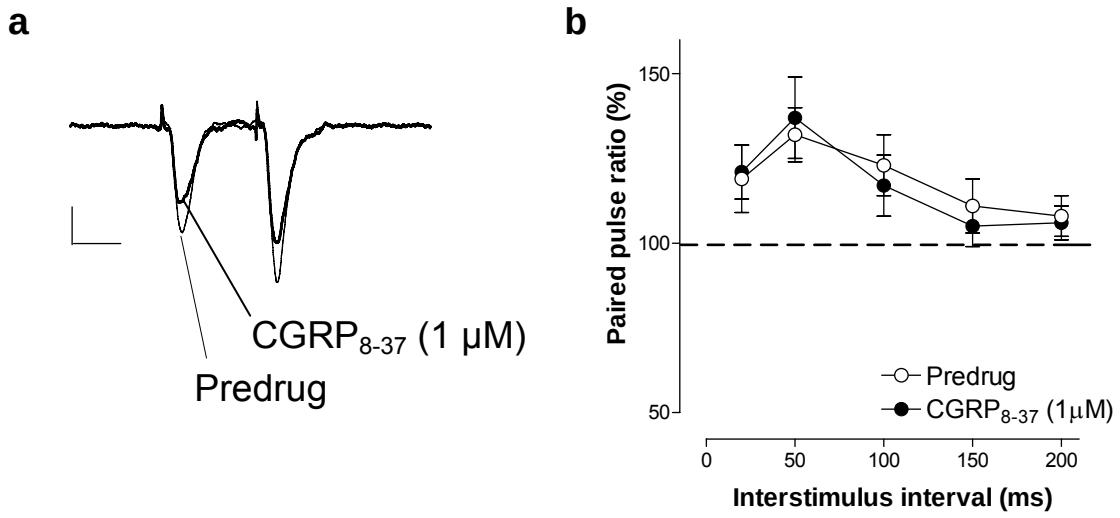

Figure 14

PPF analysis argues against pre-synaptic effects of $CGRP_{8-37}$.

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 synaptic 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. **(a)** 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 $CGRP_{8-37}$ (scale bar, 25 ms/50 pA). $CGRP_{8-37}$ had similar inhibitory effects on the first and second EPSC. **(b)** $CGRP_{8-37}$ had no significant effect on PPF at various stimulus intervals in the whole sample of neurons ($n = 6$, $P > 0.05$, paired t-test). Symbols and error bars represent mean ± SE. Neurons were recorded in voltage-clamp at -60 mV.

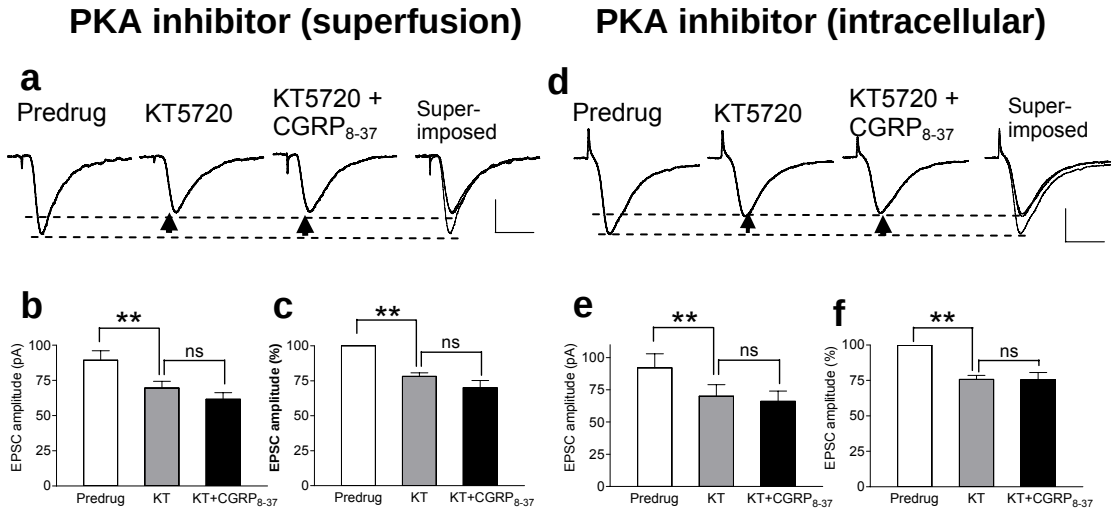

Figure 15

Effects of $CGRP_{8-37}$ are occluded by a PKA inhibitor.

(a-c) Superfusion of the slices with a selective membrane-permeable PKA inhibitor (KT5720; 1 μM) decreased synaptic plasticity and abolished the inhibitory effects of $CGRP_{8-37}$ (1 μM). **(a)** Individual traces show monosynaptic EPSCs (average of 8-10 EPSCs) in a CeLC neuron from an arthritic rat. Recordings were made before drug application, in the presence of KT5720 alone and during co-application of $CGRP_{8-37}$ and KT5720. Averaged raw **(b)** and normalized **(c)** data show the significant inhibitory effect of KT5720 but no further inhibition by $CGRP_{8-37}$ when coapplied with KT5720. **(d-f)** Direct intracellular application of the PKA inhibitor through the patch pipette filled with internal solution containing KT5720 (1μM) also occluded the inhibitory effects of $CGRP_{8-37}$. **(d)** EPSCs were measured immediately after whole-cell patch configuration was obtained (predrug). EPSC amplitude decreased 10 min after the patch formation when KT5720 had entered the cell. Co-application of $CGRP_{8-37}$ (superfusion) caused no further inhibition. Averaged raw **(e)** and normalized **(f)** data show the significant inhibitory effect of KT5720 (intracellular application) but no further inhibition by $CGRP_{8-37}$. Scale bars in **a** and **d**, 20 ms/ 40 pA. ** $P < 0.01$ (repeated measures ANOVA with Tukey post test)

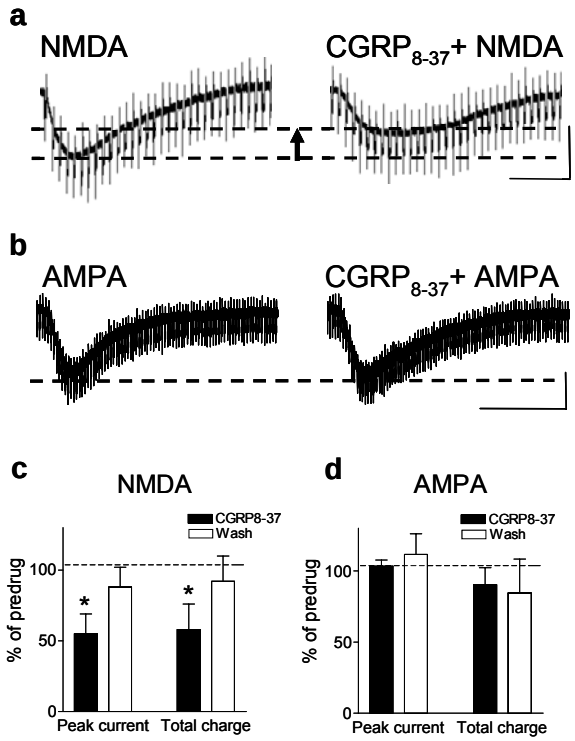

Figure 16

$CGRP_{8-37}$ inhibits NMDA, but not AMPA, receptor function.

(a) $CGRP_{8-37}$ decreased the inward current evoked by the application of NMDA (1 mM in the recording chamber) in a CeLC neuron from an arthritic rat (scale bars, 20 s/ 200 pA). Arrow indicates difference of peak amplitude of NMDA current before and during $CGRP_{8-37}$ application. **(b)** $CGRP_{8-37}$ had no effect on the inward current evoked by the application of AMPA (30 μM in the chamber) in a CeLC neuron from an arthritic rat (scale bars 100 s/ 200 pA), **(c)** Averaged data show the significant inhibitory effect of $CGRP_{8-37}$ on NMDA-evoked membrane currents in terms of peak current and area under the curve (n = 5). **(d)** AMPA-evoked membrane currents were not affected significantly by $CGRP_{8-37}$ (n = 5). Bar histogram and error bars represent mean ± SE. Drug effects were expressed as percentage of predrug value (set to 100%). Neurons were recorded in voltage-clamp at -60 mV. * $P < 0.05$ (paired t-test).

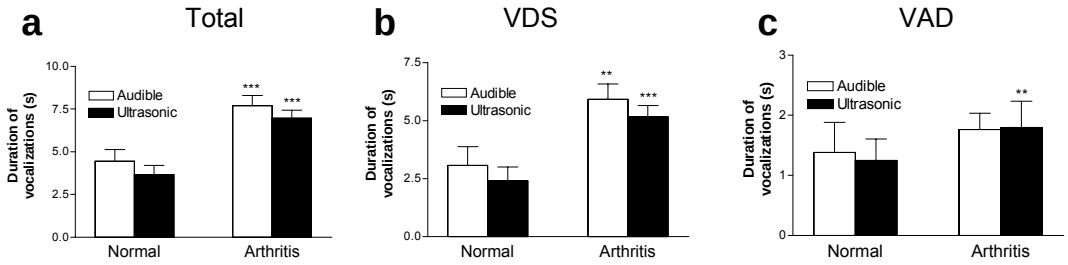

Figure 17

Increased audible and ultrasonic vocalizations in a model of arthritic pain.

Audible and ultrasonic vocalizations were measured before and after arthritis induction. Duration of vocalizations was measured as the arithmetic sum of the duration of each individual vocalization event as described previously (Han and Neugebauer, 2005). Vocalizations during stimulation (VDS) and vocalization afterdischarges (VAD), which are organized in the brain stem and the limbic forebrain, respectively (Borszcz and Leaton, 2003; Han and Neugebauer, 2005), were evoked by noxious (2000 g/30 mm^2) stimulation of the knee. Mechanical stimuli were applied for 15 s; duration of the recording period was 1 min. **(a)** Total duration of audible and ultrasonic vocalizations increased significantly 6 h after induction of arthritis compared to the values measured in the same animals before arthritis (n = 16). **(b)** Duration of VDS also increased significantly 6h after arthritis induction (n = 16). **(c)** Duration of ultrasonic, but not audible, VAD following increased significantly in the arthritis pain model in this set of animals (n = 16). Symbols and error bars represent mean ± SE. ** $P < 0.01$, *** $P < 0.001$ (paired t-test).

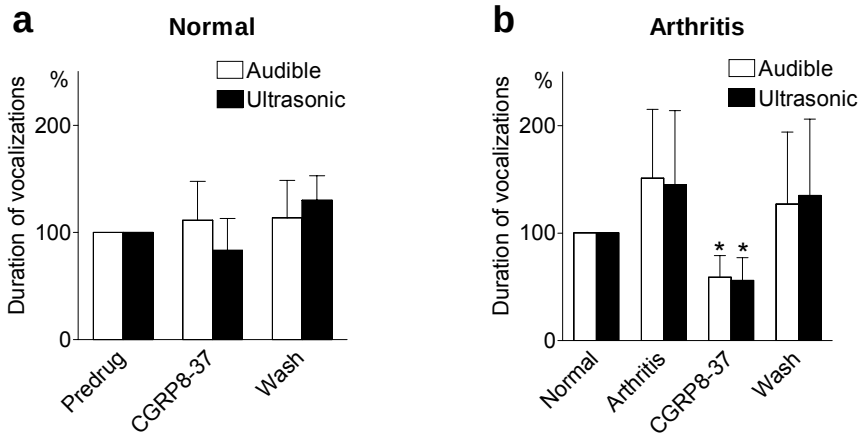

Figure 18

$CGRP_{8-37}$ inhibits vocalization afterdischarges (VAD) in animals with arthritis but not in normal animals.

Audible and ultrasonic vocalizations were measured in normal rats **(a)** and arthritic rats **(b)**. Duration of vocalizations was measured as the arithmetic sum of the duration of each individual vocalization event as described previously (Han and Neugebauer, 2005). Vocalization afterdischarges (VAD) were evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the knee. **(a)** Application of $CGRP_{8-37}$ (100 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC of normal rats had no significant effect ($n = 4$, $P > 0.05$, paired t-test). **(b)** In arthritic animals (6 h postinduction), $CGRP_{8-37}$ (100 μM) significantly reduced audible and ultrasonic VAD ($n = 9$, $P < 0.05$, paired t-test). Vocalizations of arthritic animals were expressed as percentage of vocalizations of the same animals before arthritis induction (normal, set to 100 %). Bar histograms and error bars represent mean ± SE. * $P < 0.05$.

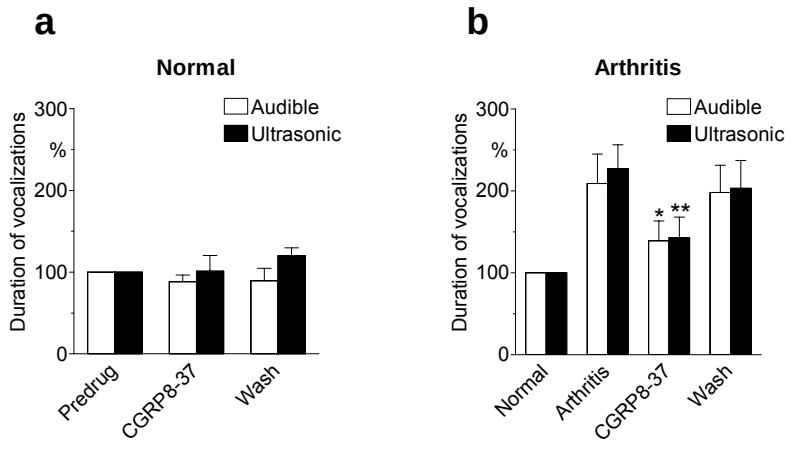

Figure 19

$CGRP_{8-37}$ inhibits vocalizations during stimulation (VDS) in animals with arthritis but not in normal animals.

Audible and ultrasonic vocalizations during stimulation (VDS), which are organized at the medullary brainstem level (Borszcz and Leaton, 2003; Han and Neugebauer, 2005), were evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the knee. Administration of $CGRP_{8-37}$ (100 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC did not affect the duration of audible and ultrasonic VDS in normal animals (**a**, n = 4, P > 0.05, paired t-test) but significantly inhibited VDS of arthritic rats (**b**, n = 9, P < 0.05, paired t-test). Bar histograms and error bars represent mean ± SE. * P < 0.05, ** P < 0.01.

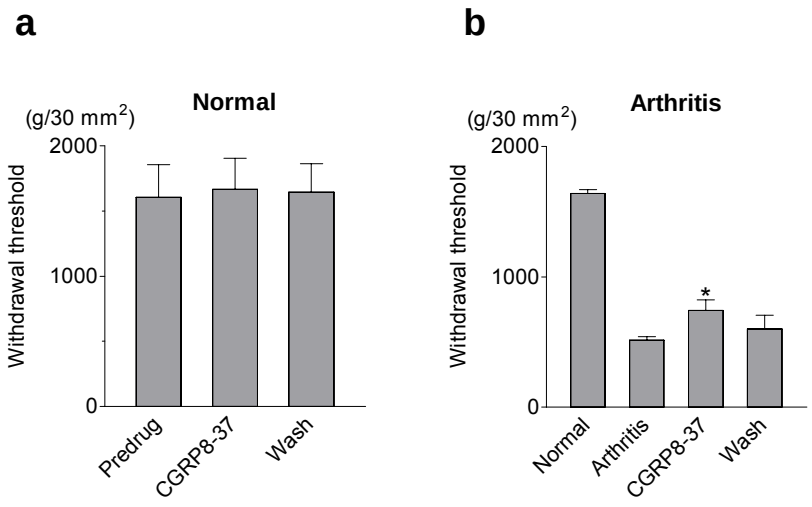

Figure 20

$CGRP_{8-37}$ increased hind limb withdrawal thresholds in animals with arthritis but not in normal animals.

Spinally organized hindlimb withdrawal reflexes were evoked by mechanical stimulation (compression) of the knee (15 s) with increasing intensity (steps of 50 g/30 mm^2). Withdrawal thresholds were defined as the minimum stimulus intensity that evoked a withdrawal reflex. Thresholds decreased 6 h after arthritis induction, indicating increased sensitivity. Application of $CGRP_{8-37}$ into the CeLC significantly increased the reduced thresholds in arthritic animals (**b**, n = 6, $P < 0.05$, paired t-test), but had no effect in normal rats (**a**, n = 4, $P > 0.05$, paired t-test). Bar histograms and error bars represent mean ± SE. * $P<0.05$.

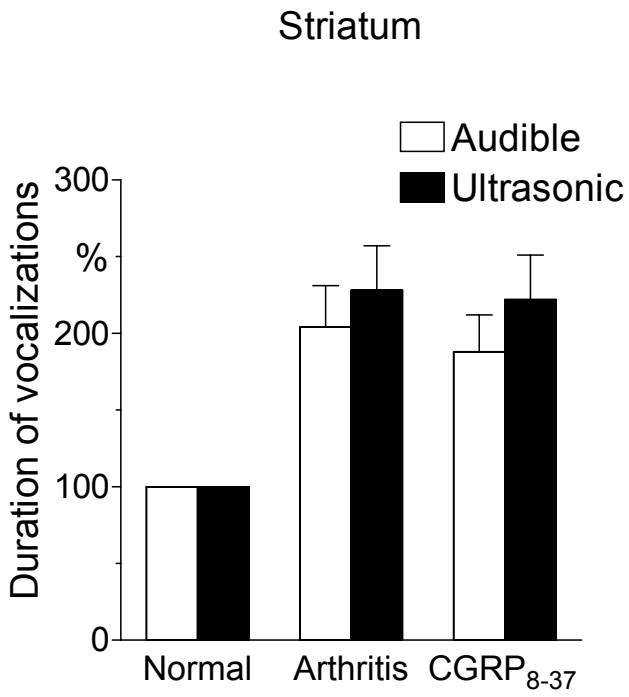

Figure 21

Placement control experiments.

Application of $CGRP_{8-37}$ into the striatum (caudate-putamen, dorsolateral to CeLC) did not produce significant changes of audible and ultrasonic vocalizations in arthritic animals ($n = 5$, $P > 0.05$, paired t-test). Vocalization data represent the total duration of vocalizations. Since neither VAD nor VDS were inhibited by $CGRP_{8-37}$ the data were pooled for simplification. $CGRP_{8-37}$ (100 μM) was administered by microdialysis for 15-20 min. Bar histograms and error bars represent mean ± SE.

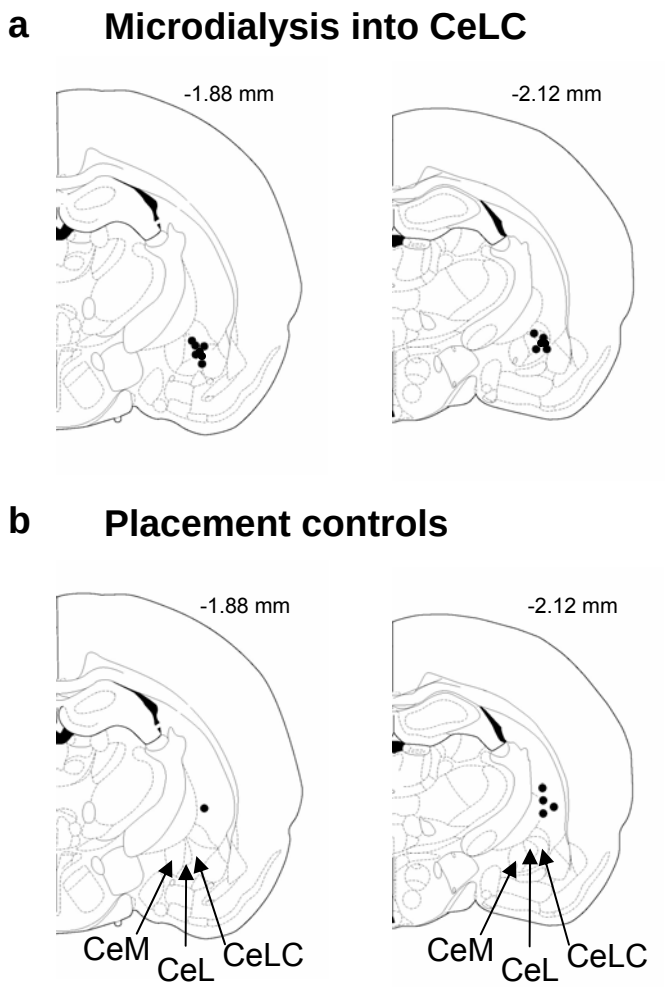

Figure 22

Histological verification of drug application sites.

(a) Sites of drug application into the CeLC by microdialysis in the vocalization experiments. **(b)** Sites of drug application into the striatum by microdialysis as placement controls for any effects due to drug diffusion. CeM, CeL, CeLC: medial, lateral and latero-capsular divisions of the central nucleus of the amygdala. Numbers indicate distance from bregma. Each symbol indicates the location of the tip of one microdialysis probe.

CHAPTER 4: EXOGENOUS ACTIVATION OF CGRP RECEPTORS PRODUCES PAIN-RELATED PLASTICITY AND BEHAVIOR

SYNAPTIC PLASTICITY AND ENHANCED EXCITABILITY IN THE CELC DURING EXOGENOUS APPLICATION OF CGRP

Whole-cell voltage-clamp recordings of CeLC neurons were made in brain slices from normal rats (no arthritis). Superfusion of the slices with CGRP significantly enhanced synaptic transmission, mimicking synaptic plasticity observed in the arthritic pain model. Peak amplitudes of monosynaptic EPSCs at the nociceptive PB-CeLC synapse were increased by CGRP in a concentration-dependent manner (EC_{50} = 28.3 nM; **Fig. 23**). These data show the presence of functional CGRP receptors in the CeLC under normal conditions.

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 (pA) as a function of afferent fiber volley stimulus intensity (μA) for each neuron (**Fig. 24**). The input-output function of the PB-CeLC synapse was significantly increased in CeLC neurons by CGRP (n = 9) as evidenced by the steeper slope and upward shift at higher stimulus intensities (**Fig. 24**, $P < 0.0001$, $F_{1,140} = 18.00$, two-way ANOVA). The sites of synaptic stimulation and patch-clamp recording in the amygdala brain slice are schematically illustrated in **Fig. 3**.

To determine the effect of CGRP on neuronal excitability, 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. CGRP significantly increased the input-output function of CeLC neurons in slices from normal animals (**Fig. 25**, n = 6, $P < 0.05$, $F_{1,080} = 6.14$, two-way ANOVA), mimicking the increased excitability observed in the arthritis pain model (see Chapter 3).

POST- RATHER THAN PRE-SYNAPTIC ACTION OF CGRP

To determine whether CGRP acts on pre- or post-synaptic sites in the CeLC we used a number of electrophysiological parameters, including the analysis of neuronal excitability (**Fig. 25**), amplitude and frequency of spontaneous mEPSCs (**Fig. 26**), and PPF (**Fig. 27**). These parameters were measured in slices from normal (untreated) animals before and during application of CGRP in amygdala brain slices from normal rats (no arthritis).

CGRP increased the amplitude, but not frequency, of mEPSCs recorded in the presence of TTX (1 μM) (**Fig. 26**). This postsynaptic effect is illustrated in the current traces recorded in an individual CeLC neuron (**Fig. 26a**). In the whole sample of neurons (n = 4), the normalized mean mEPSC amplitude was significantly increased ($P < 0.005$, Kolmogorov-Smirnov test) (**Fig. 26b**). $CGRP_{8-37}$ had no effect on the frequency of mEPSCs (see normalized cumulative inter-event interval distribution and mean mEPSC frequency, **Fig. 26c**; $P > 0.05$, paired t-test, n = 4).

Further arguing against a presynaptic site of action, CGRP had no significant effect on PPF. PPF was calculated as the ratio of the second and the first of two consecutive EPSCs evoked by electrical stimuli of equal intensity. CGRP application did not significantly change PPF at different interstimulus intervals (n = 12, $P > 0.05$, paired t-test) (**Fig. 27**). Increased neuronal excitability shown in **Fig. 25** also suggests a post-synaptic mechanism. Taken together, the analysis of PPF, mEPSCs and neuronal excitability suggests that exogenous CGRP receptor activation occurs at a post- rather than pre-synaptic site in CeLC neurons.

CGRP ADMINISTRATION INTO THE CELC MIMICS ARTHRITIC PAIN-RELATED BEHAVIORAL CHANGES

To validate the behavioral significance of CGRP receptor activation in the CeLC, we analyzed the effect of CGRP on supraspinally (vocalizations) and spinally (hindlimb withdrawal reflexes) organized behavior in awake normal animals. Pain-related vocalizations in the audible and ultrasonic range were measured in the same animal

before and during administration of CGRP into the CeLC. As described before, CGRP effects were determined separately for vocalizations during stimulation (VDS), which are organized at the medullary brainstem level, and vocalization afterdischarges (VAD), which outlast the actual stimulus and are organized in the limbic forebrain, particularly the amygdala (Borszcz and Leaton, 2003; Han and Neugebauer, 2005).

Administration of CGRP (10 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC increased the duration of audible and ultrasonic VAD and VDS (**Fig. 28**) evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the arthritic knee (n=4). The facilitatory effects of CGRP were statistically significant ($P < 0.01$-0.05; paired t-test). Predrug vocalizations were measured during administration of ACSF through the microdialysis probe, thus serving as vehicle controls. 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. 29**). Mechanical stimuli of increasing intensity (steps of 50 g/30 mm^2) were applied to the knee joint. Withdrawal threshold was defined as the minimum stimulus intensity that evoked a withdrawal reflex (Han and Neugebauer, 2005). Administration of CGRP (10 μM; 15 min) into the CeLC decreased the withdrawal thresholds measured in naïve (non-arthritic) animals, indicating increased sensitivity. Drugs were administered into the right CeLC because our previous electrophysiological in vivo and in vitro studies showed pain-related plasticity in the right amygdala (CeLC) (Neugebauer and Li, 2003; Neugebauer et al., 2003) and our behavioral data showed that the right amygdala is coupled to pain facilitation in the arthritis pain model (Han and Neugebauer, 2005). 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. 30**). The concentration of CGRP was selected based on the data from in vitro electrophysiology (see above) and adjusted for the concentration gradient (×100) across the dialysis membrane.

The vocalization data show that activation of the CeLC by CGRP produces pain responses organized in the brainstem (VDS) and limbic forebrain (VAD).

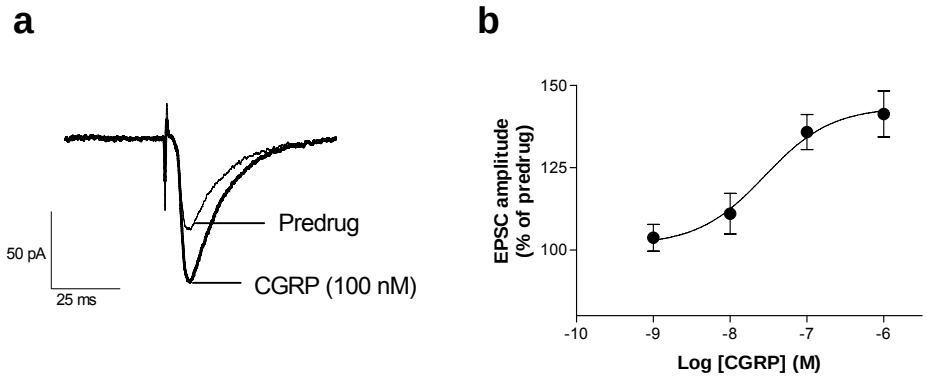

Figure 23

CGRP enhances synaptic transmission in the CeLC in slices from normal animals.

Individual example **(a)** and concentration-response relationship **(b)** show that CGRP enhanced synaptic transmission at the PB-CeLC synapse in a concentration dependent manner in slices from naïve animals (no arthritis). Peak amplitudes of monosynaptic EPSCs during each concentration of CGRP were averaged and expressed as percent of predrug (baseline) control (set to 100 %). CGRP was applied for at least 15 min and measurements were made at 12 min. Traces in **(a)** are the average of 10 EPSCs. Symbols and error bars in **(b)** represent mean ± SE.

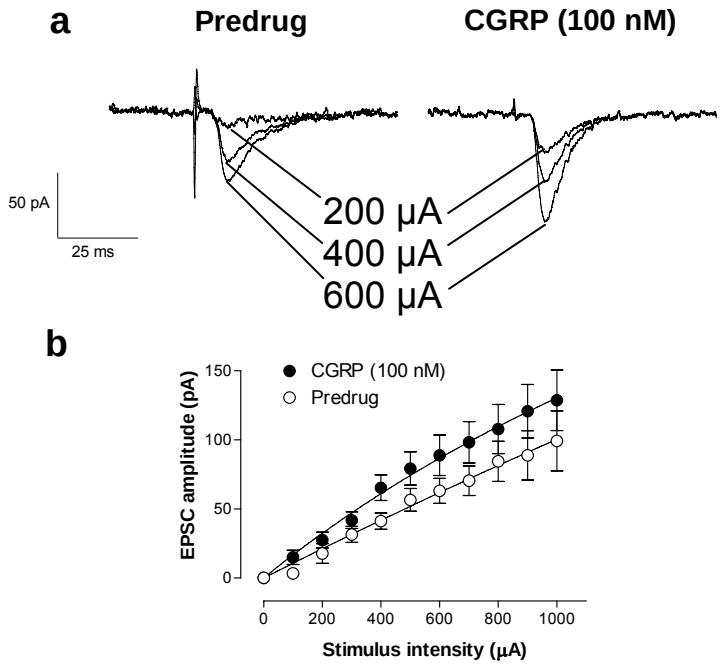

Figure 24

CGRP increases input-output function of the PB-CeLC synapse in slices from normal animals.

Input-output function of the PB-CeLC synapse was measured by increasing the stimulus intensity in 100 µA steps. CGRP (100 nM) significantly increased EPSC amplitudes evoked by different stimulation intensities ($n = 9$, $P < 0.0001$, $F_{1,140} = 18.00$, two-way ANOVA). Whole-cell voltage-clamp recordings were made from CeLC neurons held at -60 mV in slices from naïve animals (no arthritis). Symbols and error bars in **(b)** represent mean ± SE.

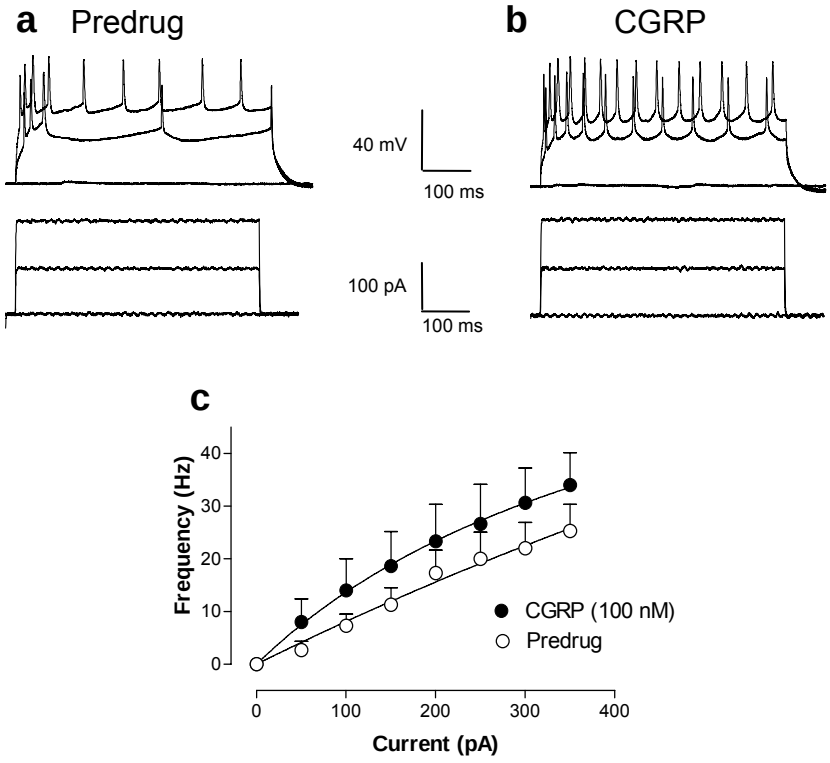

Figure 25

CGRP enhances neuronal excitability of CeLC neurons in slices from normal animals.

Action potentials were evoked in CeLC neurons by direct (through the patch electrode) intracellular injections of current pulses (500 ms) of increasing magnitude (50 pA steps) before and during CGRP administration. **(a,b)** Original traces show action potential firing rate increased during superfusion of CGRP (100 nM). **(c)** CGRP increased input-output functions significantly ($n = 6$, $P < 0.05$, $F_{1,080} = 6.14$, two-way ANOVA), suggesting a direct postsynaptic membrane effect. For the measurement of action potential firing in current clamp, neurons were recorded at -60 mV. Symbols and error bars in **c** represent mean ± SE.

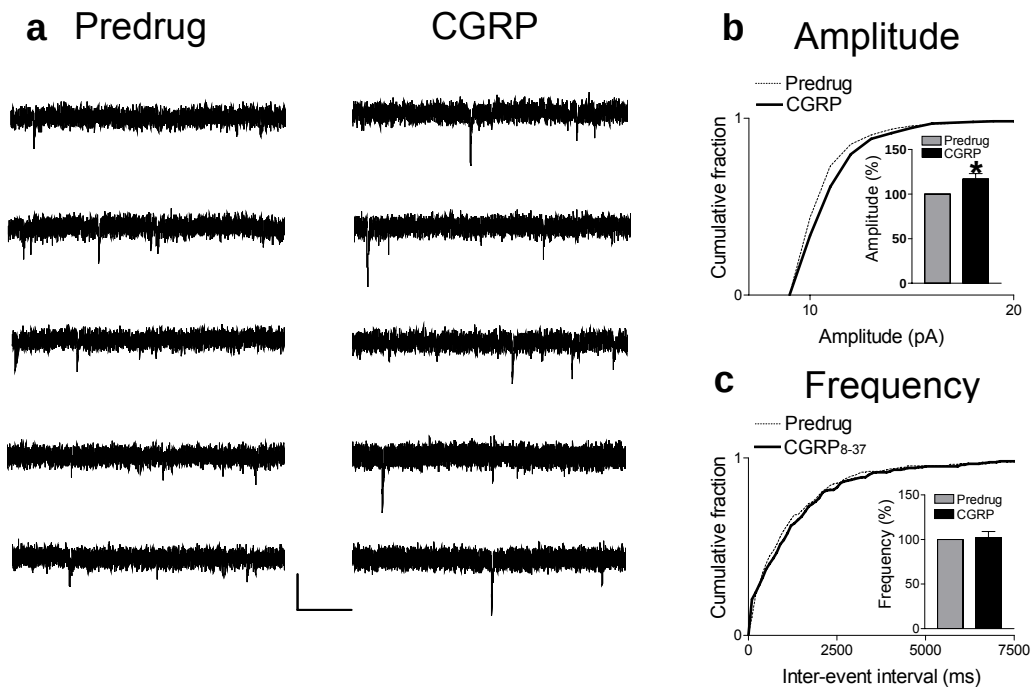

Figure 26

Miniature EPSC (mEPSC) analysis suggests post- rather than pre-synaptic effects of CGRP.

(a) Original current traces of mEPSC recordings in an individual CeLC neuron in the presence of TTX (1 μM) show that CGRP (100 nM) increased the amplitude but not frequency of mEPSCs (scale bars, 1 s/ 20 pA). **(b, c)** The cumulative distribution analysis of mEPSC amplitude and frequency showed that CGRP caused a significant shift toward larger amplitudes **(b)** ($P < 0.005$, maximal difference in cumulative fraction = 0.122, Kolmogorov-Smirnov test) but had no effect on the interevent interval (frequency) distribution **(c)**. CGRP selectively increased the mean mEPSC amplitude ($P < 0.05$, paired t-test) but not mEPSC frequency (events/s) in the sample of neurons (n = 4; see bar histograms in **b,c**). Recordings were made in slices from naïve (untreated) animals. * $P < 0.05$.

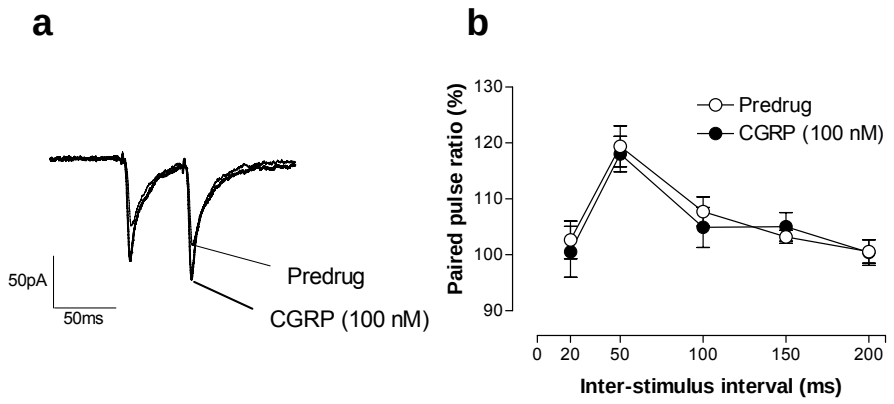

Figure 27

PPF analysis shows no evidence for pre-synaptic effects of CGRP.

PPF, a measure of presynaptic mechanisms, was not affected by CGRP. 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 inter-stimulus intervals. Peak EPSC amplitudes were measured as the difference between the current level before the stimulus artifact and the peak of the EPSC. **(a)** Current traces (average of 8-10 EPSCs) recorded in an individual CeLC neuron illustrate that PPF evoked at the 50 ms interval was not affected by CGRP. **(b)** CGRP had no significant effect on PPF at various stimulus intervals in the whole sample of neurons (n = 12, $P > 0.05$, paired t-test). Symbols and error bars represent mean ± SE. Neurons were recorded in voltage-clamp at -60 mV.

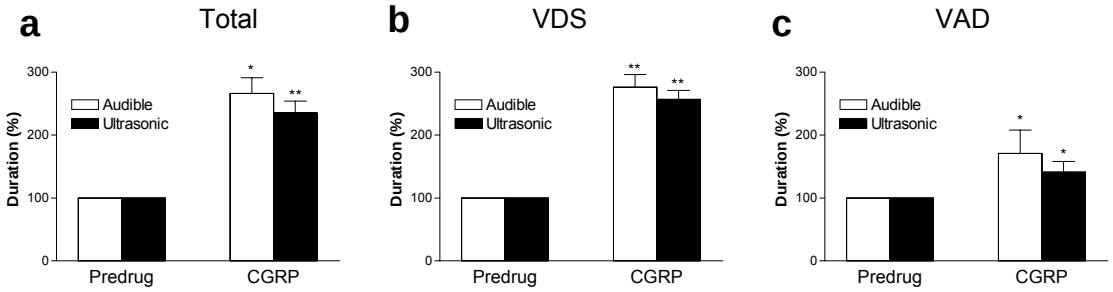

Figure 28

CGRP increases audible and ultrasonic vocalizations in normal animals.

Total duration of vocalizations and vocalizations during and after stimulation (VDS and VAD, respectively) were analyzed separately. Duration of audible and ultrasonic VAD and VDS increased significantly during CGRP administration (10 μM) through the microdialysis probe implanted in the CeLC (n = 4, paired t-test). Noxious mechanical stimuli (2000 g/30 mm^2) were applied for 15 s; duration of the recording period was 1 min. Symbols and error bars represent mean ± SE. *P < 0.05, ** P < 0.01 (paired t-test).

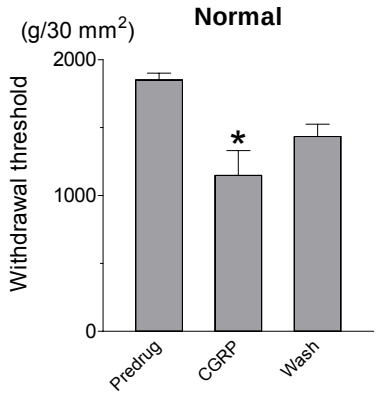

Figure 29

CGRP decreased hind limb withdrawal thresholds in normal animals.

Spinally organized hindlimb withdrawal reflexes were evoked by mechanical stimulation (compression) of the knee (15 s) with increasing intensity. Application of CGRP into the CeLC significantly decreased the withdrawal thresholds in normal animals (n = 4, $P < 0.05$, paired t-test). Bar histograms and error bars represent mean ± SE. * $P < 0.05$.

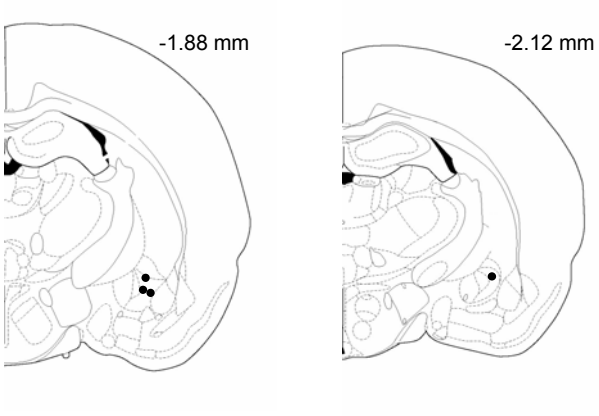

Figure 30

Histological verification of drug application sites.

Sites of CGRP application into the CeLC by microdialysis in the vocalization experiments. Numbers indicate distance from bregma. Each symbol indicates the location of the tip of one microdialysis probe.

CHAPTER 5: ACTIVATION OF PKA AND ERK1/2, BUT NOT PKC, IS INVOLVED IN ARTHRITIS- AND CGRP-INDUCED PLASTICITY AND BEHAVIOR

INHIBITION OF PKA AND ERK1/2, BUT NOT PKC, REDUCES ARTHRITIC PAIN-RELATED SYNAPTIC PLASTICITY IN THE CELC

To identify signal transduction mechanisms involved in arthritic pain-related synaptic plasticity, whole-cell voltage-clamp recordings of CeLC neurons were made in brain slices from untreated normal rats and from rats in which an arthritis pain state had been induced 6 h before. EPSC amplitudes at the PB-CeLC synapse were measured before and during application of PKA, PKC and MEK (ERK1/2, extracellular signal-regulated kinase) inhibitors to identify mechanisms of pain related synaptic plasticity. A selective PKA inhibitor (KT5720, 1 μM) inhibited synaptic transmission in neurons from arthritic rats (**Fig. 31**, $n = 7$, $P < 0.05$, paired t-test) but not normal synaptic transmission in neurons from naïve rats ($n = 7$, $P > 0.05$). Likewise, a MEK inhibitor (U0126, 1 μM) decreased EPSC amplitudes in neurons from arthritic rats (**Fig. 32**, $n = 6$, $P < 0.05$, paired t-test) but not in neurons from normal rats ($n = 6$, $P > 0.05$). The inhibitory effects in arthritis but not under normal conditions suggest that PKA and ERK1/2 activation is involved specifically in pain-related synaptic plasticity in the CeLC. In contrast, a selective PKC inhibitor (GF109203x, 1 μM) did not significantly affect synaptic transmission at the PB-CeLC synapse in neurons from normal (**Fig. 33**, $n = 5$, $P > 0.05$, paired t-test) and arthritic rats ($n = 6$).

INHIBITION OF PKA AND ERK1/2, BUT NOT PKC, REDUCES ARTHRITIC PAIN-RELATED BEHAVIOR

Next we determined if the signaling pathways involved in synaptic plasticity also play a role in pain-related behavior. Inhibitors of PKA, PKC and MEK were administered into the CeLC in arthritic animals. Pain-induced vocalizations in the audible and

ultrasonic range were measured in the same animal before and after induction of arthritis and before and during drug administration by microdialysis. Vocalizations during stimulation (VDS) and vocalization afterdischarges (VAD) were analyzed separately as described earlier (see Chapter 2 and 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 postinduction, **Fig. 34-36**). Administration of PKA and MEK inhibitors (KT5720 and U0126, 100 μM respectively; concentration in the microdialysis probe; 15-20 min) into the CeLC decreased the duration of audible and ultrasonic VAD and VDS evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the arthritic knee. Concentrations were selected based on the data from in vitro electrophysiology (see above) and adjusted for the concentration gradient (×100) across the dialysis membrane. The inhibitory effects of KT5720 (n = 4) and U0126 (n = 5) were significant for total duration and VDS and were largely reversible (**Fig. 34 and 35**, $P < 0.001$-0.05; paired t-test). Effects on VAS were less pronounced and did not reach significance in all cases. In contrast, a PKC inhibitor (GF109203X, n = 4; 100 μM; concentration in the microdialysis probe; 15-20 min) had no significant effect on any type of vocalizations (**Fig. 36**). Predrug vocalizations were measured during 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 (Bernard and Bandler, 1998; Neugebauer et al., 2004). Also, our previous electrophysiological in vivo and in vitro studies showed pain-related plasticity in the right amygdala (CeLC) (Neugebauer and Li, 2003; Neugebauer et al., 2003). Our behavioral data also suggest that the right amygdala is coupled to pain facilitation in the arthritis pain model (Han and Neugebauer, 2005). 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 after each experiment (**Fig. 37**).

INHIBITION OF PKA AND ERK1/2, BUT NOT PKC, REVERSES CGRP-INDUCED SYNAPTIC PLASTICITY IN THE CELC

We showed previously (Bird et al., 2005) that PKA-dependent phosphorylation increases the function of postsynaptic NMDA receptors in the CeLC. The present study further suggests an important role of PKA, but not PKC, in pain-related plasticity and behavior. The major signal transduction pathway of CGRP involves cAMP formation and PKA activation (Poyner, 1996;Wimalawansa, 1996;Van Rossum et al., 1997). One of the downstream signaling molecules of PKA is the MAP kinase ERK1/2, which plays an important role in spinal pain mechanisms (Ji et al., 2002; Hu and Gereau, 2003). Therefore, we tested the hypothesis that CGRP-induced synaptic facilitation in the CeLC depends on the cAMP-PKA-ERK1/2 signaling pathway rather than PKC.

Whole-cell voltage-clamp recordings of CeLC neurons were made in brain slices from untreated naïve rats. Consistent with the data presented earlier, CGRP enhanced synaptic transmission at the nociceptive PB-CeLC synapse, mimicking synaptic plasticity observed in the arthritic pain model. Coapplication of inhibitors of PKA, MEK and PKC with CGRP were used to identify mechanisms of CGRP-induced facilitation at the PB-CeLC synapse. A selective PKA inhibitor (KT5720, 1 μM) significantly reversed the CGRP-induced increase of synaptic transmission (**Fig. 38**, n = 7). Likewise, a MEK inhibitor (U0126, 1 μM) significantly reversed the effect of CGRP (**Fig. 39**, n = 7). The effects of KT5720 and U0126 were reversible. In contrast, co-administration of a selective PKC inhibitor (GF109203x, 1 μM) did not change the synaptic facilitation by CGRP (**Fig. 40**, n = 6). These data suggest that activation of PKA and ERK1/2, but not PKC, are involved in CGRP-induced synaptic plasticity in the CeLC. The fact that the effects of KT5720 and U0126 were comparable may suggest that PKA and ERK1/2 act in a serial rather than parallel manner.

INHIBITION OF PKA REVERSES CGRP-INDUCED PAIN BEHAVIOR

Next we verified the importance of PKA-dependent CGRP-induced synaptic facilitation for CGRP-induced pain behavior. Audible and ultrasonic vocalizations were

measured before and during application of CGRP (10 μM) into the right CeLC by microdialysis. Then a PKA inhibitor (KT5720, 100 μM) was co-applied into the right CeLC. All animals had guide cannulas for the microdialysis probes implanted on the day before the behavioral tests.

The total duration of vocalizations and the duration of VAD and VDS increased during CGRP application. Co-administration of KT5720 (100 μM) and CGRP (10 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC decreased the duration of audible and ultrasonic vocalizations evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the knee (**Fig. 41**, n = 4). Predrug vocalizations were measured during administration of ACSF through the microdialysis probe, thus serving as vehicle controls. The positions of the microdialysis probes in the CeLC were verified histologically after each experiment (**Fig. 30**). These behavioral data suggest that PKA plays an important role in CGRP-induced pain behavior.

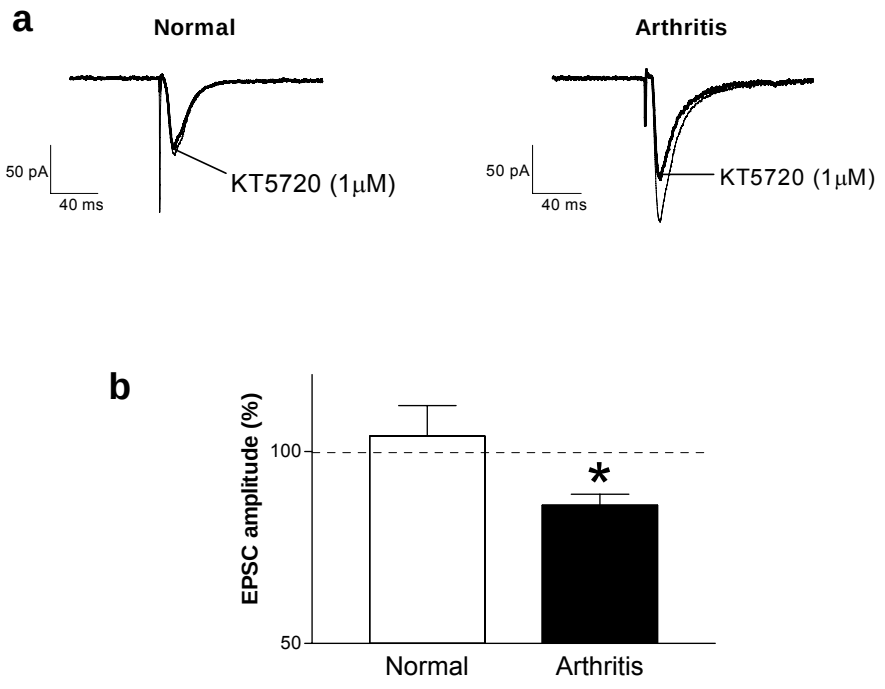

Figure 31

A PKA inhibitor (KT5720) reduces arthritic pain-related synaptic plasticity.

Monosynaptic EPSCs were evoked at the PB-CeLC synapse in slices from normal rats (n = 7) and arthritic rats (n = 7) before and during drug application. **(a)** Individual traces (averages of 8-10 EPSCs) show that KT5720 reduced synaptic plasticity at the PB-CeLC synapse in a CeLC neuron from an arthritic rat but not in a neuron from a normal rat. **(b**) Peak amplitudes of EPSCs during drug application were expressed as percentage of predrug control values (set to 100 %) and averaged across the sample of neurons (n = 7 in each group). Bar histograms and error bars represent mean ± SE. * $P < 0.05$ (paired t-test)

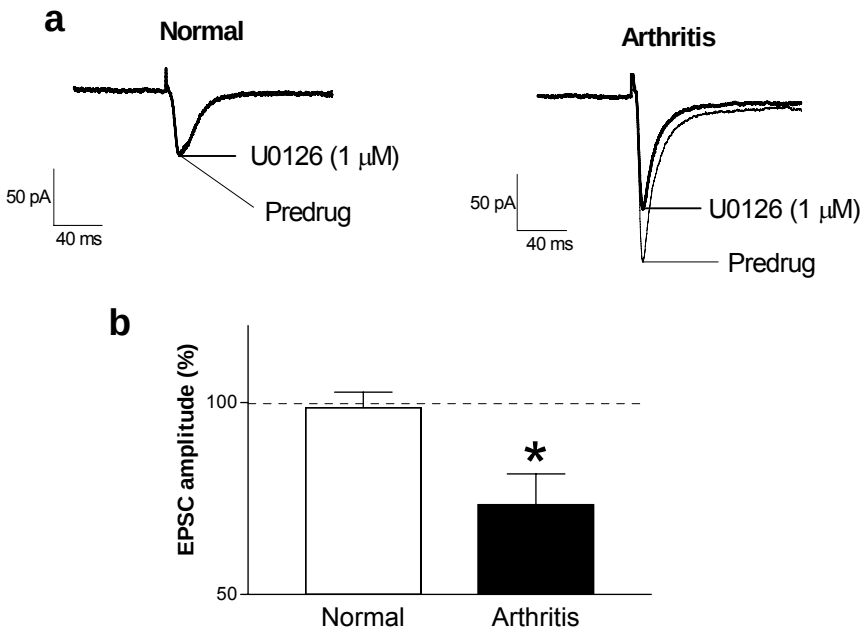

Figure 32

A MEK inhibitor (U0126) reduces arthritic pain-related synaptic plasticity.

Monosynaptic EPSCs were evoked at the PB-CeLC synapse in brain slices from normal (n = 6) and arthritic rats (n = 6) before and during drug application. **(a)** Individual traces (averages of 8-10 EPSCs) show that U0126 (1 μM) reduced synaptic plasticity at the PB-CeLC synapse in a neuron from an arthritic rat, but not in a neuron from a normal rat. **(b)** Peak EPSC amplitudes recorded during drug application are expressed as percentage of predrug control values (set to 100 %) and averaged across the sample of neurons. Bar histograms and error bars represent mean ± SE. *P < 0.05 (paired t-test).

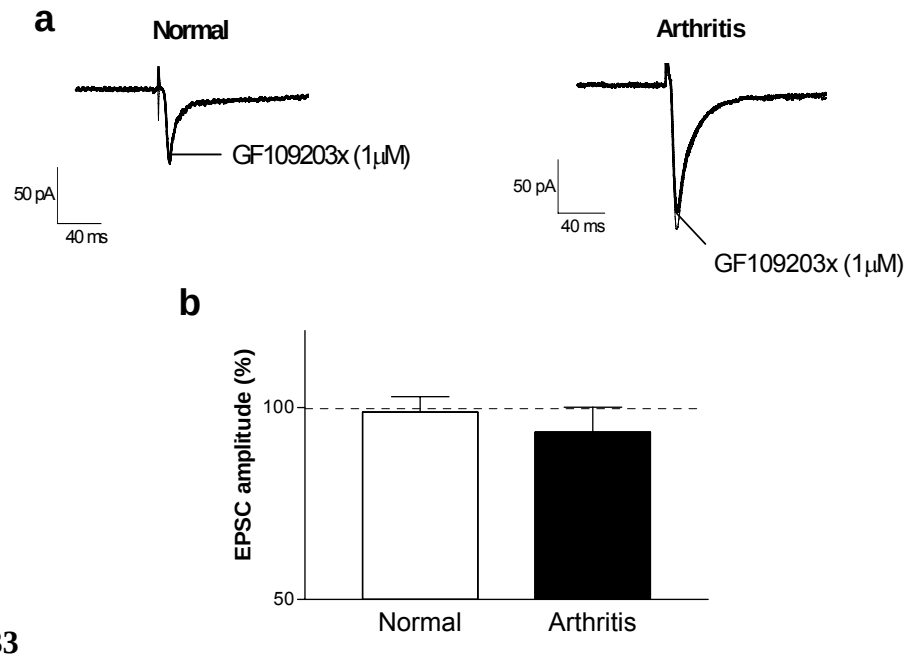

Figure 33

A PKC inhibitor (GF109203X) has no effect on arthritic pain-related synaptic plasticity.

Monosynaptic EPSCs were evoked at the PB-CeLC synapse in normal (n = 5) and arthritic rats (n = 6) before and during drug application. **(a)** Individual traces (averages of 8-10 EPSCs) show that GF109203X (1 μM) did not significantly change EPSC amplitudes at the PB-CeLC synapse in a neuron from an arthritic and another one from a normal rat. **(b)** Peak amplitudes of EPSCs recorded during drug application are expressed as percentage of predrug control values (set to 100 %) and averaged across the sample of neurons. Bar histograms and error bars represent mean ± SE.

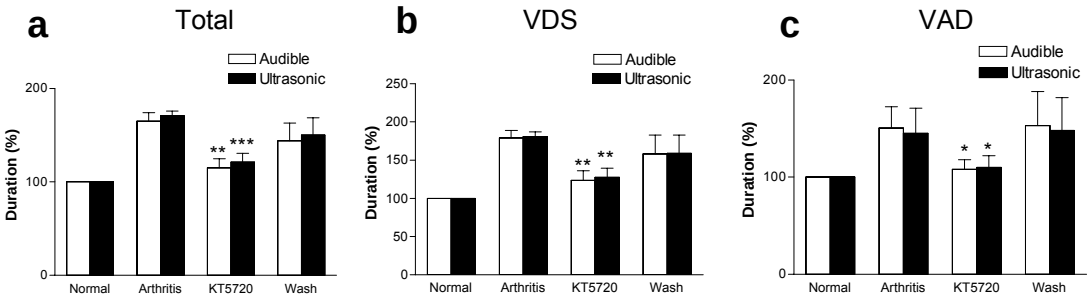

Figure 34

KT5720 inhibits vocalizations of arthritic animals.

Audible and ultrasonic vocalizations were measured before and 6 h after arthritis induction (n = 4). Total duration **(a)** and durations of VDS **(b)** and VAD **(c)** were analyzed separately. Duration of vocalizations was measured as the arithmetic sum of the duration of each individual vocalization event. Vocalizations were evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the knee. Application of KT5720 (100 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC inhibited vocalizations of arthritic rats. Vocalizations of arthritic animals were expressed as percentage of vocalizations of the same animals before arthritis induction (normal, set to 100 %). Bar histograms and error bars represent mean ± SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (paired t-test).

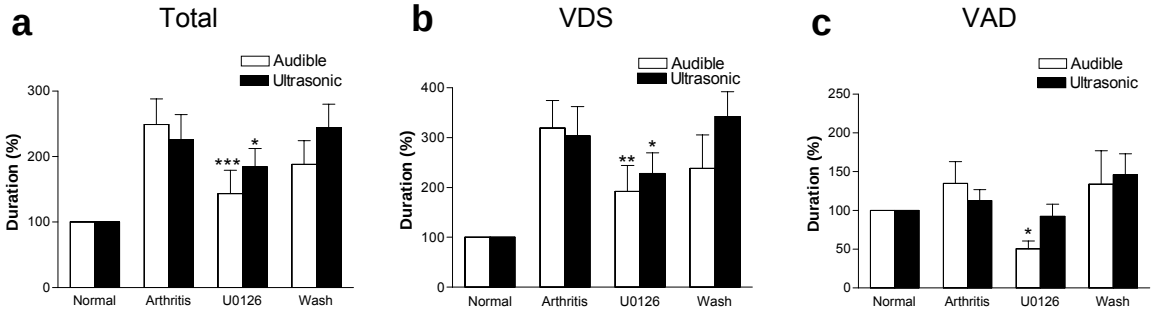

Figure 35

U0126 inhibits vocalizations of arthritic animals.

Audible and ultrasonic vocalizations were measured before and 6 h after arthritis induction (n = 5). Total duration **(a)** and duration of VDS **(b)** and VAD **(c)** were analyzed separately. **(a)**. Duration of vocalizations was measured as the arithmetic sum of the duration of each individual vocalization event. Vocalizations were evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the knee. Application of U0126 (100 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC inhibited vocalizations of arthritic rats. Vocalizations of arthritic animals were expressed as percentage of vocalizations of the same animals before arthritis induction (normal, set to 100 %). Bar histograms and error bars represent mean ± SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (paired t-test).

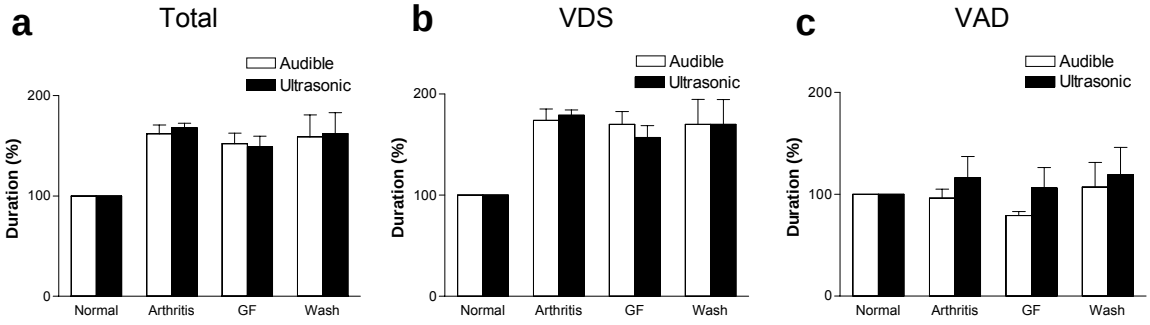

Figure 36

GF109203X has no significant effect on vocalizations of arthritic animals.

Audible and ultrasonic vocalizations were measured before and 6 h after arthritis induction (n = 4). Total duration **(a)** and durations of VDS **(b)** and VAD **(c)** were analyzed separately. Duration of vocalizations was measured as the arithmetic sum of the duration of each individual vocalization event. Vocalizations were evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the knee. Application of GF109203X (GF, 100 µM, concentration in the microdialysis probe; 15-20 min) into the CeLC of arthritic rats did not affect vocalizations ($P > 0.05$, paired t-test). Vocalizations of arthritic animals were expressed as percentage of vocalizations of the same animals before arthritis induction (normal, set to 100 %). Bar histograms and error bars represent mean ± SE.

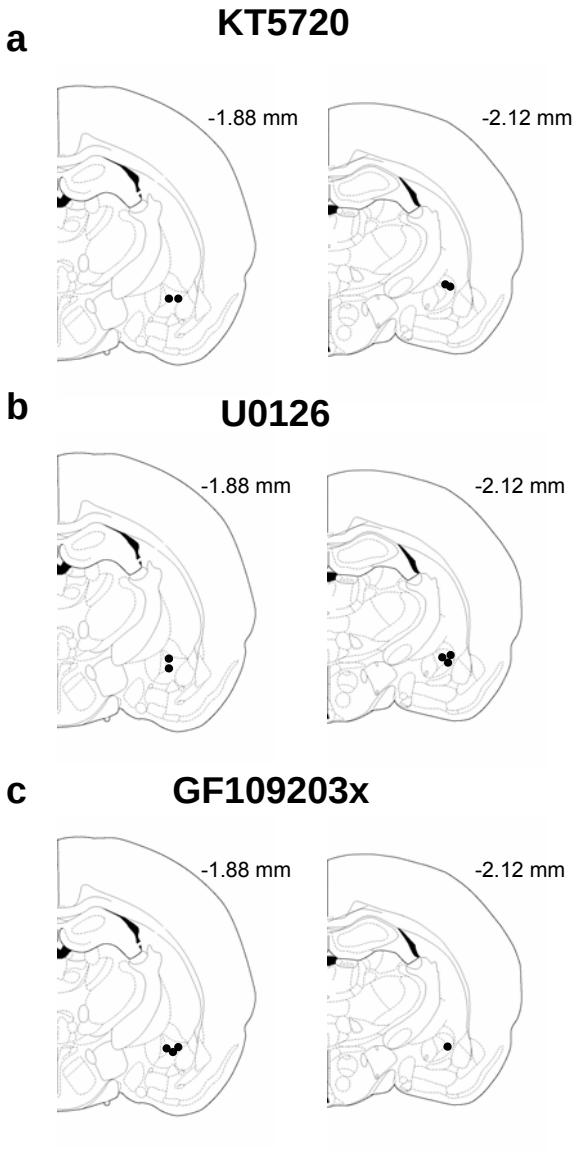

Figure 37

Histological verification of drug application sites.

Sites of KT5720 **(a)**, U0126 **(b)** and GF109203x **(c)** applications into the CeLC by microdialysis in the vocalization experiments. Numbers indicate distance from bregma. Each symbol shows the location of the tip of one microdialysis probe.

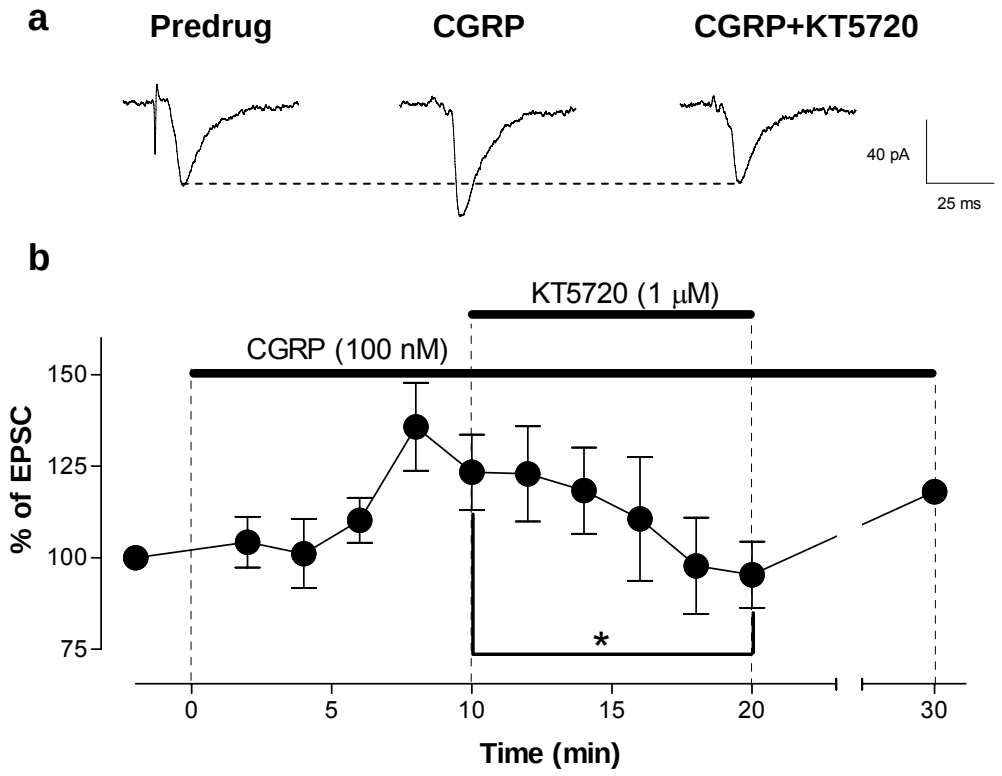

Figure 38

KT5720 reverses CGRP-induced synaptic plasticity.

Monosynaptic EPSCs were evoked at the PB-CeLC synapse in neurons (n = 7) from naïve rats before and during drug applications. CGRP (100 nM) increased EPSC amplitudes. Co-administration of KT5720 (1 μM) reversed the effect of CGRP. **(a)** Original recordings in one individual neuron. Traces are averages of 8-10 EPSCs. **(b)** Time course of CGRP-induced facilitation and reversal by KT5720 (1 μM). Peak amplitudes of EPSCs recorded during drug application were expressed as percentage of predrug control values (set to 100 %). Symbols and error bars represent mean ± SE. * $P < 0.05$ (paired t-test).

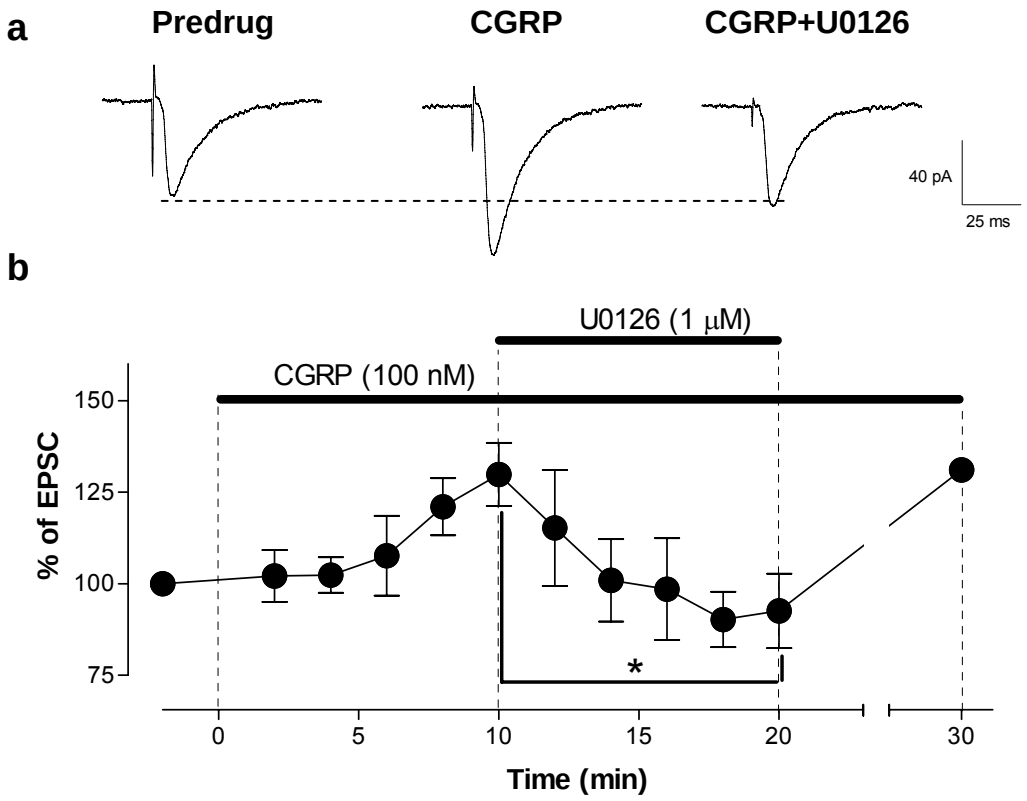

Figure 39

U0126 reverses CGRP-induced synaptic plasticity.

Monosynaptic EPSCs were evoked at the PB-CeLC synapse in neurons (n = 7) from naïve rats before and during drug applications. CGRP (100 nM) increased EPSC amplitudes. Co-administration of U0126 (1 μM) reversed the effect of CGRP. **(a)** Original recordings in one individual neuron. Traces are averages of 8-10 EPSCs. **(b)** Time course of CGRP-induced facilitation and reversal by U0126 (1 μM). Peak amplitudes of EPSCs recorded during drug application were expressed as percentage of predrug control values (set to 100 %). Symbols and error bars represent mean ± SE. * $P < 0.05$ (paired t-test).

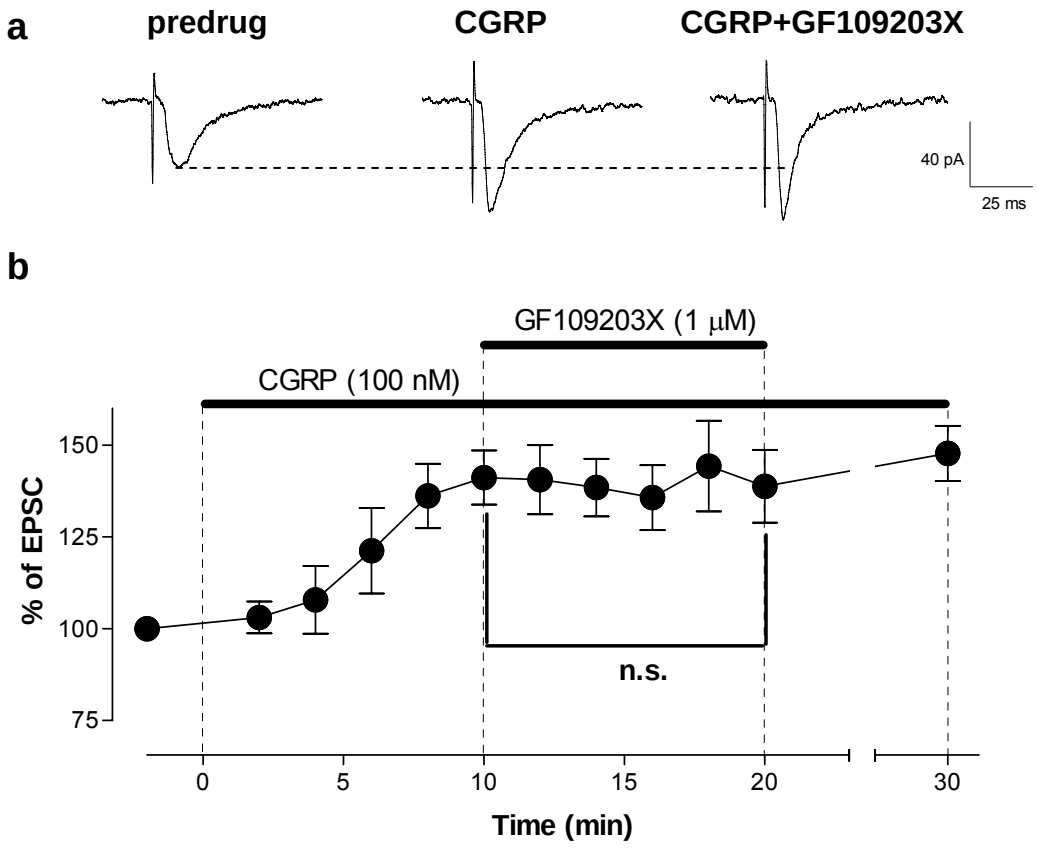

Figure 40

GF109203X has no effect on CGRP-induced synaptic plasticity.

Monosynaptic EPSCs were evoked at the PB-CeLC synapse in neurons (n = 6) from naïve rats before and during drug applications. CGRP (100 nM) increased EPSC amplitudes. Co-administration of GF109203X (1 μM) failed to reverse the increased synaptic transmission by CGRP **(a)** Original recordings in one individual neuron. Traces are averages of 8-10 EPSCs. **(b)** Peak amplitudes of EPSCs recorded during drug application were expressed as percentage of predrug control values (set to 100 %). Symbols and error bars represent mean ± SE. n.s., $P > 0.05$ (paired t-test)

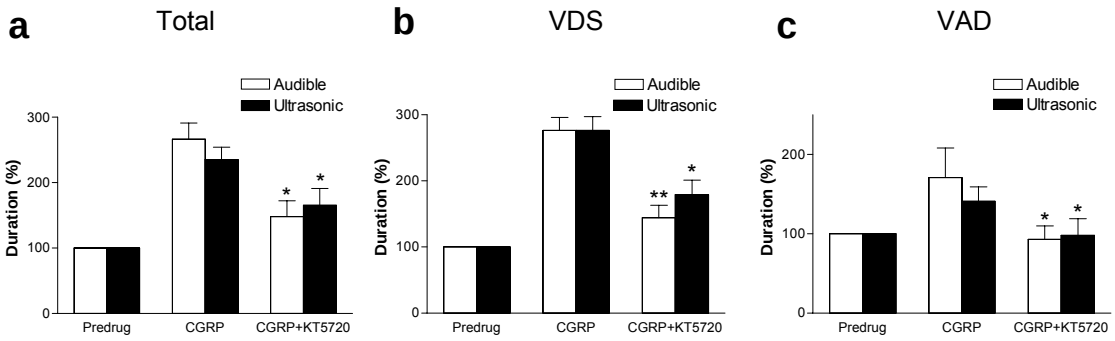

Figure 41

KT5720 reverses CGRP-induced vocalizations in normal animals.

Audible and ultrasonic vocalizations were measured in naïve rats (n = 4). Total duration **(a)** and durations of VDS **(b)** and VAD **(c)** were analyzed separately. Duration of vocalizations was measured as the arithmetic sum of the duration of each individual vocalization event. Vocalizations were evoked by noxious (2000 g/30 mm^2) stimulation (15 s) of the knee. CGRP (10 μM, concentration in the microdialysis probe; 15-20 min) into the CeLC increased vocalizations of normal rats. Co-application of a PKA inhibitor (KT5720, 100 μM) reversed the effects of CGRP significantly. Vocalizations during drug administration were expressed as percentage of vocalizations of the same animals before drug administration (predrug, set to 100 %). Bar histograms and error bars represent mean ± SE. * $P < 0.05$, ** $P < 0.01$ (paired t-test).

CHAPTER 6: DISCUSSION

SUMMARY OF RESULTS

This research project determined the role of a non-opioid neuropeptide (CGRP) in the amygdala, a brain area that is emerging as an important neuronal substrate for the emotional-affective component of pain. Our studies show for the first time that CGRP in the nociceptive amygdala (CeLC) contributes critically to pain-related synaptic plasticity and consequently to pain behavior. CGRP acts through a postsynaptic mechanism that involves PKA, ERK1/2 and NMDA receptors. The integrative approach of *in vitro* electrophysiology and behavioral analysis allowed the assessment of the cellular mechanisms of CGRP function and their significance at the systems level.

The major findings of this study 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 a model of arthritis pain induced in vivo. (2) Analysis of spontaneous miniature EPSCs (mEPSCs), paired-pulse facilitation (PPF) and membrane effects indicates a post- rather than presynaptic function of CGRP1 receptors. (3) The occlusion of $CGRP_{8-37}$ effects by a PKA inhibitor and the direct inhibition of NMDA, but not AMPA, receptor activation by $CGRP_{8-37}$ suggests that CGRP1 receptors couple to PKA activation and NMDA receptor function. (4) Chemical inactivation of the CeLC by $CGRP_{8-37}$ inhibited spinally (withdrawal reflexes) and supraspinally (vocalizations) organized pain behavior in awake animals. (5) Exogenous CGRP facilitated synaptic transmission at the PB-CeLC synapse, mimicking pain-related synaptic plasticity. (6) CGRP effects are mediated by a postsynaptic mechanism as evidenced by mEPSC and PPF analysis. (7) Exogenous application of CGRP into the CeLC increased pain-related vocalizations in awake animals. (8) Selective inhibitors of PKA and MEK (ERK1/2), but not PKC, inhibited synaptic plasticity and behavioral changes in the arthritis pain model. (9) Inhibitors of PKA and MEK (ERK1/2), but not PKC, reversed CGRP-induced synaptic facilitation and behavioral changes.

ROLE OF THE AMYGDALA IN PAIN PROCESSING

The amygdala is well positioned to play an important role in the clinically relevant and well-documented reciprocal relationship between pain and emotional-affective states (Rhudy and Meagher, 2001; Gallagher and Verma, 2004; Rhudy et al., 2005). The amygdala is 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 (see Neugebauer et al., 2004). Neuroimaging pain studies using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have repeatedly identified pain-related signal changes in the amygdala in animals and humans (Porro, 2003). The experimental conditions included the application of brief noxious heat stimuli to the skin of humans (Derbyshire et al., 1997; Becerra et al., 1999; Bingel et al., 2002; Bornhovd et al., 2002), vascular pain induced in humans by balloon dilatation of a dorsal foot vein (Schneider et al., 2001), noxious colorectal stimulation in patients with irritable bowel syndrome (Bonaz et al., 2002; Naliboff et al., 2003; Mayer et al., 2005), and mechanical allodynia in neuropathic pain patients (Petrovic et al., 1999) and in a rat model of peripheral mononeuropathy (Paulson et al., 2002). In these studies both activation and deactivation ("negative activation") were measured.

Pain modulation by the amygdala

The central nucleus of the amygdala (CeA), including its latero-capsular division (CeLC), forms direct and indirect connections with descending pain-modulating systems in the brainstem (**Fig. 2**). Descending pain control systems centered on the periaqueductal gray (PAG) and rostroventral medulla (RVM) network can be inhibitory (anti-nociceptive) as well as facilitatory (pro-nociceptive) (Heinricher and McGaraughty, 1999; Gebhart, 2004; Suzuki et al., 2004; Vanegas and Schaible, 2004). Electrical or chemical activation of the CeA has been shown to excite some neurons in the PAG and inhibit others (Da Costa Gomez and Behbehani, 1995). Activity in the amygdala can be

modified by negative and positive emotions, which in turn can reduce (acute stress, fear; pleasant music) or enhance (anxiety and depression) pain (Rhudy and Meagher, 2000; 2001; Neugebauer et al., 2004; Rhudy et al., 2005). The dependence of amygdala activity on affective state and the dual coupling of the amygdala to pain inhibition and facilitation may be an important mechanism for the differential modulation of sensory experience and pain by affective state and disorders.

Although a substantial number of studies suggested a role of the amygdala in various forms of analgesia (Helmstetter, 1992; Helmstetter and Bellgowan, 1993; Fox and Sorenson, 1994; Werka, 1997; Watkins et al., 1998; Crown et al., 2000), it has become clear that the amygdala can also contribute to the generation and enhancement of pain responses (see Neugebauer et al., 2004). Unilateral excitotoxin-induced lesions of the CeA significantly inhibited the second, but not the first, phase of formalin-induced pain behavior (Manning, 1998), although it should be noted that no significant effect was detected in earlier studies (Helmstetter, 1992; Manning and Mayer, 1995). In that study, nociceptive scores (flinches) were reduced in rats with ipsilateral CeA lesions compared to non-lesioned "sham" rats (Manning, 1998). Conversely, chronic activation of glucocorticoid and mineralocorticoid receptors by corticosterone administration into the CeA (stereotaxic implants) produced visceral hypersensitivity, which was paralleled by increased indices of anxiety (Greenwood-Van Meerveld et al., 2001). Rats with corticosterone implants spent significantly less time in the open arm of the plus maze assay than control (cholesterol implanted) rats, which is consistent with enhanced anxiety-like behavior. The elevated plus maze contains two open and two closed arms, which are arranged in the shape of a cross ("plus" sign). Animals have free access to each arm but normally prefer the closed arms. Either forced or voluntary passage onto the open arms is associated with physiological and behavioral indices of anxiety. The tendency to stay in the closed arms can be enhanced by anxiety-provoking compounds. Therefore, the time spent in the enclosed rather than the open arms serves as a measure of anxiety (Hogg, 1996; Rodgers and Dalvi, 1997). Stimulation of the CeA with corticosterone also produced increased visceromotor responses to colorectal distension in

normal rats and mimicked the visceral hypersensitivity that followed the sensitization of the colon with intracolonic acetic acid. These data suggest an important pro-nociceptive role of the amygdala, presumably the CeA, in the development of visceral hypersensitivity (Greenwood-Van Meerveld et al., 2001). They are also consistent with the hypothesis that the amygdala serves as an interface between pain and negative affect such as anxiety.

Subsequent studies provided evidence to suggest that the amygdala-evoked visceral hypersensitivity involves the sensitization of spinal dorsal horn neurons through amygdala-dependent descending facilitation. In rats with elevated glucocorticoid levels in the CeA, spinal neurons with nociceptive visceral input from the colon or the urinary bladder showed greater and longer-lasting excitatory responses to colorectal and urinary bladder distension, respectively, compared to control (cholesterol implanted) rats (Qin et al., 2003a; 2003b; 2003c). Importantly, the amygdala-evoked sensitization of spinal neurons to visceral stimulation did not require altered visceral receptor sensitivity or primary afferent sensitization since the visceral tissue was not inflamed or injured in these animals. These data add strong support to the concept that the amygdala regulates descending facilitatory pathways to increase pain processing and contributes to chronic pain through the generation and maintenance of central sensitization in the spinal cord. The results of the present research project support such a pain facilitating role of the amygdala (see below).

Electrophysiological and synaptic properties of neurons in the CeA

Relatively few studies have attempted to classify CeA neurons based on their electrophysiological properties (Schiess et al., 1993; Schiess et al., 1999; Sah et al., 2003; Lopez de Armentia and Sah, 2004). Tow major classes of neurons have been described consistently based on their action potential firing pattern (spike accommodation). Non-accommodating neurons fire continuously throughout a prolonged depolarizing current injection (500 ms) without adaptation. Accommodating neurons generate only a few action potentials during the initial stage of a prolonged current injection. A third type of

neurons (late firing) shows a similar firing pattern as non-accommodating neurons except for a noticeable delay before the first action potential. One common finding among those studies is the fact that the vast majority of neurons in the CeA show no spike accommodation. There is some evidence for at least 2 subgroups of non-accommodating neurons based on their resting membrane potential, size of afterdepolarization and kinetics of afterhyperpolerization (Zhu and Pan, 2004). These neurons are morphologically similar to the peptide-containing neurons that send projections to the basal forebrain, midbrain and brainstem nuclei (Schiess et al., 1999). The neurons in the present study were non-accommodating (see **Fig. 7 and 11**). Importantly, at least one third of neurons that are targeted by CGRP containing terminals of afferents from the parabrachial area (PB) also contain corticotropin-releasing factor (Harrigan et al., 1994). Taken together, the neurons described in this project are likely peptidergic projection neurons. It is not known, however, if these neurons also contain glutamate or γ-amino butyric acid (GABA) and form excitatory or inhibitory connections.

The different cell types do not appear to have different synaptic properties (Zhu and Pan, 2004). Anatomical, neurochemical and electrophysiological evidence (Nose et al., 1991; Swanson and Petrovich, 1998; Sah et al., 2003) suggests a dominant glutamatergic projections from the BLA to the CeA. The pharmacology of synaptic inputs from the PB has not been described before, but immunohistochemical studies showed nerve fibers from the PB to the CeA contain CGRP, substance P, neurotensin and enkephalin (Yamano et al., 1988; Block et al., 1989; Harrigan et al., 1994). Our previous electrophysiological studies showed the PB-CeA synapse is also glutamatergic (Neugebauer et al., 2003; Han et al., 2004; Bird et al., 2005). The glutamatergic synaptic inputs from the BLA and PB are largely mediated by non-NMDA receptors with little or no contribution of NMDA receptors (Zhu and Pan, 2004; Bird et al., 2005). Some studies showed also a GABAergic inhibitory postsynaptic current (IPSC) evoked in CeA neurons by stimulation of the BLA, possibly through GABAergic cells in the intercalated cell mass (Royer et al., 1999; Collins and Pare, 1999). The GABAergic IPSC may require high intensity stimulation (Zhu and Pan, 2004) and was not apparent in the present study.

Other inhibitory inputs presumably come from the bed nucleus of the stria terminalis (Dong et al., 2001). This pathway forms somatic synapses that express only $GABA_A$ receptors (Delaney and Sah, 2001). The subdivisions of the central nucleus also have extensive intradivisional connections (Jolkkonen and Pitkanen, 1998). Many of these neurons are thought to be GABAergic (Nitecka and Ben-Ari, 1987; McDonald and Augustine, 1993). However, the functional importance of these intradivisional connections is not well understood.

Mechanisms of pain-related plasticity in the CeLC

Our previous studies were the first to show neuro-plastic changes in the amygdala in persistent pain (Neugebauer and Li, 2003; Neugebauer et al., 2003; Han and Neugebauer, 2004; Li and Neugebauer, 2004a; Li and Neugebauer, 2004b; Bird et al., 2005). Using the arthritis pain model, 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.

Electrophysiological studies of CeLC neurons in anesthetized animals in vivo (Li and Neugebauer, 2004a; Li and Neugebauer, 2004b) and in brain slices in vitro (Neugebauer et al., 2003; Han et al., 2004; Bird et al., 2005) showed important roles of G-protein coupled metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors of the NMDA-type. Agonists for mGluR1/5 (DHPG) and mGluR5 (CHPG) potentiated normal synaptic transmission in vitro and increased responses of CeLC neurons to peripheral stimuli in vivo. In slices from arthritic rats (6 h postinduction), the effects of DHPG, but not CHPG, increased, suggesting an enhanced function of mGluR1 rather than mGluR5 in pain-related synaptic plasticity in the CeLC (Neugebauer et al., 2003). Likewise, DHPG, but not CHPG, increased the responses of sensitized CeLC neurons during arthritis more potently than under normal conditions (Li and Neugebauer, 2004a). Importantly, these agents had no direct effect on membrane properties and neuronal excitability but reduced paired-pulse facilitation (PPF), suggesting a pre- rather

than post-synaptic site of action (Neugebauer et al., 2003). A selective mGluR1 antagonist (CPCCOEt) had no effect on normal synaptic transmission in CeLC neurons in slices from normal rats but inhibited synaptic plasticity in slices from arthritic rats. A selective mGluR5 antagonist (MPEP) inhibited normal synaptic transmission as well as synaptic plasticity (Neugebauer et al., 2003). A parallel in vivo study showed more pronounced changes of CPCCOEt than MPEP effects in sensitized CeLC neurons in the arthritis pain state (Li and Neugebauer, 2004a). Thus, enhanced endogenous activation of presynaptic mGluR1 appears to be an important mechanism of pain-related synaptic plasticity in the CeLC.

A group III agonist (LAP4) inhibited synaptic transmission more potently in CeLC neurons from arthritic rats than in control neurons from normal animals (Han et al., 2004). LAP4 had no significant effects on membrane properties but increased paired-pulse facilitation (PPF), suggesting a presynaptic site of action. The analysis of mEPSC further verified a presynaptic action by showing that mEPSC frequency, but not amplitude, changed. The inhibitory effects of LAP4 were reversed by a selective group III mGluR antagonist (UBP1112). These data suggest that presynaptic group III mGluRs can modulate pain-related synaptic plasticity in the CeLC. The role of group II mGluRs in the CeLC remains to be determined. Presynaptic modulation of mGluRs can regulate the release of glutamate and other transmitters and modulators, including neuropeptides such as CGRP (Cartmell and Schoepp, 2000).

Mechanisms of pain-related synaptic plasticity in the CeLC also involve postsynaptic changes of NMDA receptor function in the arthritis pain model (Bird et al., 2005). Synaptic plasticity in brain slices from arthritic animals, but not normal synaptic transmission in control neurons, was inhibited by a selective NMDA receptor antagonist (AP5). Electrophysiological recordings in vivo showed that AP5 inhibited the enhanced responses of sensitized CeLC neurons to noxious stimulation in the arthritis pain state (Li and Neugebauer, 2004b). An NMDA-receptor-mediated synaptic component was recorded in CeLC neurons from arthritic animals, but not in control neurons, and was blocked by inhibitors of PKA (KT5720) but not PKC (GF109203X). Exogenous NMDA

evoked a larger inward current in neurons from arthritic animals than in control neurons, indicating enhanced function of postsynaptic receptors. PPF, a measure of presynaptic mechanisms, was not affected by AP5. Western blot analysis showed increased levels of phosphorylated NMDA-receptor 1 (NR1) subunit protein, but not of total NR1, in the CeA of arthritic rats compared to controls. These results suggest that pain-related synaptic plasticity and sensitization in the CeLC are accompanied by PKA-mediated activation of postsynaptic NMDA-receptor function and increased phosphorylation of NR1 subunits (Li and Neugebauer, 2004b; Bird et al., 2005).

Behavioral consequences of pain-related plasticity

The behavioral consequences of amygdala activity in persistent pain were largely unknown until recently. Chemical inactivation of the CeLC by agents that are now known to inhibit pain-related sensitization and synaptic plasticity also inhibited spinally and supraspinally organized behavioral responses in a model of arthritic pain (Han and Neugebauer, 2005). Spinal withdrawal reflexes and supraspinally organized audible and ultrasonic vocalizations increased in the arthritis pain state. Audible vocalizations evoked by noxious stimuli represent a nocifensive response whereas ultrasonic vocalizations reflect affect-related behavior (see discussion in Han et al., 2005; Han and Neugebauer, 2005). Administration of an antagonist selective for mGluR1 (CPCCOEt) into the CeLC inhibited vocalizations during stimulation (VDS), which are organized at the brainstem level, and vocalizations that continue after stimulation (VAD; afterdischarges), which are organized in the limbic forebrain, particularly the amygdala (Borszcz and Leaton, 2003; Han and Neugebauer, 2005). CPCCOEt in the CeLC also increased the threshold of spinally mediated hindlimb withdrawal reflexes. Block of mGluR5 in the CeLC with a selective antagonist (MPEP) inhibited only VAD but had no effect on VDS and withdrawal reflexes. These findings suggest pharmacologically distinct mechanisms in the CeLC are involved in the modulation of pain behavior organized at different levels of the pain neuraxis (spinal cord, brainstem, limbic forebrain). These data also show that the

endogenous activation of the CeLC in persistent pain contributes to enhanced nociceptive processing and pain behavior.

The present research project shows that manipulations of another important molecule, the non-opioid neuropeptide CGRP and its receptors, can modulate pain-related behavior. Chemical inactivation of the CeLC by a CGRP1 receptor antagonist inhibited nocifensive and affective behaviors organized at supra-spinal and spinal levels, further suggesting a descending facilitatory role of the amygdala in pain-related plasticity. Chemical activation of the CeLC by exogenous CGRP produced pain behavior, mimicking the effect of the arthritis pain state. Although differences in experimental conditions, pain models, pain tests, and outcome measures need to be considered in this and previous studies, it appears that the amygdala, including the CeLC, has a dual pain-modulating function, which may play an important role in the reciprocal relationship between pain and negative affect (see above).

Contribution of the amygdala to the emotional component of pain

The amygdala is a key player in emotionality. Emotions are complicated collections of chemical, physiological and behavioral responses to changes in the internal and external bodily environment (Damasio, 2000; Adolphs, 2002; Craig, 2005). Pain is a multi-dimensional experience that includes nociceptive ("pain sensation") and emotional-affective ("pain affect") components (Damasio, 2000; Meagher et al., 2001; Craig, 2005). As mentioned earlier, the CeLC is well positioned to serve as an integrative brain structure of sensory and affective components of pain. The CeLC receives nociceptive subcortical inputs from the brainstem and spinal cord whereas highly processed polymodal information comes from the cortex and thalamus through the LA and BLA nuclei, which are part of the fear-anxiety circuitry (**Fig. 1**). Therefore, different components of pain-related information merge in the CeLC.

The CeA is also the common output nucleus of the amygdalar complex. The CeLC forms widespread connections with the brainstem, hypothalamus, midline and mediodorsal thalamic nuclei, forebrain structures and cortical areas (**Fig. 2**) (Pitkanen et

al., 1997; Price, 2003). Projections to the hypothalamus may be related to the autonomic and endocrine responses to emotional stimuli and pain such as changes in blood pressure, heart rate, respiratory pattern and corticosteroid release (Davis, 1998; LeDoux, 2000; Sah et al., 2003). Projections to the brainstem, including the PAG and RVM, are important for the expression of emotional behaviors and can modulate spinal nociceptive processing as part of a well documented descending pain control system (Da Costa Gomez and Behbehani, 1995; Manning, 1998; Heinricher and McGaraughty, 1999; Gebhart, 2004). The CeLC projects extensively to the substantia innominata dorsalis, which forms widespread connections with the cholinergic and noradrenergic basal forebrain nuclei, orbital and medial prefrontal cortices, agranular insular cortex and bed nucleus of the stria terminalis (Bourgeais et al., 2001; Price, 2003). These connections may transmit information related to affective states such as fear, anxiety, depression, stress, anger and resignation, which are known consequences of persistent pain. Pain-related affective content can also alter the level of attention, vigilance and arousal and may gain access to memory processes. Conversely, cortical interaction with the amygdala may be a mechanism by which negative and positive emotions and emotions with low and high arousal can modulate pain and pain behavior (Rhudy and Meagher, 2001; Rhudy et al., 2005). Therefore, the reciprocal connections with the cortex allow the amygdala to contribute to the experience and evaluation of pain and to the conscious and cognitive modulation of behavior to avoid pain (Flor et al., 1990; Davidson et al., 1999; LeDoux, 2000). The prefrontal cortex is known to be important for conscious decision-making processes to obtain reward, avoid punishment and implement long-term plans (Rolls, 2000). This hypothesis is supported by the fact that the CeA is essential for the expression of fear conditioned behavior and fear memories, which are often associated with noxious stimuli (LeDoux, 2000; Sah et al., 2003).

ROLE OF CGRP IN THE AMYGDALA IN PAIN-RELATED PLASTICITY AND BEHAVIOR

CGRP and its receptors

CGRP is a 37 amino acid peptide derived from the calcitonin (CT)/CGRP gene in chromosome 11. Alternative splicing of the primary RNA transcript leads to tissue-specific translation to two distinct peptides, CGRP and CT (Wimalawansa, 1996). CGRP is often colocalized with other substances. Colocalization with substance P in the CNS and periphery has been reported frequently (Goodman and Iversen, 1986; Poyner, 1992; Ma et al., 2001). Colocalization with vasoactive intestinal peptide, somatostatin and neuropeptide Y also has been observed (Ekblad et al., 1988; Kummer and Heym, 1991). CGRP containing primary afferent terminals form synapses on spinothalamic tract cells in the spinal cord (Carlton et al., 1990). Some glutamatergic primary afferents which contain dense-core vesicles coexpress substance P and/or CGRP (De and Rustioni, 1988; Merighi et al., 1991). Thus, glutamate and these peptides are presumably co-released in the spinal cord (De and Rustioni, 1990). The extensive codistribution with other substances has been taken to suggest a role of CGRP as a neuromodulator rather than an independent neurotransmitter. The present study shows that CGRP modulates glutamatergic transmission. The relatively rapid time course of CGRP effects in this study may suggest that CGRP can also act as a transmitter.

The half-life of CGRP in mammalian plasma is approximately 10 minutes (Kraenzlin et al., 1985; Struthers et al., 1986). Many target cells for CGRP contain a cell surface enzyme, the neutral endopeptidase (enkephalinase), that can cleave CGRP (Katayama et al., 1991; Wimalawansa, 1996; McDowell et al., 1997; Kramer et al., 2005). The other major substrates for this enzyme include substance P, neurontensin and neurokinin-A (Wimalawansa, 1996). The competition for the same degradation pathway may be an underlying mechanism by which CGRP potentiates the actions of other neuropeptides.

CGRP activates adenylyl cyclase and PKA through G-protein-coupled receptors, including the CGRP1 receptor for which selective antagonists are available (see Chapter

1) (Wimalawansa, 1996). Pharmacologically two classes of CGRP receptors have been proposed, termed $CGRP_1$ and $CGRP_2$ receptors, respectively, which have no significant affinity for calcitonin-like peptides (Poyner, 1996; Wimalawansa, 1996; Oliver et al., 1998; Hasbak et al., 2003). Antagonists selective for the CGRP1 receptor include the C-terminal peptide fragment $CGRP_{8-37}$ and the novel non-peptide compound BIBN4096BS (Poyner, 1996; Wimalawansa, 1996; Van Rossum et al., 1997; Doods et al., 2000).

Pain modulation by CGRP

The role of CGRP in spinal pain mechanisms is well-documented in a variety of experimental pain models with histological, electrophysiological and behavioral approaches, including inflammatory pain (Sluka et al., 1992; Sluka and Westlund, 1993; Neugebauer et al., 1996; Schaible, 1996; Zhang et al., 2001; Sun et al., 2003; Winston et al., 2005), neuropathic pain (Carlton and Coggeshall, 1996) and spinal cord injury (Christensen and Hulsebosch, 1997; Bennett et al., 2000). Spinal application of CGRP induced sensitization of spinal dorsal horn neurons to stimulation of the skin and deep tissue, which was reversed by a CGRP1 receptor antagonist ($CGRP_{8-37}$) (Neugebauer et al., 1996; Ebersberger et al., 2000; Sun et al., 2004a). Spinal application of $CGRP_{8-37}$ prevented and reversed the central sensitization of dorsal horn neurons induced by intradermal capsaicin injection (Sun et al., 2004a). Focal ionophoretic administration of $CGRP_{8-37}$ into the dorsal horn also reduced the responses of sensitized dorsal horn neurons to mechanical stimulation of the knee and other deep tissues in the arthritis pain model (Neugebauer et al., 1996). In behavioral studies, intrathecal administration of an antiserum against CGRP reversed heat and mechanical hyperalgesia induced by intraplantar adjuvant or subcutaneous carrageenan (Kawamura et al., 1989). Spinal administration of $CGRP_{8-37}$ by microdialysis reduced secondary mechanical allodynia and hyperalgesia induced by intradermal capsaicin injection (Sun et al., 2003).

Some evidence from immunohistochemical and electrophysiological studies also suggests a pronociceptive role of CGRP in primary afferents (dorsal root ganglion cells) (Ryu et al., 1988; Galeazza et al., 1995; Kilo et al., 1997; Natura et al., 2005). Still, most

information about pain modulation by CGRP has come from studies on spinal pain mechanisms. The role of CGRP in the brain is largely unknown, although CGRP and CGRP1 receptors are present in the brain with particularly high levels in the CeLC (Schwaber et al., 1988; Kruger et al., 1988; Harrigan et al., 1994; de Lacalle and Saper, 2000).

Role of CGRP in the amygdala

CGRP and its receptors typically couple to the activation of the cAMP-PKA signaling pathway. PKA-dependent enhanced function of postsynaptic NMDA receptors plays an important role in pain-related plasticity in the CeLC (Bird et al., 2005). Therefore, this research project tested the hypothesis that CGRP serves as a critical molecule that links pre- and postsynaptic mechanisms of pain-related plasticity in the CeLC and contributes to pain behavior organized at different levels of the pain neuraxis. The predominant if not exclusive source of CGRP in the amygdala (CeA) is the lateral parabrachial area (Schwaber et al., 1988; Kruger 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 PAG (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 due to CGRP release from the spino-parabrachio-amygdaloid pain pathway but not from intrinsic circuits.

Our electrophysiological data show the critical involvement of CGRP in the synaptic transmission of pain-related information 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 (Neugebauer et al., 2004; Gebhart, 2004; Vanegas and Schaible, 2004). Accordingly, exogenous CGRP administration into the CeLC had pronociceptive effects, increasing transmission at the nociceptive PB-CeLC synapse and producing pain behavior.

Our data further suggest that CGRP1 receptor activation occurs at a post- rather than presynaptic site. $CGRP_{8-37}$ inhibited action potential firing evoked by direct depolarizing current injections into the neuron (postsynaptic site) while exogenous CGRP enhanced action potential firing. Also, $CGRP_{8-37}$ inhibited the membrane currents evoked by the application of NMDA, but not AMPA.

Paried pulse facilitation (PPF), a measure of transmitter release probability was not affected by either CGRP or $CGRP_{8-37}$. PPF is directly dependent on the intracellular calcium level. A transient increase of the residual calcium level in the presynaptic terminal is generally thought to be the mechanism underlying PPF (Katz and Miledi, 1968; Charlton et al., 1982; Wu and Saggau, 1994). Presynaptic manipulations such as decreasing the external $[Mg^{2+}]/[Ca^{2+}]$ ratio, have been shown to decrease PPF (Katz and Miledi, 1968; Creager et al., 1980; Kuhnt and Voronin, 1994), while postsynaptic manipulations that affect the amplitude of excitatory postsynaptic currents (EPSCs) had little influence on PPF (Manabe et al., 1993).

Amplitude distribution (quantal size), but not frequency, of spontaneous miniature EPSCs (mEPSCs) was altered by CGRP and $CGRP_{8-37}$. The analysis of spontaneous miniature synaptic potentials was used to discover the quantal nature of synaptic transmission when recordings were made at the neuro-muscular junction (Fatt and Katz, 1952; Del and Katz, 1954). These studies provided evidence that transmitter is released in packets or "quanta," each of them containing thousands of transmitter molecules. Each quantum of transmitter produces a postsynaptic potential of fixed size, called quantal synaptic potential. The total postsynaptic potential is made up from an integral number of quantal responses. According to the quantal transmitter release theory, presynaptic changes can be attributable to changes in the number of release sites and/or probability of release from those sites. Changes in release probability can be assessed by analysis of

frequency, while postsynaptic changes modulate the size of the postsynaptic response to one vesicle of transmitter, which alters the quantal amplitude (Fatt and Katz, 1952). Taken together, these findings suggest a postsynaptic action of CGRP through modulation of NMDA receptor channel activity.

The low nanomolar affinities of CGRP and $CGRP_{8-37}$ measured in the present study are consistent with the binding to the calcitonin receptor-like receptor (CRLR) component of the CGRP1 receptor (see Chapter 1; Oliver et al., 1998; Hasbak et al., 2003). The change in efficacy of $CGRP_{8-37}$ in the arthritis pain model can be the result of increased CGRP release as well as CGRP-receptor component protein (RCP)-mediated enhanced coupling of the CGRP1 receptor to second messengers, including PKA activation.

Whereas our studies suggest that endogenous activation of CGRP1 receptors in the amygdala produces pain behavior through descending facilitation, one behavioral study reported antinociceptive effects of exogenous CGRP administration into the central nucleus of the amygdala (Xu et al., 2003). However, these experiments were done in normal rats (not in a pain model). Furthermore, 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 if lateralization or differences between normal conditions and persistent pain can account for this difference.

SIGNAL TRANSDUCTION MECHANISMS OF CGRP IN THE AMYGDALA

Role of PKA, PKC and ERK1/2 in pain modulation

PKA, PKC and ERK1/2 have been shown to be involved in central sensitization (Hu and Gereau, 2003; Ji et al., 2003). Spinal application of a PKA activator induced allodynia and hyperalgesia in behavioral experiments (Sluka, 1997). Activation of PKA also increased the responses of spinothalamic tract cells to mechanical stimuli (Lin et al., 2002). Enhanced responses of spinothalamic tract neurons were observed with spinal microdialysis application of a PKC activator whereas an inhibitor of PKC prevented central sensitization (Lin et al., 1996). Similarly, in vitro preparations showed that PKA and PKC produce a long-lasting enhancement of excitatory responses of dorsal horn neurons (Chen and Huang, 1991; Chen and Huang, 1992; Cerne et al., 1992; Yang et al., 2004). PKA and PKC can induce NMDA receptor phosphorylation in the spinal cord and modulate A-type K^+ currents, thus enhancing neuronal excitability (Zou et al., 2002; Hu and Gereau, 2003; Hu et al., 2003; Zou et al., 2004). Inhibition of PKA and PKC has been shown to block the development of mechanical allodynia and thermal hyperalgesia in models of inflammatory pain (Sluka et al., 1997a; Sluka et al., 1997b; Sun et al., 2004b; Jones and Sorkin, 2005).

PKA and PKC are also upstream activators of the MAP kinases ERK1/2 (Kolch, 2000; Hu et al., 2003). ERK1/2 have been shown to mediate changes of neuronal excitability and ion channel activity by PKA and PKC (Hu and Gereau, 2003). In the nervous system, ERK1 and ERK2 are activated by the upstream kinases MEK1/2 and transduce extracellular signals into intracellular functional changes by transcriptional and non-transcriptional (phosphorylation) modifications (Cano and Mahadevan, 1995; Impey et al., 1999). ERK1/2 play an important role in various forms of synaptic plasticity in the central nervous system, including long-term potentiation (LTP) in the hippocampus (English and Sweatt, 1996; English and Sweatt, 1997; Atkins et al., 1998; Impey et al., 1999; Sweatt, 2004). ERK1/2 are also involved in central sensitization in a variety of pain models. Intense noxious peripheral stimuli or electrical C-fiber activation induced

ERK1/2 activation most predominantly in lamina I and II of the dorsal horn. Conversely, inhibition of ERK1/2 phosphorylation by a MEK inhibitor reduced the second phase of formalin-induced pain behavior, which reflects central sensitization (Ji et al., 1999). The involvement of ERK1/2 activation has been observed in models of neuropathic pain (Ma and Quirion, 2002; Kominato et al., 2003) as well as inflammatory pain (Ji et al., 1999; Karim et al., 2001; Ji et al., 2002; Adwanikar et al., 2004). These studies suggest an important role of ERK1/2 and its upstream activation in nociceptive plasticity.

Interestingly, activation of ERK1/2 has also been observed in the central nucleus of the amygdala by peripheral inflammation (Gereau lab, unpublished observation). Our data show that PKA and ERK1/2 activation in the amygdala is necessary for the development of pain behavior and synaptic plasticity in the arthritis pain model. Inhibition of PKA and MEK in naïve animals did not affect synaptic transmission and behavioral responses. However, arthritis-induced synaptic plasticity and behavioral changes were reversed by PKA and MEK inhibitors. This observation is consistent with in vivo studies in the spinal cord, which showed that a PKA inhibitor increased mechanical paw withdrawal thresholds only in the state of CGRP- or capsaicin-induced hyperalgesia but not under normal conditions (Sun et al., 2004a; Sun et al., 2004b). Therefore, PKA and ERK1/2 do not appear to play major roles under normal conditions but undergo plastic changes in central sensitization and contribute to synaptic plasticity and behavioral changes in persistent pain states.

Intracellular signaling of CGRP

Several mechanisms of cellular effects of CGRP have been proposed. CGRP enhanced the excitatory effects of substance P (SP) on dorsal horn neurons (Biella et al., 1991). CGRP application in slice preparations of the spinal dorsal horn and cultured dorsal root ganglion (DRG) cells caused enhanced excitability and a slow, dose-dependent depolarization that was not prevented by blockage of Na^+ and K^+ currents (Ryu et al., 1988). CGRP potentiated both amplitude and duration of Ca^{2+}-spikes in DRG neurons, which may be responsible for increased synaptic transmission (Ryu et al., 1988).

Neuronal plasticity involving changes in activity of ion-channels, cellular excitability and synaptic transmission is mediated by various intracellular transduction mechanisms such as the PKA and PKC pathways (Evans et al., 2001; Hu et al., 2003; Yang et al., 2004). PKA and PKC can serve as upstream regulators of MEK and ERK1/2, resulting in ion channel activity changes and/or transcriptional modifications (Kolch, 2000; Hu et al., 2003).

The present study shows that CGRP serves as the critical molecule to link pre- and postsynaptic mechanisms of pain-related plasticity in the CeLC and contributes to pain behavior organized at different levels of the pain neuraxis. In the spinal cord, it has been demonstrated that CGRP-induced hyperalgesia and central sensitization are attenuated by inhibition of PKA and PKC (Sun et al., 2003; Sun et al., 2004b). Our previous (Bird et al., 2005) and present patch-clamp studies in CeLC neurons show that PKA is involved in the postsynaptic activation of NMDA receptors, CGRP-induced plasticity and behavior and pain-related synaptic plasticity and behavior. Importantly, the synaptic component inhibited by block of PKA and/or CGRP1 receptors was comparable to 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 $CGRP_{8-37}$ on NMDA-, but not AMPA-, evoked membrane currents. Importantly, a differential effect of CGRP8-37 on NMDA- versus AMPA-evoked responses has also been observed in spinal dorsal horn neurons (Ebersberger et al., 2000).

Enhanced synaptic transmission and vocalizations evoked by exogenous CGRP were reversed by inhibition of PKA and MEK, further supporting the involvement of the PKA-MEK1/2-ERK1/2 pathway in the actions of CGRP. Although mechanisms of synaptic plasticity mediated through this proposed pathway remain unknown, possible consequences of intracellular activation of PKA and ERK1/2 can be the phosphorylation of several ligand- or voltage-gated ion channels, including NMDA receptors and A-type K^+ channels (Hu et al., 2003; Bird et al., 2005). In contrast, inhibition of PKC had no significant effect on CGRP- and arthritic pain-induced plasticity and behavior. Lack of

effect of PKC inhibition in our study needs further consideration. A recent study suggested that both PKA and PKC are involved in CGRP-evoked hyperalgesia and increased responses spinal dorsal horn neurons (Sun et al., 2004b). It remains to be determined if this discrepancy suggests different mechanisms of CGRP action at different levels of the pain neuraxis.

CONCLUSIONS

The present study shows for the first time that CGRP and CGRP1 receptors in the CeLC are critically involved in pain-related plasticity of CeLC neurons via the PKA-MEK-ERK1/2 pathway. The consequences of the CGRP-activated signal transduction cascade are increased nocifensive and affective responses (audible and ultrasonic vocalizations, respectively). Therefore, CGRP and intracellular signaling messengers in the amygdala may be important therapeutic targets for pain relief. The present study also makes a major contribution to the better understanding of the pain-affect relationship by providing valuable novel information about mechanisms of plasticity in the nociceptive amygdala and their behavioral consequences. Affective disorders such as anxiety and depression can increase the duration and intensity of pain (Huyser and Parker, 1999). They can also modify activity in the amygdala, particularly the circuitry in the lateral and basolateral nuclei (Davidson et al., 1999; Lin et al., 2000). If affective states and disorders can mimic pain-related plasticity in the amygdala, they would be able to gain access to pain modulation through the nociceptive amygdala. Although this hypothesis awaits further exploration, the present study offers possible cellular mechanisms and behavioral consequences of the pain-affect interaction in the nociceptive amgydala. Our proposed model (**Fig. 42**) summarizes mechanisms of pain-related plasticity in the CeLC.

Pain-related plasticity in the amygdala

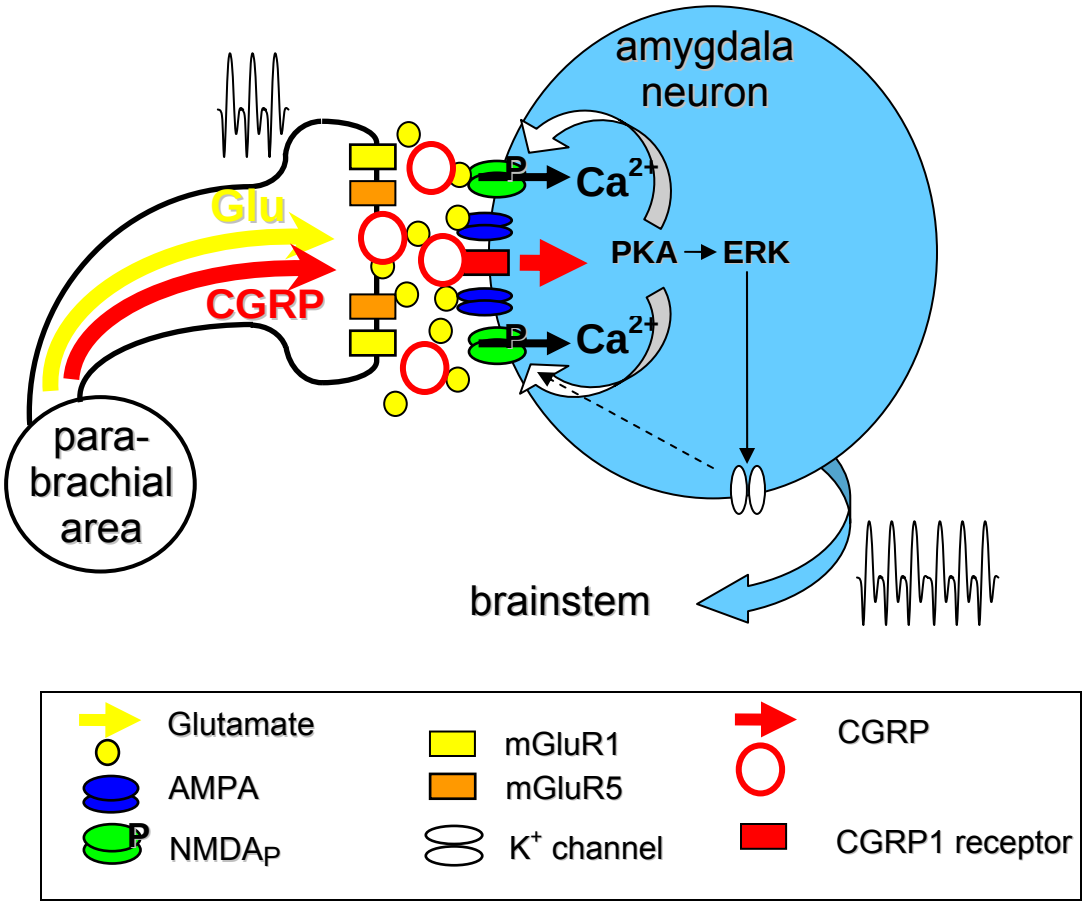

Figure 42

Figure 42 (continued)

Proposed pre- and post-synaptic mechanisms of pain-related plasticity in the CeLC.

Increased nociceptive signals in the spino-parabrachio-amygdaloid pain pathway (Neugebauer et al., 2004) lead to enhanced presynaptic release of glutamate (Glu) through metabotropic glutamate receptor subtypes mGluR1 and mGluR5 (Neugebauer et al., 2003; Li and Neugebauer, 2004a). Glutamate acts postsynaptically to activate N-methyl-D-aspartate (NMDA) receptors, which are "silent" under normal conditions but become functional through receptor phosphorylation ($NMDA_P$) by PKA but not PKC (Bird et al., 2005). The present study shows that CGRP is a critical molecule to link pre- and postsynaptic mechanisms of pain-related plasticity in the CeLC through the PKA-ERK1/2 pathway. Endogenous CGRP release activates postsynaptic CGRP1 receptors coupled to PKA to increase NMDA, but not AMPA, receptor function through phosphorylation ($NMDA_P$), resulting possibly in increased calcium (Ca^{2+}) influx (Li and Neugebauer, 2004b; Bird et al., 2005). NMDA receptor function can also be increased by ERK1/2 activation through phosphorylation of K^+ channels and membrane depolarization (Hu et al., 2003). The overall consequences are synaptic plasticity, increased neuronal excitability and pain responses.

REFERENCES

Adolphs R (2002) Neural systems for recognizing emotion. Curr Opin Neurobiol 12: 169-177.

Adwanikar H, Karim F, Gereau IV (2004) Inflammation persistently enhances nocifensive behaviors mediated by spinal group I mGluRs through sustained ERK activation. Pain 111: 125-135.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. Nat Neurosci 1: 602-609.

Becerra LR, Breiter HC, Stojanovic M, Fishman S, Edwards A, Comite AR, Gonzalez RG (1999) Human brain activation under controlled thermal stimulation and habituation of noxious heat: an fMRI study. Mag Reson in Med 41: 1044-1057.

Bennett AD, Chastain KM, Hulsebosch CE (2000) Alleviation of mechanical and thermal allodynia by CGRP8-37 in a rodent model of chronic central pain. Pain 86: 163-175.

Bernard J-F, Bandler R (1998) Parallel circuits for emotional coping behaviour: new pieces in the puzzle. J Comp Neurol 401: 429-436.

Bernard J-F, Bester H, Besson JM (1996) Involvement of the spino-parabrachio - amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog Brain Res 107: 243-255.

Biella G, Panara C, Pecile A, Sotgiu ML (1991) Facilitatory role of calcitonin gene-related peptide (CGRP) on excitation induced by substance P (SP) and noxious stimuli in rat spinal dorsal horn neurons. An iontophoretic study in vivo. Brain Res 559: 352-356.

Bingel U, Quante M, Knab R, Bromm B, Weiller C, Buchel C (2002) Subcortical structures involved in pain processing: evidence from single-trial fMRI. Pain 99: 313-321.

Bird GC, Lash LL, Han JS, Zou X, Willis WD, Neugebauer V (2005) PKA-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in amygdala neurons. J Physiol (Lond) 564.3: 907-921.

Block CH, Hoffman G, Kapp BS (1989) Peptide-containing pathways from the parabrachial complex to the central nucleus of the amygdala. Peptides 10: 465-471.

Bonaz B, Baciu M, Papillon E, Bost R, Gueddah N, Le Bas J-F, Fournet J, Segebarth C (2002) Central processing of rectal pain in patients with irritable bowel syndrome: an fMRI study. Am J Gastroenterol 97: 654-661.

Bornhovd K, Quante M, Glauche V, Bromm B, Weiller C, Buchel C (2002) Painful stimuli evoke different stimulus-response functions in the amygdala, prefrontal, insula and somatosensory cortex: a single-trial fMRI study. Brain 125: 1326-1336.

Borszcz GS, Johnson CP, Fahey KA (1994) Comparison of motor reflex and vocalization thresholds following systemically administered morphine, fentanyl, and diazepam in the rat: assessment of sensory and performance variables. Pharmacol Biochem Behav 49: 827-834.

Borszcz GS, Leaton RN (2003) The effect of amygdala lesions on conditional and unconditional vocalizations in rats. Neurobiol Learn Mem 79: 212-225.

Bourgeais L, Gauriau C, Bernard J-F (2001) Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. Eur J Neurosci 14: 229-255.

Braz JM, Nassar MA, Wood JN, Basbaum AI (2005) Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. Neuron 47: 787-793.

Burstein R, Potrebic S (1993) Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. J Comp Neurol 335: 469-485.

Cabell L, Audesirk G (1993) Effects of selective inhibition of protein kinase C, cyclic AMP- dependent protein kinase, and Ca(2+)-calmodulin-dependent protein kinase on neurite development in cultured rat hippocampal neurons. Int J Dev Neurosci 11: 357-368.

Cahill L (1999) A neurobiological perspective on emotionally influenced, long-term memory. Semin Clin Neuropsychiatry 4: 266-273.

Cano E, Mahadevan LC (1995) Parallel signal processing among mammalian MAPKs. Trends Biochem Sci 20: 117-122.

Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002) Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev 26: 321-352.

Carlton SM, Coggeshall RE (1996) Stereological analysis of galanin and CGRP synapses in the dorsal horn of neuropathic primates. Brain Res 711: 16-25.

Carlton SM, Westlund KN, Zhang DX, Sorkin LS, Willis WD (1990) Calcitonin gene-related peptide containing primary afferent fibers synapse on primate spinothalamic tract cells. Neurosci Lett 109: 76-81.

Cartmell J, Schoepp DD (2000) Regulation of neurotransmitter release by metabotropic glutamate receptors. J Neurochem 75: 889-907.

Cerne R, Jiang M, Randic M (1992) Cyclic adenosine 3'5'-monophosphate potentiates excitatory amino acid and synaptic responses of rat spinal dorsal horn neurons. Brain Res 596: 111-123.

Charlton MP, Smith SJ, Zucker RS (1982) Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse. J Physiol 323:173-93.: 173-193.

Chen L, Huang LY (1992) Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. Nature 356: 521-523.

Chen L, Huang L-YM (1991) Sustained potentiation of NMDA receptor-mediated glutamate responses through activation of protein kinase C by a μ opioid. Neuron 7: 319-326.

Christensen MD, Hulsebosch CE (1997) Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn in the rat. Exp Neurol 147: 463-475.

Collins DR, Pare D (1999) Spontaneous and evoked activity of intercalated amygdala neurons. Eur J Neurosci 11: 3441-3448.

Craig KD (2005) Textbook of pain: Emotions and Pscychology of pain.5th edition: Elsevier: 231-241.

Creager R, Dunwiddie T, Lynch G (1980) Paired-pulse and frequency facilitation in the CA1 region of the in vitro rat hippocampus. J Physiol 299:409-24.: 409-424.

Crown ED, King TE, Meagher MW, Grau JW (2000) Shock-induced hyperalgesia: III. Role of the bed nuclus of the stria terminalis and amygdaloid nuclei. Behav Neurosci 114: 561-573.

Da Costa Gomez TM, Behbehani MM (1995) An electrophysiological characterization of the projection from the central nucleus of the amygdala to the periaqueductal gray of the rat: the role of opioid receptors. Brain Res 689: 21-31.

Damasio A (2000) The feelining of what happens: Emotion and feeling.1st edition: Harcourt Inc.: 51-55.

Davidson RJ, Abercrombie H, Nitschke JB, Putnam K (1999) Regional brain function, emotion and disorders of emotion. Curr Opin Neurobiol 9: 228-234.

Davidson RJ (2002) Anxiety and affective style: role of prefrontal cortex and amygdala. Biol Psychiatry 51: 68-80.

Davis M (1998) Anatomic and physiologic substrates of emotion in an animal model. J Clin Neurophysiol 15: 378-387.

de Lacalle S, Saper CB (2000) Calcitonin gene-related peptide-like immunoreactivity marks putative visceral sensory pathways in human brain. Neuroscience 100: 115-130.

De BS, Rustioni A (1988) Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. Proc Natl Acad Sci U S A 85: 7820-7824.

De BS, Rustioni A (1990) Ultrastructural immunocytochemical localization of excitatory amino acids in the somatosensory system. J Histochem Cytochem 38: 1745-1754.

Del CJ, Katz B (1954) Quantal components of the end-plate potential. J Physiol 124: 560-573.

Delaney AJ, Sah P (2001) Pathway-specific targeting of GABA(A) receptor subtypes to somatic and dendritic synapses in the central amygdala. J Neurophysiol 86: 717-723.

Derbyshire SWG, Jones AKP, Gyulai F, Clark S, Townsend D, Firestone LL (1997) Pain processing during three levels of noxious stimulation produces differential patterns of central activity. Pain 73: 431-445.

Dong HW, Petrovich GD, Watts AG, Swanson LW (2001) Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. J Comp Neurol 436: 430-455.

Doods H, Hallermayer G, Wu D, Entzeroth M, Rudolf K, Engel W, Eberlein W (2000) Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. Br J Pharm 129: 420-423.

Ebersberger A, Charbel Issa P, Vanegas H, Schaible H-G (2000) Differential effects of calcitonin gene-related peptide and calcitonin gene-related peptide 8-37 upon responses to N-methyl--aspartate or (R,S)-[alpha]-amino-3-hydroxy-5-methylisoxazole-4-propionate in spinal nociceptive neurons with knee joint input in the rat. Neuroscience 99: 171-178.

Ekblad E, Ekman R, Hakanson R, Sundler F (1988) Projections of peptide-containing neurons in rat colon. Neuroscience 27: 655-674.

English JD, Sweatt JD (1996) Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. J Biol Chem 271: 24329-24332.

English JD, Sweatt JD (1997) A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J Biol Chem 272: 19103-19106.

Evans DI, Jones RS, Woodhall G (2001) Differential actions of PKA and PKC in the regulation of glutamate release by group III mGluRs in the entorhinal cortex. J Neurophysiol 85: 571-579.

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol 117: 109-128.

Flor H, Birbaumer N, Turk DC (1990) The psychobiology of chronic pain. Advances in Behavioural Research and Therapy 12: 47-84.

Fox RJ, Sorenson CA (1994) Bilateral lesions of the amygdala attenuate analgesia induced by diverse environmental challenges. Brain Res 648: 215-221.

Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS (1995) Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. Neuroscience 66: 443-458.

Gallagher M, Schoenbaum G (1999) Functions of the amygdala and related forebrain areas in attention and cognition. Ann N Y Acad Sci 877: 397-411.

Gallagher RM, Verma S (2004) Mood and anxiety disorders in chronic pain. Progress in Pain Res and Management 27: 139-178.

Gauriau C, Bernard J-F (2004) A comparative reappraisal of projections from the superficial laminae of the dorsal horn in the rat: the forebrain. J Comp Neurol 468: 24-56.

Gauriau C, Bernard J-F (2002) Pain pathways and parabrachial circuits in the rat. Exp Physiol 87: 251-258.

Gebhart GF (2004) Descending modulation of pain. Neurosci Biobehav Rev 27: 729-737.

Goodman EC, Iversen LL (1986) Calcitonin gene-related peptide: novel neuropeptide. Life Sci 38: 2169-2178.

Greenwood-Van Meerveld B, Gibson M, Gunder W, Shepard J, Foreman R, Myers D (2001) Stereotaxic delivery of corticosterone to the amygdala modulates colonic sensitivity in rats. Brain Res 893: 135-142.

Han JS, Bird GC, Li W, Neugebauer V (2005) Computerized analysis of audible and ultrasonic vocalizations of rats as a standarized measure of pain-related behavior. Neurosci Meth 141: 261-269.

Han JS, Bird GC, Neugebauer V (2004) Enhanced group III mGluR-mediated inhibition of pain-related synaptic plasticity in the amygdala. Neuropharmacology 46: 918-926.

Han JS, Neugebauer V (2005) mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. Pain 113: 211-222.

Han JS, Neugebauer V (2004) Synaptic plasticity in the amygdala in a visceral pain model in rats. Neuroscience Letters 361: 254-257.

Harrigan EA, Magnuson DJ, Thunstedt GM, Gray TS (1994) Corticotropin releasing factor neurons are innervated by calcitonin gene-related peptide terminals in the rat central amygdaloid nucleus. Brain Res Bull 33: 529-534.

Hasbak P, Opgaard OS, Eskesen K, Schifter S, Arendrup H, Longmore J, Edvinsson L (2003) Investigation of CGRP Receptors and Peptide Pharmacology in Human Coronary Arteries. Characterization with a Nonpeptide Antagonist. J Pharmacol Exp Ther 304: 326-333.

Hebert MA, Ardid D, Henrie JA, Tamashiro K, Blanchard DC, Blanchard RJ (1999) Amygdala lesions produce analgesia in a novel, ethologically relevant acute pain test. Phys and Behav 67: 99-105.

Heinricher MM, McGaraughty S (1999) Pain-modulating neurons and behavioral state. 487-503.

Helmstetter FJ (1992) The amygdala is essential for the expression of conditional hypoalgesia. Behav Neurosci 106: 518-528.

Helmstetter FJ, Bellgowan PS (1993) Lesions of the amygdala block conditional hypoalgesia on the tail flick test. Brain Res 612: 253-257.

Hogg S (1996) A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 54: 21-30.

Hu HJ, Gereau RW (2003) ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. II. Modulation of neuronal excitability. J Neurophysiol 90: 1680-1688.

Hu HJ, Glauner KS, Gereau RW (2003) ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. I. Modulation of A-type K+ currents. J Neurophysiol 90: 1671-1679.

Huyser BA, Parker JC (1999) Negative affect and pain in arthritis. Rheum Dis Clin North Am 25: 105-121.

Impey S, Obrietan K, Storm DR (1999) Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. Neuron 23: 11-14.

Ji RR, Baba H, Brenner GJ, Woolf CJ (1999) Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 2: 1114-1119.

Ji RR, Kohno T, Moore KA, Woolf CJ (2003) Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci 26: 696-705.

Ji RR, Woolf CJ (2001) Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. Neurobiol Dis 8: 1-10.

Ji R-R, Befort K, Brenner GJ, Woolf CJ (2002) ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. J Neurosci 22: 478-485.

Jolkkonen E, Pitkanen A (1998) Intrinsic connections of the rat amygdaloid complex: projections originating in the central nucleus. J Comp Neurol 395: 53-72.

Jones TL, Sorkin LS (2005) Activated PKA and PKC, but not CaMKIIalpha, are required for AMPA/Kainate-mediated pain behavior in the thermal stimulus model. Pain 117: 259-270.

Kang W, Wilson MA, Bender MA, Glorioso JC, Wilson SP (1998) Herpes virus-mediated preproenkephalin gene transfer to the amygdala is antinociceptive. Brain Res 792: 133-135.

Karim F, Wang CC, Gereau RW (2001) Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. J Neurosci 21: 3771-3779.

Katayama M, Nadel JA, Bunnett NW, Di Maria GU, Haxhiu M, Borson DB (1991) Catabolism of calcitonin gene-related peptide and substance P by neutral endopeptidase. Peptides 12: 563-567.

Katz B, Miledi R (1968) The role of calcium in neuromuscular facilitation. J Physiol 195: 481-492.

Kawamura M, Kuraishi Y, Minami M, Satoh M (1989) Antinociceptive effect of intrathecally administered antiserum against calcitonin gene-related peptide on thermal and mechanical noxious stimuli in experimental hyperalgesic rats. Brain Res 497: 199-203.

Kilo S, Harding-Rose C, Hargreaves KM, Flores CM (1997) Peripheral CGRP release as a marker for neurogenic inflammation: a model system for the study of neuropeptide secretion in rat paw skin. Pain 73: 201-207.

Kolch W (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 351 Pt 2:289-305.: 289-305.

Kominato Y, Tachibana T, Dai Y, Tsujino H, Maruo S, Noguchi K (2003) Changes in phosphorylation of ERK and Fos expression in dorsal horn neurons following noxious stimulation in a rat model of neuritis of the nerve root. Brain Res 967: 89-97.

Kraenzlin ME, Ch'ng JL, Mulderry PK, Ghatei MA, Bloom SR (1985) Infusion of a novel peptide, calcitonin gene-related peptide (CGRP) in man. Pharmacokinetics and effects on gastric acid secretion and on gastrointestinal hormones. Regul Pept 10: 189-197.

Kramer HH, Schmidt K, Leis S, Schmelz M, Sommer C, Birklein F (2005) Inhibition of neutral endopeptidase (NEP) facilitates neurogenic inflammation. Exp Neurol 195: 179-184.

Kruger L, Sternini C, Brecha NC, Mantyh PW (1988) Distribution of calcitonin gene-related peptide immunoreactivity in relation to the rat central somatosensory projection. J Comp Neurol 273: 149-162.

Kuhnt U, Voronin LL (1994) Interaction between paired-pulse facilitation and long-term potentiation in area CA1 of guinea-pig hippocampal slices: application of quantal analysis. Neuroscience 62: 391-397.

Kummer W, Heym C (1991) Different types of calcitonin gene-related peptide-immunoreactive neurons in the guinea-pig stellate ganglion as revealed by triple-labelling immunofluorescence. Neurosci Lett 128: 187-190.

LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23: 155-184.

Li W, Neugebauer V (2004a) Differential roles of mGluR1 and mGluR5 in brief and prolonged nociceptive processing in central amygdala neurons. J Neurophysiol 91: 13-24.

Li W, Neugebauer V (2004b) Block of NMDA and non-NMDA receptor activation results in reduced background and evoked activity of central amygdala neurons in a model of arthritic pain. Pain 110: 112-122.

Lin HC, Wang SJ, Luo MZ, Gean PW (2000) Activation of group II metabotropic glutamate receptors induces long- term depression of synaptic transmission in the rat amygdala. J Neurosci 20: 9017-9024.

Lin Q, Peng YB, Willis WD (1996) Possible role of protein kinase C in the sensitization of primate spinothalamic tract neurons. J Neurosci 16: 3026-3034.

Lin Q, Wu J, Willis WD (2002) Effects of Protein Kinase A Activation on the Responses of Primate Spinothalamic Tract Neurons to Mechanical Stimuli. J Neurophysiol 88: 214-221.

Lopez de Armentia M, Sah P (2004) Firing properties and connectivity of neurons in the rat lateral central nucleus of the amygdala. J Neurophysiol 00211.

Ma QP, Hill R, Sirinathsinghji D (2001) Colocalization of CGRP with 5-HT1B/1D receptors and substance P in trigeminal ganglion neurons in rats. Eur J Neurosci 13: 2099-2104.

Ma W, Quirion R (2002) Partial sciatic nerve ligation induces increase in the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in astrocytes in the lumbar spinal dorsal horn and the gracile nucleus. Pain 99: 175-184.

Manabe T, Wyllie DJ, Perkel DJ, Nicoll RA (1993) Modulation of synaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus. J Neurophysiol 70: 1451-1459.

Manning BH (1998) A lateralized deficit in morphine antinociception after unilateral inactivation of the central amygdala. J Neurosci 18: 9453-9470.

Manning BH, Mayer DJ (1995) The central nucleus of the amygdala contributes to the production of morphine antinociception in the formalin test. Pain 63: 141-152.

Maren S (1999) Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. Trends Neurosci 22: 561-567.

Mayer EA, Berman S, Suyenobu B, Labus J, Mandelkern MA, Naliboff BD, Chang L (2005) Differences in brain responses to visceral pain between patients with irritable bowel syndrome and ulcerative colitis. Pain 115: 398-409.

McDonald AJ, Augustine JR (1993) Localization of GABA-like immunoreactivity in the monkey amygdala. Neuroscience 52: 281-294.

McDowell G, Coutie W, Shaw C, Buchanan KD, Struthers AD, Nicholls DP (1997) The effect of the neutral endopeptidase inhibitor drug, candoxatril, on circulating levels of two of the most potent vasoactive peptides. Br J Clin Pharmacol 43: 329-332.

McKernan MG, Shinnick-Gallagher P (1997) Fear conditioning induces a lasting potentiation of synaptic currents in vitro. Nature 390: 607-611.

Meagher MW, Arnau RC, Rhudy JL (2001) Pain and emotion: effects of affective picture modulation. Psychosom Med 63: 79-90.

Merighi A, Polak JM, Theodosis DT (1991) Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin-gene related peptide. Neuroscience 40: 67-80.

Millan MJ (1999) The induction of pain: an integrative review. Prog Neurobiol 57: 1-164.

Min SS, Han JS, Kim YI, Na HS, Yoon YW, Hong SK, Han HC (2001) A novel method for convenient assessment of arthritic pain in voluntarily walking rats. Neurosci Lett 308: 95-98.

Naliboff BD, Berman S, Chang L, Derbyshire SWG, Suyenobu B, Vogt BA, Mandelkern M, Mayer EA (2003) Sex-related differences in IBS patients: central processing of visceral stimuli. Gastroenterology 124: 1738-1747.

Natura G, von Banchet GS, Schaible HG (2005) Calcitonin gene-related peptide enhances TTX-resistant sodium currents in cultured dorsal root ganglion neurons from adult rats. Pain 116: 194-204.

Neugebauer V, Li W (2003) Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain. J Neurophysiol 89: 716-727.

Neugebauer V, Li W (2002) Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. J Neurophysiol 87: 103-112.

Neugebauer V, Li W, Bird GC, Bhave G, Gereau RW (2003) Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J Neurosci 23: 52-63.

Neugebauer V, Li W, Bird GC, Han JS (2004) The amygdala and persistent pain. Neuroscientist 10: 221-234.

Neugebauer V, Lucke T, Schaible H-G (1993) N-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists block the hyperexcitability of dorsal horn neurons during development of acute arthritis in rat's knee joint. J Neurophysiol 70: 1365-1377.

Neugebauer V, Rumenapp P, Schaible H-G (1996) Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn neurons during development of acute inflammation. Neuroscience 71: 1095-1109.

Nitecka L, Ben-Ari Y (1987) Distribution of GABA-like immunoreactivity in the rat amygdaloid complex. J Comp Neurol 266: 45-55.

Nose I, Higashi H, Inokuchi H, Nishi S (1991) Synaptic responses of guinea pig and rat central amygdala neurons in vitro. J Neurophysiol 65: 1227-1241.

Oliver KR, Wainwright A, Heavens RP, Hill RG, Sirinathsinghji DJS (1998) Distribution of novel $CGRP_1$ receptor and adrenomedullin receptor mRNAs in the rat central nervous system. Mol Brain Res 57: 149-154.

Pare D, Quirk GJ, Ledoux JE (2004) New Vistas on Amygdala Networks in Conditioned Fear. J Neurophysiol 92: 1-9.

Paulson PE, Casey KL, Morrow TJ (2002) Long-term changes in behavior and regional cerebral blood flow associated with painful peripheral mononeuropathy in the rat. Pain 95: 31-40.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates.4th: New York Academic

Petrovic P, Ingvar M, Stone-Elander S, Petersson KM, Hansson P (1999) A PET activation study of dynamic mechanical allodynia in patients with mononeuropathy. Pain 83: 447-457.

Pitkanen A, Savander V, LeDoux JE (1997) Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. Trends Neurosci 20: 517-523.

Porreca F, Ossipov MH, Gebhart GF (2002) Chronic pain and medullary descending facilitation. Trends Neurosci 25: 319-325.

Porro CA (2003) Functional imaging and pain: behavior, perception, and modulation. Neuroscientist 9: 354-369.

Poyner D (1996) Pharmacology of receptors for calcitonin gene-related peptide and amylin. Trends Pharmacol Sci 16: 424-429.

Poyner DR (1992) Calcitonin gene-related peptide: multiple actions, multiple receptors. Pharmacol Ther 56: 23-51.

Price JL (2003) Comparative aspects of amygdala connectivity. Ann N Y Acad Sci 57: 50-58.

Qin C, Greenwood-Van Meerveld B, Foreman RD (2003b) Visceromotor and spinal neuronal responses to colorectal distension in rats with aldosterone onto the amygdala. J Neurophysiol 90: 2-11.

Qin C, Greenwood-Van Meerveld B, Foreman RD (2003a) Spinal Neuronal Responses to Urinary Bladder Stimulation in Rats With Corticosterone or Aldosterone Onto the Amygdala. J Neurophysiol 90: 2180-2189.

Qin C, Greenwood-Van Meerveld B, Myers DA, Foreman RD (2003c) Corticosterone Acts Directly at the Amygdala to Alter Spinal Neuronal Activity in Response to Colorectal Distension. J Neurophysiol 89: 1343-1352.

Rhudy JL, Meagher MW (2001) The role of emotion in pain modulation. Curr Opin Psychiatry 14: 241-245.

Rhudy JL, Meagher MW (2000) Fear and anxiety: divergent effects on human pain thresholds. Pain 84: 65-75.

Rhudy JL, Williams AE, McCabe K, Nguyen MATV, Rambo P (2005) Affective modulation of the nociception at spinal and supraspinal level. Psychophysiology 42: 579-587

Rodgers RJ, Dalvi A (1997) Anxiety, defence and the elevated plus-maze. Neurosci Biobehav Rev 21: 801-810.

Rodrigues SM, Schafe GE, Ledoux JE (2004) Molecular Mechanisms Underlying Emotional Learning and Memory in the Lateral Amygdala. Neuron 44: 75-91.

Rolls ET (2000) Memory systems in the brain. Annu Rev Psychol 51: 599-630.

Royer S, Martina M, Pare D (1999) An inhibitory interface gates impluse taffic between the input and output stations of the amygdala. J Neurosci 19: 10575-10583.

Ryu PD, Murase K, Gerber G, Randic M (1988) Actions of calcitonin gene-related peptide on rat sensory ganglion neurones. Physiol Bohemoslov 37: 259-265.

Sah P, Faber ES, Lopez De AM, Power J (2003) The amygdaloid complex: anatomy and physiology. Physiol Rev 83: 803-834.

Salter MW (2005) Cellular signalling pathways of spinal pain neuroplasticity as targets for analgesic development. Curr Top Med Chem 5: 557-567.

Schaible H-G (1996) On the role of tachykinins and calcitonin gene-related peptide in the spinal mechanisms of nociception and in the induction and maintenance of inflammation-

evoked hyperexcitability in spinal cord neurons (with special reference to nociception in joints). Prog Brain Res 113: 423-441.

Schiess MC, Asprodini EK, Rainnie DG, Shinnick-Gallagher P (1993) The central nucleus of the rat amygdala: in vitro intracellular recordings. Brain Res 604: 283-297.

Schiess MC, Callahan PM, Zheng H (1999) Characterization of the electrophysiological and morphological properties of rat central amygdala neurons in vitro. J Neurosci Res 58: 663-673.

Schneider F, Habel U, Holthusen H, Kessler C, Posse S, Muller-Gartner HW, Arndt JO (2001) Subjective ratings of pain correlate with subcortical-limbic blood flow: an fMRI study. Neuropsychobiology 43: 175-185.

Schwaber JS, Sternini C, Brecha NC, Rogers WT, Card JP (1988) Neurons containing calcitonin gene-related peptide in the parabrachial nucleus project to the central nucleus of the amygdala. J Comp Neurol 270: 416-426.

Shi C, Davis M (1999) Pain pathways involved in fear conditioning measured with fear-potentiated startle: lesion studies. J Neurosci 19: 420-430.

Skofitsch G, Jacobowitz DM (1985) Calcitonin gene-related peptide: detailed immunohistochemical distribution in the central nervous system. Peptides 6: -721.

Sluka KA (1997) Activation of the cAMP transduction cascade contributes to the mechanical hyperalgesia and allodynia induced by intradermal injection of capsaicin. Br J Pharmacol 122: 1165-1173.

Sluka KA, Dougherty PM, Sorkin LS, Willis WD, Westlund KN (1992) Neural changes in acute arthritis in monkeys. III. Changes in substance P, calcitonin gene-related peptide and glutamate in the dorsal horn of the spinal cord. Brain Res Brain Res Rev 17: 29-38.

Sluka KA, Rees H, Chen PS, Tsuruoka M, Willis WD (1997a) Capsaicin-induced sensitization of primate spinothalamic tract cells is prevented by a protein kinase C inhibitor. Brain Res 772: 82-86.

Sluka KA, Rees H, Chen PS, Tsuruoka M, Willis WD (1997b) Inhibitors of G-proteins and protein kinases reduce the sensitization to mechanical stimulation and the desensitization to heat of spinothalamic tract neurons induced by intradermal injection of capsaicin in the primate. Exp Brain Res 115: 15-24.

Sluka KA, Westlund KN (1993) Spinal cord amino acid release and content in an arthritis model: the effects of pretreatment with non-NMDA, NMDA, and NK1 receptor antagonists. Brain Res 627: 89-103.

Stefanacci L, Amaral DG (2000) Topographic organization of cortical inputs to the lateral nucleus of the macaque monkey amygdala: a retrograde tracing study. J Comp Neurol 421: 52-79.

Struthers AD, Brown MJ, Macdonald DW, Beacham JL, Stevenson JC, Morris HR, Macintyre I (1986) Human calcitonin gene related peptide: a potent endogenous vasodilator in man. Clin Sci (Lond) 70: 389-393.

Sun RQ, Lawand NB, Lin Q, Willis WD (2004a) Role of Calcitonin Gene-Related Peptide in the Sensitization of Dorsal Horn Neurons to Mechanical Stimulation After Intradermal Injection of Capsaicin. J Neurophysiol 92: 320-326.

Sun RQ, Lawand NB, Willis WD (2003) The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. Pain 104: 201-208.

Sun RQ, Tu YJ, Lawand NB, Yan JY, Lin Q, Willis WD (2004b) Calcitonin Gene-Related Peptide Receptor Activation Produces PKA- and PKC-Dependent Mechanical Hyperalgesia and Central Sensitization. J Neurophysiol 92: 2859-2866.

Suzuki R, Rygh LJ, Dickenson AH (2004) Bad news from the brain: descending 5-HT pathways that control spinal pain processing. Trends in Pharmacological Sciences 25: 613-617.

Swanson LW, Petrovich GD (1998) What is the amygdala? Trends Neurosci 21: 323-331.

Sweatt JD (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol 14: 311-317.

Van Rossum D, Hanish U-K, Quirion R (1997) Neuroanatomical Localization, Pharmacological Characterization and Functions of CGRP, Related Peptides and Their Receptors. Neurosci Biobehav Rev 21: 649-678.

Vanegas H, Schaible HG (2004) Descending control of persistent pain: inhibitory or facilitatory? Brain Res Rev 46: 295-309.

Wang C-C, Willis WD, Westlund KN (1999) Ascending projections from the area around the spinal cord central canal: a *phaseolus vulgaris* leucoagglutinin study in rats. J Comp Neurol 415: 341-367.

Watkins LR, Wiertelak EP, McGorry M, Martinez J, Schwartz B, Sisk D, Maier SF (1998) Neurocircuitry of conditioned inhibition of analgesia: effects of amygdala, dorsal raphe, ventral medullary, and spinal cord lesions on antianalgesia in the rat. Behav Neurosci 112: 360-378.

Wei F, Zhuo M (2001) Potentiation of sensory responses in the anterior cingulate cortex following digit amputation in the anaesthetised rat. J Physiol 532: 823-833.

Werka T (1997) The effects of the medial and cortical amygdala lesions on post-stress analgesia in rats. Behav Brain Res 86: 59-65.

Willis WD (2001) Role of neurotransmitters in sensitization of pain responses.933: 142-156.

Wimalawansa SJ (1996) Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr Rev 17: 533-585.

Winston JH, He ZJ, Shenoy M, Xiao SY, Pasricha PJ (2005) Molecular and behavioral changes in nociception in a novel rat model of chronic pancreatitis for the study of pain. Pain 117: 214-222.

Wu LG, Saggau P (1994) Presynaptic calcium is increased during normal synaptic transmission and paired-pulse facilitation, but not in long-term potentiation in area CA1 of hippocampus. J Neurosci 14: 645-654.

Wyllie DJ, Manabe T, Nicoll RA (1994) A rise in postsynaptic Ca2+ potentiates miniature excitatory postsynaptic currents and AMPA responses in hippocampal neurons. Neuron 12: 127-138.

Xu W, Lundeberg T, Wang YT, Li Y, Yu L-C (2003) Antinociceptive effect of calcitonin gene-related peptide in the central nucleus of amygdala: activating opioid receptors through amygdala-periaqueductal gray pathway. Neuroscience 118: 1015-1022.

Yamano M, Hillyard CJ, Girgis S, Macintyre I, Emson PC, Tohyama M (1988) Presence of a substance P-like immunoreactive neurone system from the parabrachial area to the central amygdaloid nucleus of the rat with reference to coexistence with calcitonin gene-related peptide. Brain Res 451: 179-188.

Yang HW, Hu XD, Zhang HM, Xin WJ, Li MT, Zhang T, Zhou LJ, Liu XG (2004) Roles of CaMKII, PKA, and PKC in the Induction and Maintenance of LTP of C-Fiber-Evoked Field Potentials in Rat Spinal Dorsal Horn. J Neurophysiol 91: 1122-1133.

Zald DH (2003) The human amygdala and the emotional evaluation of sensory stimuli. Brain Res Rev 41: 88-123.

Zhang L, Hoff AO, Wimalawansa SJ, Cote GJ, Gagel RF, Westlund KN (2001) Arthritic calcitonin/[alpha] calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity. Pain 89: 265-273.

Zhu W, Pan ZZ (2004) Synaptic properties and postsynaptic opioid effects in rat central amygdala neurons. Neuroscience 127: 871-879.

Zou X, Lin Q, Willis WD (2002) Role of protein kinase A in phosphorylation of NMDA receptor 1 subunits in dorsal horn and spinothalamic tract neurons after intradermal injection of capsaicin in rats. Neuroscience 115: 775-786.

Zou X, Lin Q, Willis WD (2004) Effect of protein kinase C blockade on phosphorylation of NR1 in dorsal horn and spinothalamic tract cells caused by intradermal capsaicin injection in rats. Brain Res 1020: 95-105.

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Publications

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Min SS, Han JS, Kim YI, Na HS, Yoon YW, Hong SK, and Han HC. A novel method for convenient assessment of arthritic pain in voluntarily walking rats, *Neuroscience Letters,* 308: 95-98, 2001.

Hong SK, Han JS, Min SS, Hwang JM, Kim YI, Na HS, Yoon YW and Han HC. Local neurokinin-1 receptor in the knee joint contributes to the induction, but not maintenance, of arthritic pain in the rat. *Neuroscience Letters*, 322: 21-24, 2002.

Han JS, Bird GC and Neugebauer V. Enhanced group III mGluR-mediated inhibition of pain-related synaptic plasticity in the amygdala. *Neuropharmacology*, 46: 918-926, 2004.

Han JS and Neugebauer V. Synaptic plasticity in the amygdala in a visceral pain model in rats. *Neuroscience Letters*, 361: 254-257, 2004.

Han JS, Bird GC, Li W, Jones J and Neugebauer V. Computerized analysis of audible and ultrasonic vocalizations of rats as a standardized measure of pain-related behavior. *Journal of Neuroscience Method*. 141: 262-269, 2005.

Han JS, Bird GC, Li W, Jones J and Neugebauer V. mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. *Pain*. 113:211-22, 2005.

Bird GC, Lash LL, Han JS, Zou X, Willis WD and Neugebauer V. PKA-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in amygdala neurons. *Journal of Physiology*. 564: 907-921, 2005

Han JS, Li W and Neugebauer V. Critical role of CGRP1 receptors in the amygdala in synaptic plasticity and pain behavior. *Journal of Neuroscience.* 25: 10717-10728

Han JS, Bird GC and Neugebauer V. Enhanced group II mGluR-mediated inhibition of pain-related synaptic plasticity in the amygdala. *Neuropharmacology* (in preparation).