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CB1 augments mGluR5 function in medial prefrontal cortical neurons to inhibit amygdala hyperactivity in an arthritis pain model

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Abstract

The medial prefrontal cortex (mPFC) serves executive control functions and forms direct connections with subcortical areas such as the amygdala. Our previous work showed abnormal inhibition of mPFC pyramidal cells and hyperactivity of amygdala output neurons in an arthritis pain model. To restore mPFC activity hence control pain-related amygdala hyperactivity this study focused on CB1 and mGluR5 receptors, which are important modulators of cortical functions. Extracellular single-unit recordings of infralimbic mPFC pyramidal cells and of amygdala output neurons in the laterocapsular division of the central nucleus (CeLC) were made in anesthetized adult male rats. mPFC neurons were classified as “excited” or “inhibited” based on their response to brief innocuous and noxious test stimuli. After arthritis pain induction, background activity and evoked responses of excited neurons and background activity and inhibition of inhibited neurons decreased. Stereotaxic application of an mGluR5 positive allosteric modulator (VU0360172) into the mPFC increased background and evoked activity of excited, but not inhibited, mPFC neurons under normal conditions but not in arthritis. A selective CB1 receptor agonist (ACEA) alone had no effect but restored the facilitatory effects of VU0360172 in the pain model. Coactivation of CB1 and mGluR5 in the mPFC inhibited the pain-related activity increase of CeLC neurons but had no effect under normal conditions. The data suggest that excited mPFC neurons are inversely linked to amygdala output (CeLC) and that CB1 can increase mGluR5 function in this subset of mPFC neurons to engage cortical control of abnormally enhanced amygdala output in pain.

Keywords

endocannabinoids; metabotropic glutamate receptor; pyramidal cell; cognitive processes; rat

Introduction

The medial prefrontal cortex (mPFC) serves executive functions such as top-down cognitive control that has been established in experimental models of behavioral “extinction” of negative emotions (Myers and Davis 2007; Quirk et al 2010; Hartley and Phelps 2010; Herry et al 2010). The infralimbic mPFC region inhibits amygdala output to suppress

(“extinguish”) aversive behaviors (Likhtik et al 2005; Pape and Pare 2010; Sotres-Bayon and Quirk 2010; Herry et al 2010; Orsini and Maren 2012; Marek et al 2013). Decreased infralimbic activity has been linked to extinction deficits (Hefner et al 2008; Kim et al 2010; Chang and Maren 2010; Sierra-Mercado et al 2011; Wei et al 2012) and behavioral disinhibition (Dalley et al 2011).

Functional and structure abnormalities in the mPFC have also been found in human pain patients (Apkarian et al 2004a) and in animal pain models (Metz et al 2009; Ji et al 2010; Cardoso-Cruz et al 2013), and they correlate with cognitive decision-making deficits in gambling tasks (Apkarian et al 2004b; Pais-Vieira et al 2009; Ji et al 2010). Our previous study showed that pain-related amygdala hyperactivity drives feedforward inhibition of mPFC pyramidal to decrease cortical output (Ji et al 2010). Failure to activate the mPFC is associated with visceral hypersensitivity in patients (Mayer et al 2005). Therefore, we hypothesized that restoring mPFC activity and output could be a useful strategy to mitigate cognitive deficits and control pain behaviors.

Feedforward inhibition of the mPFC critically involves activation of metabotropic glutamate receptor subtype mGluR1, but not mGluR5, presynaptic to GABAergic interneurons that inhibit mPFC pyramidal cells (Sun and Neugebauer 2011; Ji and Neugebauer 2011). Blockade of mGluR1, however, restored mPFC activity only partially (Ji and Neugebauer 2011), but activation of mGluR5 with positive allosteric modulators can increase the excitatory synaptic drive of infralimbic mPFC pyramidal cells (Kiritoshi et al 2013) and enhance hippocampal synaptic plasticity (Ayala et al 2009; Noetzel et al 2012). Both mGluR1 and mGluR5 subtypes are expressed in the PFC at pre- and postsynaptic sites (Cauli et al 2000; Muly et al 2003). Importantly, mGluR5 can interact with presynaptic endocannabinoid CB1 receptors (Guindon and Hohmann 2009) to depress inhibitory transmission (Lovinger 2008; Kano et al 2009) and produce hippocampal long-term depression (see Izumi and Zorumski 2011). In the rodent mPFC, CB1 is exclusively expressed in GABAergic interneurons (Marsicano and Lutz 1999; Wedzony and Chocyk 2009), and presynaptic CB1 receptors on axon terminals face postsynaptic mGluR5 on pyramidal cells (Lafourcade et al 2007).

Here we determined the effect of mGluR5 and CB1 activation on the activity of infralimbic mPFC cells under normal conditions and in an arthritis pain model. The infralimbic mPFC is linked to a cluster of GABAergic neurons in the intercalated cell mass (ITC) of the amygdala (McDonald 1998; Amir et al 2011; Pinard et al 2012) to exert inhibitory influences on amygdala output and control negative emotions during behavioral extinction (Jungling et al 2008; Pape and Pare 2010; Marek et al 2013). Recent work from our laboratory showed that activation of ITC cells inhibits pain-related synaptic plasticity of amygdala neurons in the laterocapsular division of the central nucleus (CeLC) and pain behaviors (Ren et al 2013c). CeLC hyperactivity accounts for emotional-affective pain behaviors (Neugebauer et al 2004; Neugebauer et al 2009). Therefore, we also evaluated the effect of mGluR5 and CB1 activation in the mPFC on the activity of CeLC neurons.

Methods

Experimental animals

Adult male Sprague Dawley rats ($n = 55$; 250–350 g; Harlan Laboratories, Indianapolis) were housed in standard plastic cages (40×20 cm) in a temperature-controlled room and maintained on a 12-h day/night cycle. Water and food were available without restriction. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Texas Medical Branch (UTMB) and conform to the Guidelines laid down by the National Institutes of Health (NIH).

Animal surgery and anesthesia

As described in our previous studies (for recent references see Ji et al 2010; Ji and Neugebauer 2011; Ji and Neugebauer 2012) the animal was anesthetized with pentobarbital sodium throughout the experiment (induction, 50 mg/kg, intraperitoneally, i.p.; maintenance, $15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, intravenously, i.v., through a catheter in the jugular vein). After insertion of a tracheal cannula the animal was paralyzed with pancuronium (0.3 mg/h, i.v.) and artificially ventilated (3–3.5 ml; 55–65 strokes/min). End-tidal CO_2 levels (kept at 4.0%), heart rate and ECG pattern were monitored continuously. Core body temperature was maintained at 37°C by means of a homeothermic blanket system. These measures ensured a constant internal state of body functions. The animal was mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and a small craniotomy was performed at the suture frontoparietalis level to allow the insertion of the recording electrode and microdialysis probe (for drug application, see “Drugs”).

Electrophysiological recordings

Extracellular recordings were made from single neurons in the infralimbic part of the mPFC and in the laterocapsular division of the central nucleus of the amygdala (CeLC) with glass-insulated carbon filament electrodes (4–6 $\text{M}\Omega$) using the following stereotaxic coordinates (Paxinos and Watson 1998): mPFC, 3.0–3.2 mm rostral to bregma, 0.5–1.0 mm lateral to midline, depth 4.5–6.0 mm; CeLC, 2.1–2.8 mm caudal to bregma; 3.8–4.5 mm lateral to midline; depth 7–9 mm. Recordings were made in the right hemisphere because of previous evidence for pain-related lateralization (Carrasquillo and Gereau 2008; Ji and Neugebauer 2009; Goncalves and Dickenson 2012). Recorded signals were amplified, band-pass filtered (300 Hz – 3 kHz), displayed on analog and digital storage oscilloscopes, fed into a window discriminator (World Precision Instruments), digitized (1401 plus interface, Cambridge Electronic Design), and recorded on a personal computer (PC) using Spike2 software (version 4; Cambridge Electronic Design) to create peristimulus rate histograms online and to store and analyze digital records of single-unit activity offline.

Identification of mPFC and amygdala (CeLC) neurons

Animals were under continuous anesthesia throughout the experiment. By the time recordings on an individual neuron started, the animals had been under anesthesia for at least 5 h. An individual neuron was identified by the configuration, shape, and height of the recorded action potentials (spikes) that occurred spontaneously (background activity) or in

response to mechanical (tissue compression) search stimuli (evoked responses). mPFC pyramidal neurons can be distinguished from interneurons based on their broader action potential waveform (peak-to-valley spike-width $>500\ \mu\text{s}$) and low baseline discharge rate ($<10\ \text{Hz}$) as described before (Ji et al 2010; Ji and Neugebauer 2011; Ji and Neugebauer 2012). CeLC neurons responding to noxious stimuli form a homogenous population of neurons characterized extensively in our previous studies (see Neugebauer et al 2004; 2009). Spikes were detected and recorded based on the waveform signal that crossed a trigger level and matched a pre-set shape or template, which was created for the individual neuron at the beginning of the recording period using Spike2 software. Only those neurons were included in this study whose spike configuration remained constant (matching the template) and could be clearly discriminated from any activity in the background throughout the experiment, indicating that the activity of one and the same one neuron was measured.

Background and evoked activity

Spontaneous “background” activity (in the absence of intentional stimulation) and responses evoked by brief (10 s) mechanical test stimuli of innocuous ($300\ \text{g}/30\ \text{mm}^2$) and noxious ($2000\ \text{g}/30\ \text{mm}^2$) intensities were recorded. Mechanical stimuli (compression) were applied to the knee joint contralateral to the recording site, using a calibrated forceps equipped with a force transducer, whose output signal was amplified, displayed in grams on a liquid-crystal display screen, digitized by the CED interface, and recorded on the Pentium PC for on- and offline analysis as described before (Ji et al 2010; Ji and Neugebauer 2011; Ji and Neugebauer 2012). For the analysis of net evoked activity, background activity in the 10-s time period preceding the 10-s stimulus was subtracted from the total activity during stimulation. The number of stimulations was kept at a minimum to avoid any confounding effects of repeated stimulation. Sufficiently long control periods were included in each experiment to establish pre-arthritis or pre-drug baseline activity.

Arthritis pain model

To determine changes in the arthritis pain model, one and the same neuron was recorded continuously before and after arthritis induction, allowing paired analysis. However, drugs were tested either before or 5–6 h after arthritis induction. The animal was anesthetized throughout the experiment. Arthritis was induced as described in detail previously (Neugebauer et al. 2007). A kaolin suspension (4%, 100 μl) was injected slowly into the joint cavity through the patellar ligament with the use of a syringe and needle (1 ml, 25 gauge, 5/8 in). After repetitive flexions and extensions of the knee for 15 min, a carrageenan solution (2%, 100 μl) was injected into the knee joint cavity, and the leg was flexed and extended for another 5 min. This treatment paradigm reliably leads to a localized inflammation confined to one knee joint within 1–3 h, persists for weeks, and is significantly associated with pain behaviors (Neugebauer et al 2007).

Drug application

Several hours before the start of the electrophysiological recordings, a microdialysis probe (CMA11, CMA Microdialysis, Sweden; membrane diameter: 250 μm ; membrane length: 1 mm) was lowered vertically into the infralimbic mPFC using the following stereotaxic coordinates (Paxinos and Watson 1998): 3.2 mm anterior to bregma; 0.8 mm lateral to

midline; depth, 5.0 mm (Ji et al 2010; Ji and Neugebauer 2011). The distance between the microdialysis probe and the recording electrode in the mPFC was 0.5–1.0 mm. The microdialysis probe was connected to an infusion pump (Harvard Apparatus, Holliston, MA) with polyethylene-50 tubing and perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125.0, KCl 2.6, NaH₂PO₄ 2.5, CaCl₂ 1.3, MgCl₂ 0.9, NaHCO₃ 21.0, and glucose 3.5, oxygenated and equilibrated to pH 7.4. ACSF was pumped through the fiber at a rate of 5 µl/min during the entire experiment and served as a vehicle control.. Drugs were administered into the mPFC at a rate of 5 µl/min for at least 15 min to establish equilibrium in the tissue. Drug concentration in the tissue is at least 100 times lower than in the microdialysis probe, due to the concentration gradient across the dialysis membrane and diffusion in the tissue (Li and Neugebauer 2004; Han and Neugebauer 2005; Ji and Neugebauer 2011). The numbers given in this article refer to the drug concentrations in the microdialysis fiber. For the analysis of drug effects, the same neuron was recorded before and during drug administration into the mPFC and background and evoked activity were measured every 5–10 min before and during drug application. This protocol was the same under normal conditions (no arthritis) and in the arthritis pain state (5–6 h postinduction). In experiments in which drugs were tested under normal conditions no arthritis was induced afterwards to avoid any confounding effects, even though all drug effects appeared to be reversible. In arthritis, drugs were tested on some neurons that had been recorded continuously during the development of arthritis and in other neurons that were recorded only in the arthritis state (5–6 h postinduction of arthritis). No apparent differences were found and the data were pooled.

Drugs

The following selective compounds were used: mGluR5 positive allosteric modulator N-cyclobutyl-6-((3-fluorophenyl)ethynyl) nicotinamide hydrochloride (VU0360172, VU¹172), a generous gift from Dr. P. Jeffrey Conn, Vanderbilt Center for Neuroscience Drug Discovery and Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN, was synthesized as described previously (Rodriguez et al 2010); CB1 agonist arachidonyl-2-chloroethylamide (ACEA) was purchased from Tocris Bioscience (R&D Systems, Inc., Minneapolis, MN). All drugs were dissolved in ACSF to their final concentration on the day of the experiment. Selectivity and target concentrations have been established in the literature (Hillard et al 1999; Rodriguez et al 2010; Pertwee 2010) and our own studies (Kiritoshi et al 2013).

Histological verification of recording and stimulation sites

At the end of each experiment, the recording site in the mPFC or CeLC was marked by injection of a direct current (250 µA for 3 min) through the carbon-filament glass electrode. Brains were removed and fixed in 4% paraformaldehyde for 6–12 h; then they were placed in 30% sucrose for 48–72 h before they were frozen-sectioned at 50 µm. For verification of the position of the recording/lesion site, sections were mounted on gel-coated slides, stained with hematoxylin and eosin, and cover slipped.

Data analysis and statistics

Single-unit action potentials were analyzed offline from peristimulus rate histograms using Spike2 software (version 4; Cambridge Electronic Design). Background and evoked activity was expressed as spikes/s (Hz). Background activity was measured by calculating the average firing rate during a recording period of 3–5 min. Net evoked activity was calculated by subtracting background activity in the 10-s time period preceding each 10-s stimulus from the total activity during stimulation. Statistical analysis was performed on the raw data (spikes/s). For multiple comparisons, one-way ANOVA was used with appropriate posttests. Student's t-test (paired where appropriate) was used to compare two sets of data that have Gaussian distribution and similar variances. Statistical significance was accepted at the level $P < 0.05$. All averaged values are given as the mean $\pm$ SE. GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA) was used for all statistical analyses.

Results

Pain-related changes in the sample of mPFC neurons

Extracellular single-unit recordings were made from a total of 41 mPFC neurons in the right hemisphere of 41 rats. The right hemisphere was chosen because of evidence for pain-related lateralization of amygdala function (Carrasquillo and Gereau 2008; Ji and Neugebauer 2009; Goncalves and Dickenson 2012) and close interactions between mPFC and amygdala (see below). 16 neurons were recorded under normal conditions only, another 16 before and during the development of arthritis, and 9 only in the arthritis pain state (5–6 h postinduction). Thus, a total of 25 neurons were recorded in the arthritis pain model. Based on their baseline firing frequency under normal conditions (<10 Hz) and broad action potential waveform (peak-to-valley spike-width >500 μ s) mPFC neurons in this study were considered pyramidal cells as described before (Ji et al 2010; Ji and Neugebauer 2011; Ji and Neugebauer 2012). Firing frequency was 2.84 ± 1.98 Hz and 2.26 ± 1.45 Hz in the sample of neurons tested under normal conditions and in arthritis, respectively. Action potential duration was 562 ± 58 μ s and 581 ± 50 μ s (sample of neurons under normal conditions and in arthritis, respectively). Histological analysis of lesions made through the recording electrode at the end of each experiment showed the location of these neurons in the deep layers (V/VI) of the infralimbic cortex (Fig. 1A, normal, $n=16$; arthritis, $n=25$).

Two types of mPFC neurons were identified. “Excited” neurons responded with an increase of background activity during brief innocuous and noxious mechanical test stimuli (compression of the knee joint; see “Background and evoked activity” in Methods). “Inhibited” neurons showed a decrease of background activity during test stimuli (see individual examples in Fig. 1C and 1F). The sample of excited neurons consisted of 7 neurons recorded under normal conditions only, 9 neurons before and after arthritis induction, and 3 neurons only after arthritis induction. Thus, a total of 16 excited neurons were tested under normal conditions and 12 were tested in arthritis. Inhibited neurons included 9 neurons recorded under normal conditions only, 7 neurons before and after arthritis induction, and 6 neurons only in arthritis. Thus, a total of 16 inhibited neurons were tested under normal conditions and 13 were tested in arthritis. Figure 1A shows the location of excited and inhibited neurons (filled and open circles, respectively). We did not find an

apparent difference in the distribution of excited and inhibited neurons. Localizations of neurons recorded before and during the development of arthritis are shown only in the arthritis group because this is when the lesions were made at the end of the experiment. The neurons' receptive fields in somatic tissues (joints, muscles and skin) were large, covering the entire body. Test stimuli were applied primarily to the knee joint contralateral to the recording side.

Background activity and evoked responses of excited neurons decreased significantly after arthritis induction ($P < 0.05$ – 0.01 , paired t-test; Fig. 2A and 2B) in the sample of neurons that were recorded continuously before and during the development of arthritis ($n = 9$). Evoked responses were measured as the difference between total activity during stimulation and background activity preceding the stimulus (see "Background and evoked activity" in Methods). Background activity and net inhibition by test stimuli (total activity minus background activity) of inhibited neurons recorded continuously before and after arthritis induction also decreased significantly ($P < 0.05$, paired t-test; $n = 7$ neurons; Fig. 2D and 2E). Changes started at 1–2 hours postinduction of arthritis and reached their maximum after 3–4 hours. The pain-related changes of excited infralimbic neurons are similar to our previous observations on prelimbic mPFC neurons (Ji et al 2010), suggesting widespread deactivation in these areas; inhibited neurons were not recorded in that study.

Effects of CB1 and mGluR5 activation on mPFC neurons

Next we tested the hypothesis that activation of CB1 and/or mGluR5 could restore pre-arthritis activity levels in mPFC neurons. A selective CB1 agonist (ACEA) and/or a positive allosteric modulator selective for mGluR5 (VU0360172, VU'172) were administered into the infralimbic mPFC by microdialysis either in normal animals or 5–6 h postinduction of arthritis. Drugs were not tested before and after arthritis induction in the same animal to avoid any potentially confounding effects. Selectivity of these drugs and appropriate target concentrations were discussed in our previous brain slice study (Kiritoshi et al 2013). Concentrations in the microdialysis probe were 100 times higher than the target concentrations in the tissue because of the concentration gradient across the dialysis membrane and diffusion in the tissue (Li and Neugebauer 2004; Han and Neugebauer 2005; Ji and Neugebauer 2011).

Infralimbic administration of ACEA (10 μ M) alone or VU'172 (100 μ M) alone or ACEA together with VU'172 were tested in 7 excited and 9 inhibited neurons under normal conditions (= 16 neurons) and in 6 excited and 9 inhibited neurons in the arthritis state (5–6 h postinduction) for a total of 15 neurons in arthritis. Six of these neurons were recorded before and during the development of arthritis, but drugs were tested only in the arthritis state. The order of application of ACEA and VU'172 alone was counterbalanced. The combination of ACEA and VU'172 was always administered last. Drugs were administered for at least 15 min to establish equilibrium in the tissue (typically 20–25 min). ACSF was administered through the microdialysis probe during the entire experiment. Interval between drug applications was 30 min.

Under normal conditions, VU'172, but not ACEA, increased background and evoked activity of excited neurons significantly ($n = 7$, $P < 0.05$, Dunnett's multiple comparison

tests; Fig. 3). The combined application of VU'172 and ACEA also had significant facilitatory effects ($P < 0.05$, Dunnett's posttest) but they were not significantly different from those of VU'172 alone, suggesting that CB1 activation did not augment the mGluR5-mediated effects under these conditions. In the arthritis pain model (5–6 h postinduction), the combined application of VU'172 and ACEA increased background and evoked activity of excited neurons significantly ($n = 6$, $P < 0.05$, Dunnett's multiple comparison tests; Fig. 4). ACEA and VU'172 alone had no effect. The data suggest that co-activation of CB1 and mGluR5 is required to increase the pain-related decreased activity of excited mPFC neurons. Inhibited neurons showed a decrease of background activity during brief (10 s) innocuous and noxious stimuli. There was no significant effect of ACEA or VU'172, applied alone or together, on inhibited neurons tested under normal conditions ($n = 9$; combination was tested in 7 neurons only; Fig. 5) and in arthritis (5–6 h postinduction; $n = 9$; combination was tested in 6 neurons only; Fig. 6).

Effects of infralimbic CB1 and mGluR5 activation on CeLC neurons

The mPFC is closely linked to the amygdala. Specifically, the infralimbic mPFC is believed to control amygdala output and negative emotions such as fear by engaging a cluster of inhibitory ITC cells interposed between (baso)lateral and central amygdala nuclei (Peters et al 2009; Sotres-Bayon and Quirk 2010; Sierra-Mercado et al 2011). We showed recently that activation of ITC cells can inhibit pain-related synaptic plasticity of CeLC neurons and pain behaviors, but their synaptic activation is decreased in the arthritis pain model (Ren et al 2013b). Therefore, we tested the hypothesis that increasing infralimbic activity pharmacologically could inhibit the pain-related enhancement of activity of CeLC neurons.

CeLC neurons were recorded either under normal conditions ($n = 6$) or in the arthritis pain model (5–6 h postinduction, $n = 8$). Our previous studies (for reviews see Neugebauer et al 2004; 2009) and work by others (Goncalves and Dickenson 2012) showed that CeLC neurons consistently develop hyperactivity in different pain models. Therefore, in this study we did not continuously record CeLC neurons during the development of arthritis but tested the effect of infralimbic drug administration on CeLC neurons either under normal conditions or 5–6 h after induction of arthritis. Under normal conditions, ACEA or VU'172, applied alone or together, had no significant effect on CeLC neurons ($n = 6$; Fig. 7). In the arthritis pain state, however, co-application of ACEA and VU'172 into the infralimbic cortex inhibited background and evoked activity of CeLC neurons significantly ($n = 8$, $P < 0.05$ – 0.001 , Dunnett's multiple comparison tests; Fig. 8). The data suggest that increasing mPFC activity pharmacologically by CB1 and mGluR5 coactivation can control enhanced amygdala activity in the pain state without affecting baseline activity under normal conditions.

Discussion

The study reports several novelties regarding pain-related function of CB1 receptors in the mPFC. They include the following key findings. Infralimbic presumed pyramidal cells in layer V/VI develop decreased background activity and responsiveness in a pain model similar to prelimbic mPFC neurons described in our previous study (Ji et al 2010). These

results add to the growing body of evidence for cortical-cognitive dysfunctions in pain (see May 2008; Neugebauer et al 2009; Apkarian et al 2009; Borsook et al 2010; Moriarty et al 2011). To restore mPFC activity we focused on CB1 and mGluR5 because they are well positioned to increase the synaptic drive of mPFC pyramidal cells (mGluR5, Kiritoshi et al 2013) while blocking synaptic inhibition (CB1, Lovinger 2008; Kano et al 2009) including GABAergic transmission onto infralimbic pyramidal cells (Lin et al 2008). We found that co-activation of CB1 and mGluR5 is required to increase mPFC activity in the arthritis pain model whereas under normal conditions mGluR5 activation alone has facilitatory effects. The data suggest that CB1 activation allows mGluR5 to activate mPFC cells presumably by blocking pain-related abnormal inhibition that we described previously for the prelimbic mPFC (Ji et al 2010; Ji and Neugebauer 2011). Finally, coactivation of CB1 and mGluR5 in the mPFC inhibited background activity and responsiveness of amygdala neurons in the laterocapsular subdivision of the central nucleus (CeLC), which consistent with the concept of top-down cortical control of subcortical brain centers of emotions such as the amygdala.

Endocannabinoid signaling is an important modulator of synaptic transmission and plasticity in the brain and involves interactions with metabotropic receptors such as mGluR5 through the phospholipase C - diacylglycerol lipase α (DAGL α) pathway that leads to the formation of 2-arachidonoylglycerol and CB1-mediated homo- or heterosynaptic inhibition of transmitter release (Lovinger 2008; Guindon and Hohmann 2009; Kano et al 2009; Di Marzo 2011). In the rodent mPFC, CB1 receptors are exclusively expressed in GABAergic interneurons (Marsicano and Lutz 1999; Wedzony and Chocyk 2009), and CB1 activation inhibits synaptic inhibition of infralimbic pyramidal cells (Lin et al 2008). Axon terminals with CB1 receptors synapse on mPFC pyramidal cells expressing mGluR5 and DAGL α (Lafourcade et al 2007), supporting the concept that mGluR5 can provide a source for 2-arachidonoylglycerol endocannabinoids (Guindon and Hohmann 2009). In the mPFC, mGluR5 is expressed at particularly high levels mostly on postsynaptic elements (Muly et al 2003). Activation of mGluR5 increases excitatory synaptic inputs (Marek and Zhang 2008), synaptically evoked spiking (Kiritoshi et al 2013) and intrinsic bursting (Fontanez-Nuin et al 2011) in layer V mPFC pyramidal cells in brain slice preparations, which is consistent with the facilitatory effects of VU'172 on background and evoked activity of infralimbic pyramidal cells observed under normal conditions in our in vivo study.

The synaptic arrangement of presynaptic CB1 and postsynaptic mGluR5 allows for mGluR5-driven retrograde endocannabinoid signaling to inhibit synaptic inhibition (disinhibition) through a CB1 receptor-mediated mechanism, which could explain the facilitatory effect of mGluR5-CB1 coactivation on mPFC output in our pain model. ACEA and VU'172 alone had no effect in the pain state but their combination produced facilitatory effects on mPFC neurons and inhibitory effects on amygdala neurons, which is consistent with a synergistic interaction, although this possibility was not specifically tested here. Concentrations were chosen based on published data as discussed below (see also Methods). A potential synergistic mechanism could be the combination of CB1-mediated inhibition of abnormal synaptic inhibition and mGluR5-mediated increase of excitatory drive onto pyramidal cells.

Relevant to the present study is also the observation that activation of CB1 receptors in the infralimbic mPFC facilitates fear extinction whereas antagonists for CB1 (Lin et al 2009) or mGluR5 (Fontanez-Nuin et al 2011) impair extinction, suggesting a critical role of these receptors in the cortical control of negative emotions such as fear. Increased infralimbic activity correlates with successful extinction of negative emotions (Mickley et al 2005; Knapska and Maren 2009; Kim et al 2010; Chang et al 2010) and decreased activity with cognitive control deficits in models of behavioral extinction (Mickley et al 2007; Hefner et al 2008; Kim et al 2010; Chang and Maren 2010; Sierra-Mercado et al 2011) and behavioral disinhibition (Dalley et al 2011). Therefore, restoring or increasing mPFC output is a desirable goal in disorders that are associated with mPFC deactivation or dysfunction, such as anxiety (Bishop et al 2004; Milad et al 2006; de Visser et al 2011), PTSD (Sotres-Bayon and Quirk 2010; Sierra-Mercado et al 2011; Fontanez-Nuin et al 2011), depression (Lobo et al 2012), schizophrenia (Peled 2011), and pain (Ji et al 2010).

Top-down cognitive control of emotions is centered on interactions between mPFC and amygdala (Ochsner and Gross 2005; Dalley et al 2011). The infralimbic mPFC is believed to suppress (“extinguish”) aversive behavior through inhibition of amygdala output (Maren and Quirk 2004; Likhtik et al 2005; Akirav and Maroun 2007; Sah and Westbrook 2008; Pape and Pare 2010; Sotres-Bayon and Quirk 2010; Kim and Richardson 2010; Herry et al 2010). Hyperactivity in the amygdala network of lateral-basolateral (LA-BLA) and central (CeA) nuclei has emerged as an important contributor to emotional-affective aspects of pain (Neugebauer et al 2004; 2009). The laterocapsular division of the CeA (CeLC) receives nociceptive-specific information from spinal cord and brainstem and highly processed multimodal, including nociceptive, information from thalamus and cortex through the LA-BLA network (Neugebauer et al 2004; Neugebauer et al 2009). CeLC neurons, which were selected for the present study, respond exclusively or preferentially to noxious stimuli (Neugebauer et al 2004; Gauriau and Bernard 2004; Neugebauer et al 2009). Synaptic plasticity in the CeLC is now well established for models of arthritic pain (Neugebauer et al 2003; Han et al 2005; Bird et al 2005; Fu and Neugebauer 2008; Ren and Neugebauer 2010; Ren et al 2013a), formalin-induced pain (Adedoyin et al 2010), visceral pain (Han and Neugebauer 2004) and chronic neuropathic pain (Ikeda et al 2007; Nakao et al 2012). Pain-related synaptic plasticity is associated with increased responsiveness of CeLC neurons (Neugebauer and Li 2003; Li and Neugebauer 2004; Han et al 2005; Ji and Neugebauer 2007; Ji and Neugebauer 2009; Goncalves and Dickenson 2012) and amygdala-dependent pain behaviors (see Neugebauer et al 2004; Neugebauer et al 2009). Since pain-related changes of CeLC neurons are well established we did not monitor the development of pain-related hyperactivity of CeLC neurons in the present study but sampled neurons either under normal conditions or 5–6 h postinduction of arthritis.

mPFC control of amygdala output is accomplished through the activation of GABAergic intercalated cells (ITC) that project strongly to the CeLC (for details see Amir et al 2011; Busti et al 2011) and serve as a feedforward inhibitory gate for signals to the CeA (Royer and Pare 2002; Likhtik et al 2008; Ehrlich et al 2009; Pape and Pare 2010; Amano et al 2010). The infralimbic mPFC forms a particularly strong excitatory projection to ITC cells that inhibit CeA neurons via external capsule projections (McDonald 1998; Amir et al 2011; Pinard et al 2012). The mPFC-ITC-CeA circuitry could explain the finding of the present

study that mPFC activation inhibited CeLC activity in the pain model. This concept is also supported by our recent observation that activation of ITC cells, synaptically driven from mPFC projections in the external capsule, can inhibit enhanced CeLC output in the arthritis pain model (Ren et al 2013). Importantly, it is the mPFC population of “excited” but not “inhibited” neurons that account for mPFC-driven CeLC inhibition, because CB1-mGluR5 coactivation increased activity in mPFC neurons excited by nociceptive inputs but had no effect on inhibited neurons. This result suggests that the combination of CB1 and mGluR5 activation is a useful strategy to selectively increase mPFC output to the amygdala.

A number of methodological aspects need to be considered. mPFC neurons were identified as presumed pyramidal cells only based on functional properties but not morphologically. It is generally accepted, however, that mPFC pyramidal cells can be distinguished from interneurons based on their broader action potential waveform and lower baseline discharge rate (Constantinidis and Goldman-Rakic 2002; Laviolette and Grace 2006; Homayoun and Moghaddam 2007). Based on criteria established or discussed in these references we selected neurons with peak-to-valley spike width greater than 500 μ s and a baseline discharge rate lower than 10 Hz as in our previous studies (Ji et al 2010; Ji and Neugebauer 2011; Ji and Neugebauer 2012).

Regarding selectivity of drugs used in this study, ACEA, a synthetic analog of *N*-arachidonyl ethanolamine (anandamide), is one of the most selective agonists for CB1 (Hillard et al 1999). While effects of anandamides are not only mediated through CB1 but can also involve TRPV1 (Di Marzo 2010), and high concentrations of ACEA ($>10 \mu$ M) can activate TRPV1 in the brain (Casarotto et al 2012), CB1 and TRPV1 receptors have opposing effects in the prefrontal cortex (Rubino et al 2008; Giordano et al 2011) and TRPV1 activation counteracts endocannabinoid-mediated retrograde inhibition of GABAergic transmission in the striatum (Di Marzo 2011). The concentration used in our study was 10 μ M in the microdialysis probe and the concentration achieved in the tissue is at least 100 fold lower (see “Drug application” in Methods for Discussion), which is far below that required for actions on TRPV1. Still, we cannot rule out entirely that the lack of effects of ACEA alone might be due to an opposing action on CB1 and TRPV1, resulting in a net null effect.

VU0360172 (VU’172) is a chemically optimized orally active analog of VU0092273, one of the most potent and selective mGluR5 positive allosteric modulators (Rodriguez et al 2010). In our previous study a selective negative allosteric modulator of mGluR5 (MTEP) inhibited the effects of VU’172 on synaptically evoked spiking, confirming the involvement of mGluR5 activation (Kiritoshi et al 2013). Allosteric modulators are devoid of activity in the absence of endogenous ligands and exhibit less desensitization and fewer side effects from off target interactions (Niswender and Conn 2010). Positive allosteric modulators for mGluR5 are developed for the treatment of cognitive deficits in schizophrenia (Vinson and Conn 2012) and have been shown to enhance synaptic plasticity and learning and memory (Ayala et al 2009; Cleva and Olive 2011; for VU’172 see Noetzel et al 2012). Their role as “cognitive enhancers” formed part of the rationale for the present study to activate mGluR5 to increase mPFC function and output. It should be noted that the fact that only a

subpopulation of mPFC neurons responded to CB1 and mGluR5 activation also argues against generalized non-specific excitatory effects of these drug targets.

In conclusion, the results of this study provide evidence for a novel strategy to increase and restore mPFC activity to inhibit CeLC output in an animal pain model. Co-activation of mGluR5 and CB1 in the mPFC increased the decreased activity of a subpopulation of mPFC pyramidal neurons excited by nociceptive input in arthritic rats. Underlying synaptic and cellular mechanisms and details of the mPFC-CeLC circuitry remain to be determined.

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Abbreviations

BLA	basolateral amygdala
CB1	cannabinoid receptor 1
CeA	central nucleus of the amygdala
CeLC	laterocapsular division of the central nucleus of the amygdala
ITC	intercalated cell mass
LA	lateral amygdala
mGluR	metabotropic glutamate receptor
mPFC	medial prefrontal cortex

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Figure 1. Histologically verified location of recording sites

Diagrams drawn based on histological brain slices show coronal sections through the right hemisphere at the level of the mPFC (**A**) and amygdala (**B**). Each symbol shows the location of the tip of the recording electrode in the brain of normal (**A**, $n = 16$; **B**, $n = 6$) and arthritic animals (**A**, $n = 25$; **B**, $n = 8$). Some of the recording sites in **A** include neurons that were recorded before and after arthritis induction. Filled circles, excited neurons; open circles, inhibited neurons. IL, infralimbic; PL, prelimbic; ACC, anterior cingulate cortex; LA, lateral amygdala; BLA, basolateral amygdala; CeM, CeL, CeLC, medial, lateral, laterocapsular divisions of the central nucleus of the amygdala. Diagrams on the right show the brain area that is enlarged in the diagrams on the left showing the lesion sites.

Excited mPFC neurons

Inhibited mPFC neurons

Figure 2. Activity changes of mPFC neurons in the arthritis pain model

The same neurons were recorded continuously before and after arthritis induction in the contralateral knee. (A–C) Excited mPFC neurons ($n = 9$). (D–F) Inhibited mPFC neurons ($n = 7$). (A, D) Bar histograms show spikes/s (mean $\pm$ SE) of background activity (average firing rate over 3–5 min) and responses to innocuous and noxious stimuli (compression of the contralateral knee joint for 10 s) before (“Normal”) and 5–6 h postinduction of arthritis (“Arthritis”). For evoked “net” responses, background activity (10 s) was subtracted from the total activity during stimulation (10 s) hence inhibition resulted in a negative value. (B, E) Graphs show background activity and “net” responses of individual neurons before and after arthritis induction. (C, F) Peristimulus rate histograms show individual examples. Bin width, 1 s.

Excited mPFC neurons - normal

Figure 3. Effects of CB1 and mGluR5 activation on “excited” mPFC neurons under normal conditions

(A) Background activity and responses to brief (10 s) innocuous and noxious stimuli recorded in an individual mPFC neuron before (“Predrug”) and during infralimbic administration of ACEA (10 μ M), VU'172 (100 μ M), and ACEA combined with VU'172. (B) Bar histograms show spikes/s (mean $\pm$ SE) of background (3–5 min) and evoked activity (background activity in the 10-s time period preceding each 10-s stimulus was subtracted from the total activity during stimulation) for the sample of neurons tested ($n = 7$). VU'172 alone or together with ACEA had significant facilitatory effects. ACEA did not augment the effect of VU'172 under normal conditions. * $P < 0.05$, n.s., non-significant, Dunnett's multiple comparison tests. (C) Graph shows background and “net” evoked activity for individual neurons before and during the combined application of VU'172 and ACEA.

Excited mPFC neurons - arthritis

Figure 4. Effects of CB1 and mGluR5 activation on “excited” mPFC neurons in the arthritis pain model

Drugs were tested 5–6 h postinduction of arthritis in the contralateral knee. **(A)** Background activity and responses to innocuous and noxious stimuli (10 s) recorded in an individual mPFC neuron before (“Predrug”) and during infralimbic administration of ACEA (10 μ M), VU’172 (100 μ M), and ACEA combined with VU’172. **(B)** Bar histograms show spikes/s (mean $\pm$ SE) of background (3–5 min) and evoked activity (background activity in the 10-s time period preceding each 10-s stimulus was subtracted from the total activity during stimulation) for the sample of neurons tested (n = 6). Only the combined application had significant effects. * $P < 0.05$, Dunnett’s multiple comparison tests. **(C)** Graph shows background and “net” evoked activity for individual neurons before and during the combined application of VU’172 and ACEA.

Inhibited mPFC neurons - normal

Figure 5. Lack of effects of CB1 and mGluR5 activation on “inhibited” mPFC neurons under normal conditions

(A) Background activity and activity during innocuous and noxious stimuli (10 s) recorded in an individual mPFC neuron before (“Predrug”) and during infralimbic administration of VU’172 (100 μ M), ACEA (10 μ M), and ACEA combined with VU’172. (B) Bar histograms show spikes/s (mean $\pm$ SE) of background activity (3–5 min) and evoked inhibition (background activity in the 10-s time period preceding each 10-s stimulus was subtracted from the total activity during stimulation, resulting in negative values) for the sample of neurons tested ($n = 9$). No significant effects were found. $P > 0.05$, Dunnett’s multiple comparison tests. (C) Graph shows background and inhibited activity for individual neurons ($n = 7$) before and during the combined application of VU’172 and ACEA.

Inhibited mPFC neurons - arthritis

Figure 6. Lack of effects of CB1 and mGluR5 activation on “inhibited” mPFC neurons in the arthritis pain model

Drugs were tested 5–6 h postinduction of arthritis in the contralateral knee. **(A)** Background activity and activity during innocuous and noxious stimuli (10 s) recorded in an individual mPFC neuron before (“Predrug”) and during infralimbic administration of VU’ 172 (100 μ M), ACEA (10 μ M), and ACEA combined with VU’ 172. **(B)** Bar histograms show spikes/s (mean $\pm$ SE) of background activity (3–5 min) and evoked inhibition (background activity in the 10-s time period preceding each 10-s stimulus was subtracted from the total activity during stimulation, resulting in negative values) for the sample of neurons tested ($n = 9$). No significant effects were found. $P > 0.05$, Dunnett’s multiple comparison tests. **(C)** Graph shows background activity and evoked inhibition for individual neurons ($n = 6$) before and during the combined application of VU’ 172 and ACEA.

CeA neurons - normal

Figure 7. Lack of effects of infralimbic CB1 and mGluR5 activation on CeLC neurons under normal conditions

(A) Background activity and responses to brief (10 s) innocuous and noxious stimuli recorded in an individual CeLC neuron before ("Predrug") and during infralimbic administration of VU'172 (100 μ M), ACEA (10 μ M), and ACEA combined with VU'172. (B) Bar histograms show spikes/s (mean $\pm$ SE) of background activity (3–5 min) and evoked activity (background activity in the 10-s time period preceding each 10-s stimulus was subtracted from the total activity during stimulation) for the sample of neurons tested ($n = 6$). (C) Graph shows background and "net" evoked activity for individual neurons before and during the combined application of VU'172 and ACEA.

CeA neurons - arthritis

Figure 8. Effects of infralimbic CB1 and mGluR5 activation on CeLC neurons in the arthritis pain model

Drugs were tested 5–6 h postinduction of arthritis in the contralateral knee. **(A)** Background activity and responses to innocuous and noxious stimuli (10 s) recorded in an individual CeLC neuron before (“Predrug”) and during infralimbic administration of VU’172 (100 μ M), ACEA (10 μ M), and ACEA combined with VU’172. **(B)** Bar histograms show spikes/s (mean $\pm$ SE) of background activity (3–5 min) and evoked activity (background activity in the 10-s time period preceding each 10-s stimulus was subtracted from the total activity during stimulation) for the sample of neurons tested ($n = 8$). *,*** $P < 0.05$, 0.001, Dunnett’s multiple comparison tests. **(C)** Graph shows background and evoked activity for individual neurons before and during the combined application of VU’172 and ACEA.