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Differential effects of mGluR7 and mGluR8 activation on pain-related synaptic activity in the amygdala

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Abstract

Pain-related plasticity in the latero-capsular division of the central nucleus of the amygdala (CeLC) depends on the activation of group I metabotropic glutamate receptors (mGluRs) whereas groups II and III mGluRs generally serve inhibitory functions. Recent evidence suggests differential roles of group III subtypes mGluR7 (pain enhancing) and mGluR8 (pain inhibiting) in the amygdala (Palazzo et al., 2008). Here we addressed the underlying synaptic mechanisms of mGluR7 and mGluR8 function in the CeLC under normal conditions and in an arthritis pain model. Using patch-clamp recordings in rat brain slices, we measured monosynaptic excitatory postsynaptic currents (EPSCs), mono- and polysynaptic inhibitory synaptic currents (IPSCs), and synaptically evoked action potentials (E-S coupling) in CeLC neurons. Synaptic responses were evoked by electrical stimulation in the basolateral amygdala (BLA). A selective mGluR8 agonist (DCPG) inhibited evoked EPSCs and synaptic spiking more potently in slices from arthritic rats than in slices from normal rats. In contrast, a selective mGluR7 agonist (AMN082) increased EPSCs and E-S coupling in slices from normal rats but not in the pain model. The effects of AMN082 and DCPG were blocked by a group III antagonist (MAP4). AMN082 increased frequency, but not amplitude, of spontaneous EPSCs but had no effect on miniature EPSCs (in TTX). DCPG decreased frequency, but not amplitude, of spontaneous and miniature EPSCs. The data suggest that mGluR8 acts presynaptically to inhibit excitatory transmission whereas the facilitatory effects of mGluR7 are indirect through action potential-dependent network action. AMN082 decreased evoked IPSCs and frequency, but not amplitude, of spontaneous and miniature IPSCs in slices from normal rats. DCPG had no effect on inhibitory transmission. The results suggest that presynaptic mGluR7 inhibits inhibitory synaptic transmission to gate glutamatergic transmission to CeLC neurons under normal conditions but not in pain. Presynaptic mGluR8 inhibits pain-related enhanced excitatory transmission in the CeLC.

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Introduction

The goal of this study was to advance knowledge about the role of metabotropic glutamate receptors (mGluRs) in pain-related synaptic transmission and plasticity in the amygdala. The amygdala has emerged as an important neural substrate for the emotional–affective dimension of pain and pain modulation (Neugebauer et al., 2004; Kulkarni et al., 2007; Neugebauer et al., 2009; Apkarian et al., 2009; Heinricher et al., 2009). Amygdala activity correlates positively with pain behavior in animals. Increasing activity in the amygdala can elicit or enhance pain responses even in the absence of tissue injury (Qin et al., 2003; Myers et al., 2005; Carrasquillo and Gereau, 2007; Han et al., 2010; Myers and Greenwood-Van Meerveld B., 2010; Kolber et al., 2010; Li et al., 2011). Conversely, lesions or pharmacological deactivation of the amygdala have inhibitory effects in different pain models (Manning, 1998; Hebert et al., 1999; Han and Neugebauer, 2005; Han et al., 2005; Pedersen et al., 2007; Fu and Neugebauer, 2008; Fu et al., 2008; Palazzo et al., 2008; Ji et al., 2010). Recent studies in humans also showed pain-related activity increases in the amygdala (Kulkarni et al., 2007; Baliki et al., 2008; Tillisch et al., 2010; Liu et al., 2010).

Within the amygdala the laterocapsular division of the central nucleus (CeLC) has been termed the “nociceptive amygdala” because the vast majority of its neurons respond exclusively or preferentially to stimuli of noxious intensity (Bernard et al., 1992; Neugebauer and Li, 2002; Gauriau and Bernard, 2002; Neugebauer et al., 2004). The CeLC receives purely nociceptive information from the spino-parabrachio-amygdaloid (PB) pain pathway and highly processed information with affective content through an indirect pathway from the lateral-basolateral amygdala (LA-BLA) network via posterior thalamus (Neugebauer et al., 2004; 2009). It is now well established that CeLC neurons develop pain-related central sensitization in vivo (Neugebauer and Li, 2003; Li and Neugebauer, 2004; Li and Neugebauer, 2006; Ji and Neugebauer, 2007; Ji et al., 2009; Ji and Neugebauer, 2009) and synaptic plasticity for inputs from PB and BLA in brain slices in models of inflammatory and neuropathic pain (Neugebauer et al., 2003; Han et al., 2004; Han and Neugebauer, 2004; Han et al., 2005; Bird et al., 2005; Han et al., 2006; Ikeda et al., 2007; Fu and Neugebauer, 2008; Fu et al., 2008; Adedoyin et al., 2010; Ren and Neugebauer, 2010).

mGluRs are important regulators of synaptic transmission and neuronal excitability and have emerged as therapeutic targets for neuropsychiatric disorders associated with abnormal neuroplasticity (Anwyl, 1999; Conn, 2003; Swanson et al., 2005; Pinheiro and Mulle, 2008; Niswender and Conn, 2010; Lesage and Steckler, 2010). Group I mGluRs are required for pain-related neuroplastic changes in the amygdala (Neugebauer et al., 2003; Li and Neugebauer, 2004; Ji and Neugebauer, 2010; Li et al., 2011). Exogenous or endogenous activation of group I mGluRs in the amygdala produces or enhances pain behavior (Han and Neugebauer, 2005; Ansah et al., 2010; Kolber et al., 2010; Li et al., 2011).

In contrast, group II and group III mGluRs have been shown to inhibit pain-related synaptic plasticity and responsiveness of amygdala neurons (Han et al., 2004; Li and Neugebauer, 2006; Han et al., 2006; Adedoyin et al., 2010). Evidence from recent studies, however, suggests that group III subtypes mGluR7 and mGluR8 in the amygdala (Palazzo et al., 2008), like in the brainstem (Marabese et al., 2007), can have opposing functions related to pain modulation. Activation of mGluR7 and mGluR8 with subtype selective agonists enhanced and inhibited, respectively, pain behaviors. In models of fear and anxiety opposing roles of mGluR7 (enhancing) and mGluR8 (inhibiting) were also described (Masugi et al., 1999; Linden et al., 2002; Cryan et al., 2003; Mitsukawa et al., 2005; Duvoisin et al., 2005; Schmid and Fendt, 2006; Mitsukawa et al., 2006).

The differential effects of mGluR7 and mGluR8 are somewhat puzzling because both receptors couple to the same type of G-proteins (Gi/o) and signal transduction pathways. The underlying mechanisms remain to be determined and were addressed at the synaptic level in this study. We focused on the modulation of excitatory and inhibitory synaptic transmission because both subtypes can modulate glutamate and GABA release (Swanson et al., 2005; Marabese et al., 2007; Neugebauer, 2008; Palazzo et al., 2011).

Methods

Animals

Male Sprague Dawley rats (120–250 g) were housed in a temperature controlled room and maintained on a 12 h day/night cycle. Water and food were available ad libitum. On the day of the experiment, rats were transferred from the animal facility and allowed to acclimate to the laboratory for at least 1 hr. All experimental procedures conform to the guidelines of the International Association for the Study of Pain (IASP) and of the National Institutes of Health (NIH) and were approved by the Institutional Animal Care and Use Committee (IACUC).

Arthritis pain model

A localized arthritis was induced in one knee joint as described previously (Neugebauer et al., 2007). A kaolin suspension (4%, 100 μ l) was injected into the left knee joint cavity through the patellar ligament. After repetitive flexions and extensions of the knee for 15 minutes, a carrageenan solution (2%, 100 μ l) was injected into the knee joint cavity, and the leg was flexed and extended for another 5 minutes. 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). Brain slices were obtained 5–6 h after arthritis induction. Another group of rats (“normal”) did not receive any injections but was handled and kept under the same conditions as the arthritic rats before brain slices were obtained for electrophysiological studies.

Electrophysiology

Amygdala slice preparation—Brain slices containing the basolateral and central nuclei of the amygdala (BLA and CeA) were obtained as described before (Neugebauer et al., 2003; Han et al., 2005; Fu and Neugebauer, 2008; Ji et al., 2010; Ren and Neugebauer, 2010; Li et al., 2011). Rats were decapitated, and the brains quickly were dissected out and blocked in cold (4°C) artificial cerebrospinal fluid (ACSF). ACSF contained (in mM): 117 NaCl, 4.7 KCl, 1.2 NaH₂PO₄, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, and 11 glucose. ACSF was oxygenated and equilibrated to pH 7.4 with a mixture of 95% O₂/5% CO₂. Coronal brain slices (500 μ m) were prepared using a Vibroslice (Camden Instruments, London, UK). After incubation in ACSF at room temperature (21°C) for at least 1 h, a single brain slice was transferred to the recording chamber and submerged in ACSF (31 $\pm$ 1°C), which superfused the slice at 2 ml/min.

Whole-cell patch-clamp recording—Whole-cell current- and voltage-clamp recordings were made from visually identified CeLC neurons in brain slices from normal rats and arthritic rats using DIC enhanced infrared video-microscopy as in our previous studies (Neugebauer et al., 2003; Han et al., 2005; Fu and Neugebauer, 2008; Ji et al., 2010; Ren and Neugebauer, 2010). One neuron was recorded in each slice and 1 or 2 slices were used per animal. Patch electrodes were made from borosilicate glass capillaries (1.5 mm outer diameter, 1.12 mm inner diameter; Drummond, Broomall, PA) pulled on a Flaming-Brown micropipette puller (P-97/PC; Sutter Instruments, Novato, CA). Patch electrodes had tip resistances of 4–6 M Ω . The following internal solution was used (in mM): 122 K-gluconate,

5 NaCl, 0.3 CaCl₂, 2 MgCl₂, 1 EGTA, 10 HEPES, 5 Na₂-ATP, and 0.4 Na₃-GTP; pH was adjusted to 7.2–7.3 with KOH and osmolarity to 280 mOsm/kg with sucrose. A dual four-pole Bessel filter (Warner Instruments), low-noise Digidata 1322 interface (Molecular Devices), Axoclamp-2B amplifier (Molecular Devices) and Pentium 4 personal computer were used to acquire current and voltage data that were analyzed using pCLAMP10 software (Axon Instruments). Head-stage voltage was monitored continuously on an oscilloscope to ensure precise performance of the amplifier. Neurons were voltage-clamped at –70 (near chloride reversal potential) or 0 mV (reversal potential of EPSCs) for the study of excitatory and inhibitory transmission, respectively. The calculated equilibrium potential for chloride in this system was –68.99 mV (Nernst equation, pClamp10 software). High gigaohm seal and low series (20 MΩ) resistances were checked throughout the experiment (using pClamp10 membrane test function) to ensure high-quality recordings.

Synaptic transmission—Using concentric bipolar stimulating electrodes (David Kopf Instruments), excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs, respectively) were evoked in CeLC neurons (held at –70 mV or 0 mV) by electrical stimulation (using an S88 stimulator; Grass Instruments) in the BLA (150 μs square-wave pulses). Input–output relationships 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 50% of the intensity required for maximum responses. In current clamp mode, action potentials were synaptically evoked in CeLC neurons by the stimulation in the BLA at threshold intensity. Synaptically evoked spiking (E-S coupling) was measured as the number of spikes evoked by ten subsequent stimulations.

Spontaneous and miniature (in TTX, 1 μM) EPSCs and IPSCs were recorded at –70 and 0 mV, respectively (Fu and Neugebauer, 2008; Ji et al., 2010; Ren and Neugebauer, 2010). A fixed length of traces (5 min) was analyzed for frequency and amplitude distributions using MiniAnalysis program 5.3 (Synaptosoft). The root mean square (RMS) of background noise was computed for each set of data. Detection threshold for an event was set to three to four times the RMS value. Peaks were detected automatically, but each detected event was then visually inspected to prevent the inclusion of false data.

Drugs

The following drugs were used: 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (**NBQX**; non-NMDA receptor antagonist); [*R*-(*R**,*S**)]-6-(5,6,7,8-tetrahydro-6-methyl-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl)furo[3,4-*e*]-1,3-benzodioxol-8(6H)-one (**bicuculline**; GABA_A receptor antagonist); *N,N'*-*Bis*(diphenylmethyl)-1,2-ethanediamine dihydrochloride (**AMN082**; selective mGluR7 agonist); (*S*)-3,4-dicarboxyphenylglycine (**DCPG**; selective mGluR8 agonist); and (*S*)-2-amino-2-methyl-4-phosphonobutanoic acid (**MAP4**, selective group III mGluR antagonist). Drugs were purchased from Tocris Cookson, Ellisville, MO. 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 (Schoepp et al., 1999; Thomas et al., 2001; Mitsukawa et al., 2005; Schmid and Fendt, 2006; Marabese et al., 2007; Fendt et al., 2008). Drugs were applied to the brain slice by gravity-driven superfusion in the ACSF. Drugs were applied for at least 15 min to establish equilibrium in the tissue. ACSF served as vehicle control in all experiments.

Statistical analysis

All averaged values are given as the mean ± SE. Statistical significance was accepted at the level *P* < 0.05. GraphPad Prism 3.0 software (Graph-Pad Software, San Diego, CA) was used for all statistical analysis. Input-output functions and concentration-response

relationships were compared using two-way ANOVA with Bonferroni posttests. Student's *t*-test (paired or unpaired when appropriate) was used to compare two sets of data that have Gaussian distribution and similar variances. Kolmogorov-Smirnov (K-S) test was used for cumulative distribution analysis of spontaneous and miniature synaptic events (MiniAnalysis program 5.3, Synaptosoft Inc., Decatur, GA). 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), and *D* is the slope coefficient (GraphPad Prism software).

Results

Monosynaptic excitatory postsynaptic currents (EPSCs) and monosynaptic and polysynaptic inhibitory postsynaptic currents (mono-IPSCs and poly-IPSCs, respectively) were evoked in CeLC neurons by electrical stimulation in the BLA (Fig. 1). Monosynaptic EPSCs (Fig. 1C) and mono-IPSCs (Fig. 1D), but not poly-IPSCs (Fig. 1E,F), followed high-frequency (20 Hz) synaptic stimulation reliably (*n* = 20 neurons). Latencies of poly-IPSCs were longer and more variable than those of monosynaptic EPSCs and mono-IPSCs (Fig. 1G, *n* = 10 neurons). IPSCs were blocked by bicuculline (10 μM). Monosynaptic EPSCs depended on non-NMDA receptor activation because they were completely blocked by a non-NMDA receptor antagonist NBQX (10 μM). NBQX also inhibited poly-IPSCs but not mono-IPSCs (Fig. 1F, *n* = 10 neurons), suggesting that the BLA provides not only monosynaptic inhibitory inputs to the CeLC but also non-NMDA receptor-mediated feedforward inhibition.

Differential effects of mGluR7 and mGluR8 agonists on excitatory transmission and output function of CeLC neurons

A selective mGluR7 agonist (AMN082, Fig. 2A) increased monosynaptic EPSCs under normal conditions but had significantly weaker effects in slices from arthritic rats (*n* = 5 neurons in each group, *F*_{1,40} = 28.24, *P* < 0.0001, main effect of arthritis, two-way ANOVA). In contrast, a selective mGluR8 agonist (DCPG, Fig. 2B) had inhibitory effects on monosynaptic EPSCs and was more potent in slices from arthritic rats (apparent *EC*₅₀ = 1.5 nM) than in slices from normal rats (*EC*₅₀ = 25.8 nM, *n* = 4 neurons in each group, *F*_{1,30} = 8.33, *P* < 0.01, main effect of arthritis, two-way ANOVA). Slices from arthritic rats were taken 5–6 h postinduction (see Methods). Based on the analysis of cumulative concentration-response relationships, test concentrations were selected for AMN082 (10 μM) and DCPG (10 nM).

Next we determined the effects of AMN082 and DCPG on input-output (I/O) relationships for excitatory transmission at the BLA-CeLC synapse in voltage clamp (Fig. 3A,C,E,G) and on output functions of CeLC neurons (synaptically evoked action potentials, E-S coupling) in current-clamp (Fig. 3B,D,F,H). AMN082 (10 μM) significantly increased I/O function of EPSCs (Fig. 3A, *n* = 10 neurons, *F*_{1,198} = 50.74, *P* < 0.0001, main effect of drug, two-way ANOVA) and enhanced synaptically evoked spiking (Fig. 3B, *n* = 10 neurons, *P* < 0.05, paired *t*-test) in slices from normal rats. In slices from arthritis rats, however, AMN082 (10 μM) had no significant effect on excitatory transmission (Fig. 3C, *n* = 10 neurons, *F*_{1,198} = 0.37, *P* > 0.05, main effect of drug, two-way ANOVA) and E-S coupling (Fig. 3D, *n* = 10 neurons, *P* > 0.05, paired *t*-test). The facilitatory effect of AMN082 (10 μM) in slices from normal rats was blocked by a selective group III mGluR antagonist (MAP4, 500 μM; Fig. 4A, *n* = 5 neurons, *F*_{2,110} = 1.08, *P* > 0.05, main effect of drug, two-way ANOVA).

In contrast, DCPG (10 nM) decreased excitatory synaptic transmission (Fig. 3G, *n* = 6 neurons, *F*_{1,110} = 22.90, *P* < 0.0001, main effect of drug, two-way ANOVA) and E-S coupling (Fig. 3H, *n* = 6 neurons, *P* < 0.05, paired *t*-test) in slices from arthritic rats. In slices

from normal rats DCPG (10 nM) had no significant effect on I/O functions (Fig. 3E, $n = 8$ neurons, $F_{1,154} = 3.44$, $P > 0.05$, main effect of drug, two-way ANOVA) and E-S coupling (Fig. 3F, $n = 8$ neurons, $P > 0.05$, paired t-test). The inhibitory effect of DCPG (10 nM) in slices from arthritic rats was blocked by MAP4 (500 μ M; Fig. 4B, $n = 4$ neurons, $F_{2,99} = 0.32$, $P > 0.05$, main effect of drug, two-way ANOVA).

The data show that mGluR7 and mGluR8 have differential receptor-mediated effects on excitatory synaptic transmission in CeLC neurons. Next we sought to determine if the differential drug effects involved different pre- versus post-synaptic sites of action.

mGluR7 and mGluR8 act presynaptically to modulate excitatory transmission

The analysis of spontaneous and miniature EPSCs is a well established electrophysiological approach to determine pre- versus postsynaptic mechanisms. Presynaptic changes at the transmitter release site affect frequency, whereas changes at the postsynaptic membrane would alter amplitude (quantal size) (Wyllie et al., 1994). The effects of AMN082 were determined in slices from normal rats whereas DCPG was tested in slices from arthritic rats, because under these conditions the drugs produced significant effects on evoked EPSCs and E-S coupling (Figs. 2 and 3). AMN082 (10 μ M) increased frequency (Fig. 5A), but not amplitude (Fig. 5B), of spontaneous EPSCs (sEPSCs) significantly ($n = 5$ neurons; cumulative frequency distribution, $P < 0.05$, Kolmogorov-Smirnov [K-S] test; mean frequency, $P < 0.05$, paired t-test). AMN082 had no significant effect on frequency (Fig. 5D) and amplitude (Fig. 5E) of miniature EPSCs (mEPSCs) recorded in TTX (1 μ M) ($n = 6$ neurons; cumulative distribution, $P > 0.05$, K-S test; mean frequency and amplitude, $P > 0.05$, paired t-test). The lack of effect on mEPSCs suggests an action potential-dependent mechanism of AMN082 action. DCPG (10 nM) decreased frequency, but not amplitude, of sEPSCs (Fig. 5G,H, $n = 7$ neurons) and of mEPSCs (Fig. 5J,K, $n = 5$ neurons) significantly (cumulative frequency distribution, $P < 0.05$, K-S test; mean frequency, $P < 0.05$, paired t-test). The data suggest that mGluR8 acts presynaptically on excitatory terminals at the BLA-CeLC synapse.

Differential effects of mGluR7 and mGluR8 agonists on inhibitory transmission

Since the facilitatory effect of AMN082 on excitatory transmission depends on TTX-sensitive network activity (Fig. 2) we hypothesized that a disinhibitory mechanism may be involved. Evoked inhibitory synaptic transmission from BLA to CeLC (see Fig. 1B) was recorded in voltage-clamp at 0 mV (see Fig. 1D and E). AMN082 (10 μ M) decreased I/O function of poly-IPSCs in slices from normal rats significantly (Fig. 6A, $n = 5$ neurons, $F_{1,88} = 47.49$, $P < 0.0001$) but had no effect in the arthritis pain model (Fig. 6B, $n = 5$ neurons, $F_{1,88} = 1.87$, $P > 0.05$, main effect of drug, two-way ANOVA). AMN082 had no significant effect on mono-IPSCs in slices from normal rats (Fig. 6C, $n = 5$ neurons, $F_{1,88} = 0.05$, $P > 0.05$) and from arthritic rats (Fig. 6D, $n = 5$ neurons, $F_{1,88} = 0.61$, $P > 0.05$, main effect of drug, two-way ANOVA). In contrast, DCPG (10 nM) had no significant effect on monosynaptic (Fig. 6E,F) and polysynaptic (Fig. 6E,H) inhibitory transmission in slices from normal rats (poly-IPSCs, $n = 4$ neurons, $F_{1,66} = 2.86$, $P > 0.05$; mono-IPSCs, $n = 4$ neurons, $F_{1,66} = 0.22$, $P > 0.05$) and from arthritic rats (poly-IPSCs, $n = 4$ neurons, $F_{1,66} = 0.00$, $P > 0.05$; mono-IPSCs, $n = 4$ neurons, $F_{1,66} = 0.07$, $P > 0.05$, main effect of drug, two-way ANOVA). The data suggest that mGluR7 is associated with a particular type of inhibitory input that is activated by BLA neurons and therefore polysynaptic.

To determine the site of action of mGluR7 in the polysynaptic inhibitory pathway to the CeLC we measured the effects of AMN082 (10 μ M) on spontaneous and miniature IPSCs (Fig. 7). AMN082 decreased frequency, but not amplitude, of sIPSCs (Fig. 7A–C, $n = 4$ neurons) and of mIPSCs (Fig. 7D–F, $n = 4$ neurons) in CeLC neurons significantly

(cumulative frequency distribution, $P < 0.05$, K-S test; mean frequency, $P < 0.05$ -0.01, paired t-test). The data suggest that mGluR7 regulates inhibitory synaptic transmission onto CeLC neurons through a presynaptic mechanism.

Contribution of a GABAergic mechanism to the facilitatory effect of mGluR7 on output function of CeLC neurons

The results so far indicate that mGluR7 activation increases excitatory transmission and output function of CeLC neurons through a presynaptic network effect (Figs. 2, 3 and 5). At the same time mGluR7 activation inhibits GABAergic transmission presynaptically (Figs. 6 and 7). Therefore, we tested the hypothesis that the overall excitatory effect of mGluR7 on CeLC function resulted from disinhibition (inhibition of GABAergic transmission). Recordings were made in slices from normal rats where mGluR7 effects were most pronounced (see Figs. 2 and 3). A GABA_A receptor antagonist (bicuculline, 10 μ M) increased E-S coupling at the BLA-CeLC synapse significantly (Fig. 8, $n = 4$ neurons, $P < 0.05$, Bonferroni posttest), suggesting the presence of endogenous GABAergic control of CeLC output function under normal conditions. In the presence of bicuculline, AMN082 failed to enhance E-S coupling (Fig. 8, $n = 4$ neurons, $P > 0.05$, Bonferroni posttest). The data suggest that blockade of GABAergic transmission occludes the facilitatory effects of mGluR7 on CeLC output, which is consistent with the hypothesis that the mechanism underlying the excitatory effects of mGluR7 is inhibition of GABAergic inhibition (disinhibition).

Discussion

This study is the first to analyze the synaptic effects of mGluR7 and mGluR8 agonists in the amygdala in relation to pain. Our previous study showed the differential modulation of pain behavior by mGluR7 (facilitatory) and mGluR8 (inhibitory) in the amygdala (Palazzo et al., 2008). The mechanisms remained to be determined and were addressed at the synaptic level in this study. The novel key findings are as follows. Activation of mGluR7 had facilitatory effects on excitatory transmission and on output function of CeLC neurons predominantly if not exclusively under normal conditions but not in a model of arthritis pain. The excitatory effect was due to a TTX-sensitive action in the network rather than directly on the glutamatergic terminals. mGluR7 also acted presynaptically to inhibit inhibitory responses. We interpret these findings to suggest that mGluR7 decreased GABAergic control of excitatory transmission to CeLC neurons, resulting in the disinhibition of CeLC output function. The lack of effect of an mGluR7 agonist in the presence of bicuculline (Fig. 8) is consistent with this interpretation. In contrast, mGluR8 directly inhibited the enhanced glutamatergic transmission at the BLA-CeLC synapse observed in the arthritis pain model. The mechanism was presynaptic and did not involve GABAergic modulation.

The data are generally consistent with our previous findings that in pain the GABAergic tone is lost whereas excitatory processes and output function of CeLC neurons are increased (Ren and Neugebauer, 2010). In this system mGluR7 would act as a gatekeeper to release GABAergic control and permit excitatory information to reach the CeLC under normal conditions. Our data suggest that mGluR7 specifically modulates a subset of GABAergic neurons. AMN082 affected glutamate-driven polysynaptic but not monosynaptic IPSCs (Fig. 6), implicating BLA-activated GABAergic neurons such as interneurons of the intercalated cell masses (ITC) rather than GABAergic BLA neurons. mGluR7 is distributed throughout the amygdala and mGluR7a immunoreactivity has been observed especially on axon terminals targeting dendritic profiles in the area between ITC and CeA (Kinoshita et al., 1998).

mGluR7 has been shown to couple to the inhibition of GABA release in the nucleus accumbens, thereby increasing the glutamatergic tone (Li et al., 2008). In the hippocampus, mGluR7 produced long term depression of inhibitory interneurons (Pelkey et al., 2005). Long-lasting potentiation following group III mGluR activation with LAP4 was found in the amygdala at the LA-BLA synapse (Neugebauer et al., 1997). The facilitatory effects of mGluR7 activation in our study were not due to receptor internalization or desensitization (Pelkey et al., 2007) because they were blocked with a selective group III mGluR antagonist (MAP4). AMN082-induced decrease in GABA and increase in glutamate release in the nucleus accumbens were also blocked by a group III mGluR antagonist (MSOP; Li et al., 2008). The pain-related decrease of synaptic inhibition described in our previous study (Ren and Neugebauer, 2010) and confirmed here (see Fig. 6, normal compared to arthritis) would render mGluR7 largely ineffective and could explain the loss of AMN082 effects in slices from arthritic rats. The electrophysiological data can explain the results of our previous behavioral study showing that activation of mGluR7 with AMN082 had pro-nociceptive and anxiogenic-like effects in normal animals but not in arthritis pain (Palazzo et al., 2008).

The presynaptic inhibitory effects of mGluR8 activation on excitatory transmission at the BLA-CeLC synapse are consistent with the effects described before for a broad spectrum group III agonist (LAP4) that inhibited synaptic plasticity and central sensitization of CeLC neurons in pain (Han et al., 2004; Li and Neugebauer, 2006). Different than LAP4, however, DCPG inhibited only pain-related plasticity but not baseline transmission in the amygdala, which may suggest that a subtype other than mGluR7 or mGluR8 mediated the inhibitory effects of LAP4 on baseline transmission (Han et al., 2004; Li and Neugebauer, 2006). The electrophysiological data are in agreement with our behavioral study that showed DCPG had anti-nociceptive and anxiolytic-like effects in arthritic animals but not under normal conditions (Palazzo et al., 2008). DCPG also had no effect on neurotransmitter release in the amygdala under normal conditions (Palazzo et al., 2011). Likewise, DCPG did not affect baseline synaptic transmission in hippocampal slices from adult rats (Ayala et al., 2008) and failed to modify anxiety-like behavior in normal rats after administration into the BLA (Stachowicz et al., 2005). The electrophysiological and behavioral data on mGluR8 in the amygdala are consistent with the important antinociceptive (Varney and Gereau, 2002; Neugebauer, 2008) and anxiolytic-/antidepressant-like (Palucha et al., 2004; Swanson et al., 2005) functions of group III mGluRs.

The source of the endogenous ligand(s), be it glutamate or other amino acids, deserves some consideration. Our previous studies on pain-related synaptic plasticity showed that presynaptic mechanisms, including mGluR1, drive and maintain a state of enhanced synaptic glutamate receptor-mediated transmission (Neugebauer et al., 2003; Neugebauer et al., 2009; Ren and Neugebauer, 2010). The endogenous ligand could be released from the lateral-basolateral circuitry, which plays a key role in associative processing related to emotions (Pape and Pare, 2010). Other sources of glutamate could be cortical inputs including those from the medial prefrontal cortex that are involved in the extinction of negative emotional states (Sotres-Bayon and Quirk, 2010).

Group III mGluRs typically decrease transmitter release and can inhibit glutamatergic and GABAergic synaptic transmission so that the overall effect of group III mGluR activation is a balance between facilitatory and inhibitory actions (Swanson et al., 2005; Neugebauer, 2008). Here we show that mGluR7 and mGluR8 mediate the differential effects of group III mGluRs. Several studies have shown differential contributions of mGluR7 and mGluR8 to pain and anxiety. Activation of mGluR7 or mGluR8 in the periaqueductal grey matter had opposing pro- and anti-nociceptive effects, respectively, which correlated with differential effects on the release of GABA and glutamate and on the activity of OFF and ON cells in the rostral ventromedial medulla (Marabese et al., 2007). AMN082 blocked the acquisition

of fear learning, facilitated fear extinction, and impaired long-term potentiation in the amygdala (Fendt et al., 2008). siRNA-induced knockdown of mGluR7 (intracerebro-ventricular administration) blocked extinction of conditioned fear (Fendt et al., 2008). Genetic ablation of mGluR7 caused deficits in fear response and conditioned taste aversion (Masugi et al., 1999), impaired stress response (Mitsukawa et al., 2006), and had antidepressant- and anxiolytic-like effects (Cryan et al., 2003; Swanson et al., 2005; Mitsukawa et al., 2006). In contrast, mGluR8 knockout mice showed increased anxiety-like behavior (Linden et al., 2002; Duvoisin et al., 2005). DCPG in the amygdala inhibited acquisition and expression of conditioned fear and long-term potentiation in the lateral amygdala (Schmid and Fendt, 2006). In that study (Schmid and Fendt, 2006), DCPG also inhibited basal synaptic transmission, which is different from the results of the present study where DCPG had no effect on baseline synaptic transmission from BLA to CeLC, perhaps suggesting regional differences within the amygdala. These data show that group III subtypes can exert a variety of (opposing) effects on brain functions. The underlying mechanisms remain to be determined.

The analysis of group III mGluR subtype function in the brain has been hampered in the past by the lack of selective pharmacological tools, but subtype-selective agonists for mGluR7 (AMN082) and mGluR8 (DCPG) are now available (Thomas et al., 2001; Mitsukawa et al., 2005; Conn and Niswender, 2006). Their selectivity and target concentrations have been established in the literature (Thomas et al., 2001; Mitsukawa et al., 2005; Schmid and Fendt, 2006; Marabese et al., 2007; Fendt et al., 2008; Palazzo et al., 2011). The differential effects of AMN082 and DCPG on excitatory and inhibitory transmission under normal conditions and in a pain model in this study suggest that these drugs were able to distinguish between mGluR7 and mGluR8 subtypes. The antagonist used (MAP4) is selective for mGluR4, mGluR7 and mGluR8 (Niswender and Conn, 2010). Inhibition of allosteric agonist effects by an orthosteric antagonist may be somewhat surprising but could be explained by “indirect” antagonism. For example, it has been shown that AMN082 and DL-AP4 activities were significantly greater than additive in some cell lines (Mitsukawa et al., 2005), suggesting allosteric potentiation at least under certain conditions. A group III mGluR antagonist (MSOP) blocked the differential effects of AMN082 and DCPG in a previous study (Marabese et al., 2007).

In conclusion, the present study shows opposing roles of mGluR7 and mGluR8 in the regulation of excitatory synaptic transmission and output function of CeLC neurons. Activation of mGluR7 had facilitatory effects through disinhibition under normal conditions, consistent with pro-nociceptive and anxiety-like effects in our previous study. In contrast, mGluR8 activation inhibited CeLC function in the arthritis pain model, which can explain the anti-nociceptive and anxiolytic observed previously. Antinociceptive effects of DCPG and pronociceptive effects of AMN082 in the periaqueductal grey were previously shown to correlate with opposite functions of mGluR7 and mGluR8 in glutamate and GABA release and neuronal activities in the rostral ventromedial medulla (Marabese et al., 2007). Systemic application of DCPG had antinociceptive effects in animal models of inflammatory and neuropathic pain. Taken together these findings support the potential usefulness of mGluR8, but not mGluR7, activation for pain relief (see Nicoletti et al., 2010). Normalizing activity in the amygdala and in other brain areas with mGluR8 activators such as DCPG may also be a promising strategy for neuropsychiatric disorders associated with maladaptive plasticity such as anxiety (Swanson et al., 2005; see Nicoletti et al., 2010). The situation with mGluR7 appears to be more complex. Disruption of synaptic plasticity with AMN082 in the amygdala was associated with impaired acquisition of aversive memories and facilitation of their extinction, suggesting that mGluR7 activation may have beneficial effects in anxiety disorders through actions in the amygdala and prefrontal cortex (Fendt et al., 2008). However, mGluR7 knockout also produced an anxiety-like phenotype; in addition there is

evidence for cognitive deficits in these animals as well (O'Connor et al., 2010). The analysis of receptor function in different brain areas, excitatory and inhibitory synapses, and disease models is required to determine their therapeutic value. The results of this study emphasize the importance of mGluR7 and mGluR8 as useful targets to regulate amygdala output, which is not only important for pain but also for other disorders associated with amygdala dysfunction.

Highlights

- mGluR7 acts as a gatekeeper to regulate information flow to the output nucleus.
- mGluR7 acts through disinhibition (suppression of feedforward inhibition)
- mGluR7 gatekeeper function is lost in a pain model.
- mGluR8 inhibits excitatory transmission only in the pain state.
- Both subtypes act presynaptically to amygdala output neurons

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Figure 1. Synaptic transmission between BLA and CeLC

(A) Coronal brain slices containing the amygdala were obtained from normal rats and from arthritic rats. (B) Connections between BLA and CeLC include direct glutamatergic input to the CeLC, direct GABAergic input to the CeLC, and indirect GABAergic input provided by neurons of the intercalated cell masses (ITC). Stimulation electrode was positioned in the BLA. Patch-clamp recordings were made from CeLC neurons. (C) Monosynaptic excitatory postsynaptic currents (EPSCs, recorded at -70 mV) followed high-frequency stimulation (20 Hz; 6 individual traces each; left), showed constant latency (2x threshold stimulation intensity; 10 individual traces; middle), and were blocked by NBQX (10 μ M; right). (D) Monosynaptic inhibitory postsynaptic currents (IPSCs, recorded at 0 mV) followed high-frequency stimulation (20 Hz), had constant latency (10 traces), and were not affected by NBQX. (E) Polysynaptic inhibitory postsynaptic currents (IPSCs, recorded at 0 mV) did not consistently follow high-frequency stimulation (20 Hz), had variable latencies (10 traces), and were partially inhibited by NBQX, suggesting that polysynaptic IPSCs are driven by non-NMDA receptors. (F) Bar histograms show the number of polysynaptic IPSCs evoked by the 1st, 2nd and 3rd stimulus of a high-frequency stimulus train (HFS, 20 Hz) averaged across the sample of neurons ($n = 20$; *** $P < 0.001$, compared to 1st stimulus [no failure], Bonferroni posttests). (G) Bar histograms show average latencies of EPSCs and IPSCs ($n = 10$ neurons, ** $P < 0.01$, compared to 1st stimulus, Bonferroni posttests). (H) Bar histograms show effect of NBQX (10 μ M) expressed as percent of predrug values (set to 100%; $n = 10$ neurons, *** $P < 0.001$, compared to predrug, paired t-test). Symbols and error bars represent means $\pm$ SE.

Figure 2. Differential effects of mGluR7 and mGluR8 on excitatory synaptic transmission in CeLC neurons

(A) Cumulative concentration–response relationships for a selective mGluR7 agonist (AMN082). AMN082 increased EPSC peak amplitudes under normal conditions but had significantly weaker effects in slices from arthritis rats ($n = 5$ neurons in each group). (B) Cumulative concentration–response relationships for a selective mGluR8 agonist (DCPG). DCPG inhibited EPSCs more potently in slices from arthritic rats than in slices from normal rats ($n = 4$ neurons in each group). (A, B) Symbols represent means $\pm$ SE. Sigmoid curves were fitted through the data points using nonlinear regression analysis (GraphPad Prism software; see Methods). Traces on the right show the average of 6–8 EPSCs evoked before

(Predrug) and during application of different drug concentrations in individual CeLC neurons in slices from normal and arthritic rats. Stimulus intensity, 0.9 mA. Scale bars, 50 pA, 10 ms. *, **, $P < 0.05$, 0.01 (Bonferroni posttests, arthritis compared to normal; see text for details).

Figure 3. Effects of mGluR7 and mGluR8 on excitatory transmission and synaptically evoked spiking in CeLC neurons under normal conditions and in a model of arthritic pain

A selective mGluR7 agonist (AMN082, 10 μ M) significantly enhanced input-output (I/O) functions of monosynaptic EPSCs (**A**) and the number of synaptically evoked action potentials (**B**) in CeLC neurons in slices from normal rats ($n = 10$ neurons). AMN082 had no effect on EPSCs (**C**) and on synaptically evoked action potentials (**D**) in slices from arthritic rats ($n = 10$ neurons). A selective mGluR8 agonist (DCPG, 10 nM) had no effect on EPSCs (**E**) and synaptically evoked spiking (**F**) in slices from normal rats ($n = 8$ neurons). DCPG significantly decreased I/O functions of monosynaptic EPSCs (**G**) and the number of synaptically evoked action potentials (**H**) in CeLC neurons in slices from arthritic rats ($n = 6$ neurons). (**A**, **C**, **E**, **G**) I/O relationships were obtained in voltage-clamp by measuring peak amplitudes of EPSCs as a function of afferent fiber stimulus intensity in the absence and presence of AMN082 or DCPG. Individual traces show EPSCs (average of 8–10) evoked with a stimulation intensity of 0.9 mA. Scale bars, 50 pA, 10 ms. (**B**, **D**, **F**, **H**) Bar histograms show number of action potentials (spikes) in current-clamp (initial membrane potential, -60 mV) evoked by 10 synaptic stimuli (averaged across the sample of neurons). Individual traces are synaptically evoked spikes before (Predrug) and during drug application (10 stimuli). Scale bars, 20 mV, 5 ms. Symbols and error bars represent means $\pm$ SE. *,** $P < 0.05$, 0.01 (Bonferroni posttest in A, C, E, G; paired t-test in B, D, F, H).

Figure 4. The effects of mGluR7 and mGluR8 agonists are blocked by a group III mGluR antagonist

(A) A selective group III mGluR antagonist (MAP4, 500 μ M) had no effect on its own but blocked the facilitatory effect of AMN082 (10 μ M) on I/O function of EPSCs (see Fig. 3) in slices from normal rats ($n = 5$ neurons). (B) MAP4 (500 μ M) also blocked the inhibitory effect of DCPG (10 nM) on EPSCs evoked in slices from arthritic rats ($n = 4$ neurons). (A, B) Symbols represent means $\pm$ SE. Traces show the average of 6–8 EPSCs evoked before and during drug application in individual CeLC neurons. Stimulus intensity, 0.9 mA. Scale bars, 50 pA, 10 ms.

Figure 5. Effects of mGluR7 and mGluR8 agonists on spontaneous and miniature EPSCs in CeLC neurons

(A–C) Cumulative distribution analysis of frequency (A) and amplitude (B) of spontaneous EPSCs (sEPSCs) in slices from normal rats. AMN082 (10 μ M) caused a significant shift toward smaller inter-event intervals ($P < 0.05$, Kolmogorov–Smirnov [K-S] test), thus increasing the mean frequency significantly ($n = 5$ neurons, $P < 0.05$, paired t-test), but had no effect on sEPSC amplitude. (C) Current traces of sEPSCs in an individual CeLC neuron before (Predrug) and during AMN082. (D–F) AMN082 had no significant effect on frequency (D) and amplitude (E) of miniature EPSCs (mEPSCs) ($n = 6$ neurons, $P > 0.05$, K-S test for cumulative distribution and paired t-test for mean frequency and amplitude). (F)

Current traces of mEPSCs (in TTX, 1 μ M) recorded in an individual CeLC neuron before (Predrug) and during AMN082. (**G–I**) DCPG (10 nM) decreased frequency (**G**), but not amplitude (**H**), of sEPSCs significantly ($n = 7$ neurons, cumulative frequency distribution, $P < 0.05$, K-S test; mean frequency, $P < 0.01$, paired t-test). Recordings were made in slices from arthritic rats. (**I**) Current traces of sEPSCs recorded in one CeLC neuron before (Predrug) and during DCPG. (**J–L**) DCPG significantly decreased frequency (**J**), but not amplitude (**K**) of mEPSCs ($n = 5$ neurons, cumulative frequency distribution, $P < 0.05$, K-S test; mean frequency, $P < 0.05$, paired t-test). (**L**) Current traces of mEPSCs in the presence of TTX (1 μ M) in one CeLC neuron before (Predrug) and during DCPG. Bar histograms show means $\pm$ SE. Scale bars for current traces, 10 pA, 2 s.

Figure 6. Effects of mGluR7 and mGluR8 agonists on inhibitory synaptic transmission in CeLC neurons under normal conditions and in a model of arthritic pain

I/O relationships for IPSCs were obtained in voltage-clamp at 0 mV (same analysis as in Fig. 3 for EPSCs) in slices from normal rats (A, C, E, G) and from arthritic rats (B, D, F, H). (A) AMN082 (10 μ M) significantly decreased polysynaptic inhibitory transmission in slices from normal rats ($n = 5$ neurons). (B) AMN082 had no effect on polysynaptic IPSCs in slices from arthritic rats ($n = 5$ neurons). (C, D) AMN082 (10 μ M) had no significant effect on monosynaptic IPSCs under normal conditions ($n = 5$ neurons, C) and in the arthritis model ($n = 5$ neurons, D). (E, F) DCPG (10 nM) had no significant effect on polysynaptic IPSCs in slices from normal rats ($n = 4$ neurons, E) and from arthritic rats ($n =$

4 neurons, **F**). (**G, H**) DCPG (10 nM) had no effect on monosynaptic IPSCs in slices from normal rats (n = 4 neurons, **G**) and in slices from arthritic rats (n = 4 neurons, **H**). Symbols and error bars represent means $\pm$ SE. Individual traces show IPSCs evoked in individual CeLC neurons before (Predrug) and during drug application. Stimulus intensity, 0.9 mA. Scale bars, 50 pA, 10 ms. *,*** P < 0.05, 0.001 (compared to predrug values, Bonferroni posttests).

Figure 7. Effects of an mGluR7 agonist on spontaneous and miniature IPSCs in CeLC neurons (A–C) Cumulative distribution analysis of frequency (A) and amplitude (B) of spontaneous IPSCs (sIPSCs). AMN082 (10 μ M) decreased frequency (A), but not amplitude (B), of sIPSCs significantly (cumulative frequency distribution, $P < 0.05$, K-S test; mean frequency, $P < 0.01$, paired t-test; $n = 4$ neurons). (C) Current traces of sIPSCs in an individual CeLC neuron before (Predrug) and during AMN082. (D–E) AMN082 (10 μ M) also decreased frequency (D), but not amplitude (E), of mIPSCs significantly (cumulative frequency distribution, $P < 0.05$, K-S test; mean frequency, $P < 0.05$, paired t-test; $n = 4$ neurons). (F) Current traces of mIPSCs (in TTX, 1 μ M) recorded in an individual CeLC neuron before (Predrug) and during AMN082. Bar histograms show means $\pm$ SE. Scale bars for current traces, 10 pA, 2 s. Recordings were made in slices from normal rats.

Figure 8. Blockade of GABA_A receptors occludes the facilitatory effects of mGluR7 activation on CeLC output

Current-clamp recordings (initial holding potential, -60 mV) of synaptically evoked action potentials in CeLC in slices from normal rats. **(A)** Traces show synaptically evoked responses (10 stimuli) in an individual CeLC neuron before (Predrug) and during application of a GABA_A receptor antagonist (bicuculline, 10 μ M) and during coapplication of bicuculline with AMN082 (10 μ M). Scale bars, 20 mV, 20 ms. **(B)** Bar histograms (mean $\pm$ SE) show that bicuculline (10 μ M) increased the number of synaptically evoked action potentials in CeLC neurons significantly ($n = 4$ neurons, $P < 0.05$, Bonferroni posttests).

Coapplication of AMN082 (10 μ M) with bicuculline had no significant (n.s.) additional effect ($P > 0.05$, Bonferroni posttest).