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Amygdala group II mGluRs mediate the inhibitory effects of systemic group II mGluR activation on behavior and spinal neurons in a rat model of arthritis pain

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HIGHLIGHTS

- Systemic mGluR2/3 agonist application inhibits pain behaviors and spinal nociceptive activity.
- Amygdala mGluR2/3 mediate inhibitory effects of systemic mGluR2/3 agonist application.
- Amygdala mGluR2/3 activation mimics effects of systemic mGluR2/3 agonist application.
- Data link amygdala to activity to spinal nociceptive processing.
- Amygdala mGluR2/3 play important role in pain modulation.

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ABSTRACT

The amygdala plays a critical role in emotional-affective aspects of behaviors and pain modulation. The central nucleus of amygdala (CeA) serves major output functions, and neuroplasticity in the CeA is linked to pain-related behaviors in different models. Activation of $G_{i/o}$-coupled group II metabotropic glutamate receptors (mGluRs), which consist of mGluR2 and mGluR3, can decrease neurotransmitter release and regulate synaptic plasticity. Group II mGluRs have emerged as targets for neuropsychiatric disorders and can inhibit pain-related processing and behaviors. Surprisingly, site and mechanism of antinociceptive actions of systemically applied group II mGluR agonists are not clear. Our previous work showed that group II mGluR activation in the amygdala inhibits pain-related CeA activity, but behavioral and spinal consequences remain to be determined. Here we studied the contribution of group II mGluRs in the amygdala to the antinociceptive effects of a systemically applied group II mGluR agonist (LY379268) on behavior and spinal dorsal horn neuronal activity, using the kaolin/carrageenan-induced knee joint arthritis pain model. Audible and ultrasonic vocalizations (emotional responses) and mechanical reflex thresholds were measured in adult rats with and without arthritis (5–6 h postinduction). Extracellular single-unit recordings were made from spinal dorsal horn wide dynamic range neurons of anesthetized (isoflurane) rats with and without arthritis (5–6 h postinduction). Systemic (intraperitoneal) application of a group II mGluR agonist (LY379268) decreased behaviors and activity of spinal neurons in the arthritis pain model but not under normal conditions. Stereotaxic administration of LY379268 into the CeA mimicked the effects of systemic application. Conversely, stereotaxic administration of a group II mGluR antagonist (LY341495) into the CeA reversed the effects of systemic application of LY379268 on behaviors and dorsal horn neuronal activity in arthritic rats. The data show for the first time that the amygdala is the critical site of action for the antinociceptive behavioral and spinal neuronal effects of systemically applied group II mGluR agonists.

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1. Introduction

Pain is a multidimensional experience that affects millions of people worldwide, and it is characterized by different components interacting with each other, including emotional, cognitive, sensory and motor functions. Because of the complexity of the condition, the choice of an efficient treatment remains still a challenge for physicians. The amygdala is part of the limbic brain, and is critically involved in the emotional-affective aspects of disorders such as anxiety, addiction, and pain. The amygdala plays an important role in pain states by attaching emotional significance to sensory inputs from brain regions, such as thalamus and cortex (Neugebauer et al., 2004; Thompson and Neugebauer, 2017), and then connecting to descending pain modulatory systems and others (DeBerry et al., 2015). Evidence from human clinical studies also links amygdala function to pain conditions (Kulkarni et al., 2007; Simons et al., 2014). The relevant amygdala circuitry consists of the basolateral complex (BLA), the central nucleus (CeA) and interposed between them the intercalated cell clusters (ITC). The CeA receives multimodal information from the BLA network and more specific nociceptive inputs via the spino-parabrachio-amygdaloid tract (Bernard and Besson, 1990; Neugebauer, 2015; Thompson and Neugebauer, 2017). The CeA serves as a major amygdala output region and its hyper-activity has been mechanistically linked to pain behaviors and pain modulation in preclinical studies.

The glutamatergic system is important for physiological processes such as cognition, emotions, and memory, but overactive glutamatergic neurotransmission has been observed in different brain areas under pathological conditions, including pain states (Bleakman et al., 2006; Boccella et al., 2019; Neugebauer, 2007; Wozniak et al., 2012; Zhou et al., 2011). Central and peripheral glutamatergic signaling includes ionotropic receptors (ligand gated ion channels) and metabotropic receptors (G-protein coupled mGluRs classified into three groups). $G_{i/o}$-coupled group II mGluRs, which include mGluR2 and mGluR3, are widely expressed throughout the nervous system including regions as thalamus, amygdala, hippocampus and striatum (Imre, 2007), and have been associated with neuropsychiatric and neurological disorders, such as Parkinson's (Dickerson and Conn, 2012) and Alzheimer's (Kim et al., 2014) diseases, drug addiction (Moussawi and Kalivas, 2010), depression, anxiety (Fell et al., 2011), psychosis (Muguruza et al., 2016; O'brien et al., 2014; Patil et al., 2007) as well as pain conditions (Chiechio, 2016; Mazzitelli et al., 2018; Montana and Gereau, 2011; Neugebauer, 2007). Activation of group II mGluRs can decrease neurotransmitter release in the synaptic cleft and regulate synaptic plasticity (Di Menna et al., 2018; Nicoletti et al., 2011). Therefore, pharmacological agents acting on mGluR2/3 emerged as potential strategy for therapeutic treatment.

Importantly, mGluR2 and mGluR3 are also expressed in different regions serving pain-related functions (Gu et al., 2008; Wright et al., 2013), and evidence from preclinical studies suggests their significant role in nociceptive processing and pain modulation (Mazzitelli et al., 2018; Neugebauer, 2007; Neugebauer and Carlton, 2002). Surprisingly, the main site of action of the beneficial effects of group II mGluR agonists related to pain is actually not clear (Mazzitelli et al., 2018). Systemic (Johnson et al., 2017; Simmons et al., 2002) or peripheral (subcutaneous) (Yang and Gereau, 2002, 2003) activation of group II mGluRs had antinociceptive behavioral effects in neuropathic and inflammatory pain models, whereas the modulation of group II mGluRs at spinal level by intrathecal injection showed mixed behavioral effects (Mazzitelli et al., 2018; Zhang et al., 2009). Previous work from our lab showed inhibitory effects of group II mGluR agonists (Li and Neugebauer, 2006) on neuronal activity and excitatory synaptic transmission in CeA neurons (Han et al., 2006; Kiritoshi and Neugebauer, 2015). In this study, we investigated the antinociceptive effects of group II mGluR activation in the amygdala and their contribution to the effects of a systemically applied group II mGluR agonist on pain-related behaviors and spinal nociceptive processing.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (180–350 g) with unrestricted access to food and water were housed in a temperature-controlled room under a 12 h day/night cycle. On the day of the experiment, animals were transferred from the animal facility to the laboratory to allow acclimation for about 1 h. Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas Tech University Health Sciences Center and conform to the guidelines of the International Association for the Study of Pain (IASP) and National Institutes of Health (NIH). Rats were randomly assigned to the different experimental groups. Experiments were done in a blinded fashion.

2.2. Arthritis pain model

A monoarthritis was induced to the left knee as described in detail previously (Neugebauer et al., 2007). Rats were briefly anesthetized with isoflurane (2–3%) for the separate injections of kaolin (4% in sterile saline, 100 μl) and carrageenan (2% in sterile saline, 100 μl) into the joint cavity (K/C arthritis) followed by repetitive flexions and extensions of the leg for 5 min (min) after each injection. This well-established paradigm produces an aseptic use-dependent mono-arthritis with damage to the cartilage, and localized inflammation of only one knee joint. K/C arthritis develops rapidly within hours and persists for weeks, and it is associated with pain behaviors and neural activity changes in the peripheral and central nervous system (Kiritoshi and Neugebauer, 2018; Li and Neugebauer, 2004). Naïve rats undergoing similar handling as arthritic rats but without intraarticular injection, were used as control because data from our previous studies found no differences between naïve and sham (saline injection or needle insertion) rats > 2 h postinjection, and neither of these control groups developed neuroplasticity and pain behaviors observed in the arthritis model, justifying the use of naïve rats as an appropriate control for the pain model (Gregoire and Neugebauer, 2013; Kiritoshi and Neugebauer, 2015; Neugebauer et al., 2003).

2.3. Pain-related behaviors

2.3.1. Vocalizations

Vocalizations in the audible (20 Hz–16 kHz) and ultrasonic (25 ± 4 kHz) ranges were measured in naïve and arthritic rats, 5–6 h after the induction as in our previous studies (see “Arthritis pain model”) (Han and Neugebauer, 2005; Kiritoshi et al., 2016; Neugebauer et al., 2007; Thompson et al., 2015). Rats were briefly anesthetized with isoflurane (2–3%, precision vaporizer, Harvard Apparatus) while being placed slightly restrained in a custom designed recording chamber (U.S. Patent 7,213,538) to ensure a fixed distance from the sound detectors. A microphone connected to a preamplifier was used to record audible vocalizations, and a bat detector connected to a filter and amplifier (UltraVox four-channel system; Noldus Information Technology) measured ultrasonic vocalizations. After recovery from the brief anesthesia and after habituation to the chamber for 30 min, vocalizations were evoked by brief (10 s) innocuous (500 g/30 mm^2) and noxious (1500 g/30 mm^2) stimuli applied to the left knee joint (site of arthritis induction) using a calibrated forceps equipped with a force transducer whose output was displayed in grams on an LED screen. Vocalizations were recorded for 1 min and analyzed using Ultravox 2.0 software (Noldus Information Technology). Vocalizations were measured before and after drug or vehicle application (see 2.5).

2.3.2. Mechanical (hyper-)sensitivity

Hindlimb withdrawal thresholds were evaluated after the vocalization assays while the rat was in the recording chamber as described

previously (Neugebauer et al., 2007). A calibrated forceps with force transducer (see 2.3.1) was used to compress the left knee joint with a stimulus of continuously increasing intensity until a withdrawal reflex was evoked. The average value from 2 to 3 trials was used to calculate the withdrawal threshold, which was defined as the force required for evoking a reflex response.

2.4. In vivo electrophysiology

Extracellular single-unit recordings were performed from wide dynamic range neurons in the lumbar region (L2-L4) of the spinal dorsal horn in normal naïve and arthritic rats (5–6 h post arthritis induction) as described previously (Di Cesare Mannelli et al., 2015; Pernia-Andrade et al., 2009). Each experimental group included 5–6 rats, and only one neuron was recorded in each animal.

2.4.1. Surgical preparation for electrophysiology

On the day of the experiment, the rat was anesthetized with isoflurane (2–3%, precision vaporizer, Harvard Apparatus). The spinal segments L2-L4 were exposed by laminectomy, and the dura was carefully removed. Animal were then secured in a stereotaxic frame (David Kopf Instruments) supported by clamps attached to the vertebral processes on both sides. The exposed area of the spinal cord was first framed by agar and then filled with mineral oil. Body temperature was maintained at 37 °C by using a temperature-controlled blanket system. A glass insulated carbon filament electrode (4–6 MΩ) was inserted perpendicularly to the spinal cord surface using a microdrive (David Kopf Instruments) to record the activity of the dorsal horn neurons. Anesthesia was maintained with isoflurane (2%, precision vaporizer) throughout the experiment.

2.4.2. Extracellular single-unit recordings and data analysis

Extracellular single unit recordings were made from wide dynamic range (WDR) neurons in the dorsal horn (L2-L4). WDR neurons respond to a range of stimuli of innocuous and noxious intensities (D'Mello and Dickenson, 2008). Here we used light touch applied with a painter's brush and innocuous and noxious compression with a calibrated forceps (same as in 2.3.1 and 2.3.2). The recorded signals were amplified, band-pass filtered (300 Hz–3 kHz), and processed by a data acquisition interface (CED 1401 Plus). Spike2 software (version 4; CED) was used for spike sorting, data storage, and analysis of single-unit activity. Spike (action potential) size and configuration were monitored continuously.

After a neuron was identified, a template was created for the spikes of each individual neuron during an initial recording period of 5 min, capturing the waveform within set limits of variability for parameters such as amplitude, duration, and rise time using Spike2 software. Subsequent spikes of the neuron were matched to that template (spike sorting) and only spikes within the set limits of variability were counted as signals of that particular neuron. Only neurons were included in the study whose spike configuration matched the preset template and could be clearly discriminated from background noise throughout the experiment. Only neurons were included that were identified within a depth of 1200 μm from the dorsal surface of the spinal cord, had a receptive field on the ipsilateral knee and responded more strongly to noxious (1500 g/mm^2) than innocuous (500 g/mm^2) stimuli. Mechanical test stimuli (brushing the skin or joint compression with a calibrated forceps, see 2.1.3 and 2.3.2) were applied to the left knee joint for 10 s. Interstimulus interval was 30 s.

Neuronal activity was recorded as spikes/s. Spontaneous activity (in the absence of intentional stimulation) and evoked activity (mechanical stimuli) was measured every 10 min for a period of 30 min before and for a period of 90 min after vehicle or drug application (see 2.7.2). Neuronal activity was then analyzed off line. For evoked activity, three baseline responses of a set of stimuli consisting of brush, innocuous and noxious stimuli were calculated to represent pre-drug values. Net evoked activity was calculated by subtracting an ongoing activity preceding the mechanical stimulus from the total activity during stimulation. For each neuron, the evoked activity was calculated as a percent of the respective baseline levels to allow averaging across the sample of neurons for each experimental condition and intervention.

2.4.3. Verification of the recording site

At the end of each experiment, the recording site was marked by an electrical current (500.0 μA, 5 s), and the animal was subsequently euthanized with an overdose of pentobarbital sodium (FATAL-Plus, 125 mg/kg, intravenously). For tissue fixation, the spinal cord was kept in 4% paraformaldehyde at 4 °C overnight. Spinal tissues were then transferred to 30% sucrose in 0.1 M phosphate buffer and kept at 4 °C until sectioning. Sections (20 μm-thick) were obtained using a cryostat (Vibratome UltraPro 5000). The recording site was identified by macroscopic inspection, correlating the depth or recording indicated on the micromanipulator with the spinal cord tissue. The values were then plotted on a graph showing the depth of the electrode tip from the dorsal surface of the spinal cord.

2.5. Drugs and drug application (systemic or intra-amygdala)

The following drugs were purchased from Tocris Bioscience (R&D Systems, Minneapolis, MN) and used in the experiments: LY379268, (1*R*,4*R*,5*S*,6*R*)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid disodium salt, a group II mGluR agonist; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid disodium salt, a group II mGluR antagonist. For systemic (intraperitoneal, i. p.) injections, LY379268 was dissolved in 0.9% isotonic saline, which served as vehicle, for a total volume of 1 ml. For stereotaxic drug administration by microdialysis, LY341495 saline stock solution was diluted in artificial cerebrospinal fluid (ACSF; in mM: 117 NaCl, 4.7 KCl, 1.2 NaH2PO4, 2.5 CaCl2, 1.2 MgCl2, 25 NaHCO3, and 11 glucose) to the final concentration in the microdialysis probe. Based on our previous studies, a 100-fold greater drug concentration inside the probe is needed to achieve the intended target concentration in the tissue due to the concentration gradient across the dialysis membrane and diffusion into the brain tissue (Fu and Neugebauer, 2008; Ji et al., 2013; Kiritoshi et al., 2016). The dose for systemic application was selected based on published data for LY379268 i. p. injection (Jones et al., 2005; Simmons et al., 2002). The concentrations for stereotaxic administration of LY379268 and LY341495 by microdialysis were determined based on published in vitro study from our lab (Kiritoshi and Neugebauer, 2015). According to our previous studies a 100-fold greater drug concentration for microdialysis drug application inside the probe was needed to have the same drug effects as the drug effects of the target concentration in the tissue due to the concentration gradient across the dialysis membrane and diffusion into the brain tissue (Fu and Neugebauer, 2008; Ji et al., 2013; Kiritoshi et al., 2016; Thompson et al., 2015).

Stereotaxic drug application by microdialysis is a well-established procedure for delivering the drug into a specific brain region (Kim et al., 2017; Kiritoshi et al., 2016; Thompson et al., 2015). Drug application by microdialysis was chosen because it has several advantages. There is no volume effect; drugs can be applied for an extended period and concentrations reach as steady state. Rats were anesthetized with isoflurane (2–3%) and placed in a stereotaxic frame (David Kopf Instruments). For drug applications into the CeA of awake behaving animals, a guide cannula (CMA/Microdialysis, Solna, Sweden) was implanted stereotaxically into the right CeA the day before the experiment, using the following coordinates: 2.5 mm caudal to bregma, 4 mm lateral to midline, and 6.5 mm deep. The cannula was fixed to the skull with dental acrylic (Plastic One, Roanoke, VA). Bacitracin ointment was applied to the exposed tissue to prevent infection. On the day of the experiment (next day), a microdialysis probe (CMA/Microdialysis 11) extending 1 mm beyond the cannula was inserted and connected to an infusion pump (Harvard Apparatus, Holliston, MA)

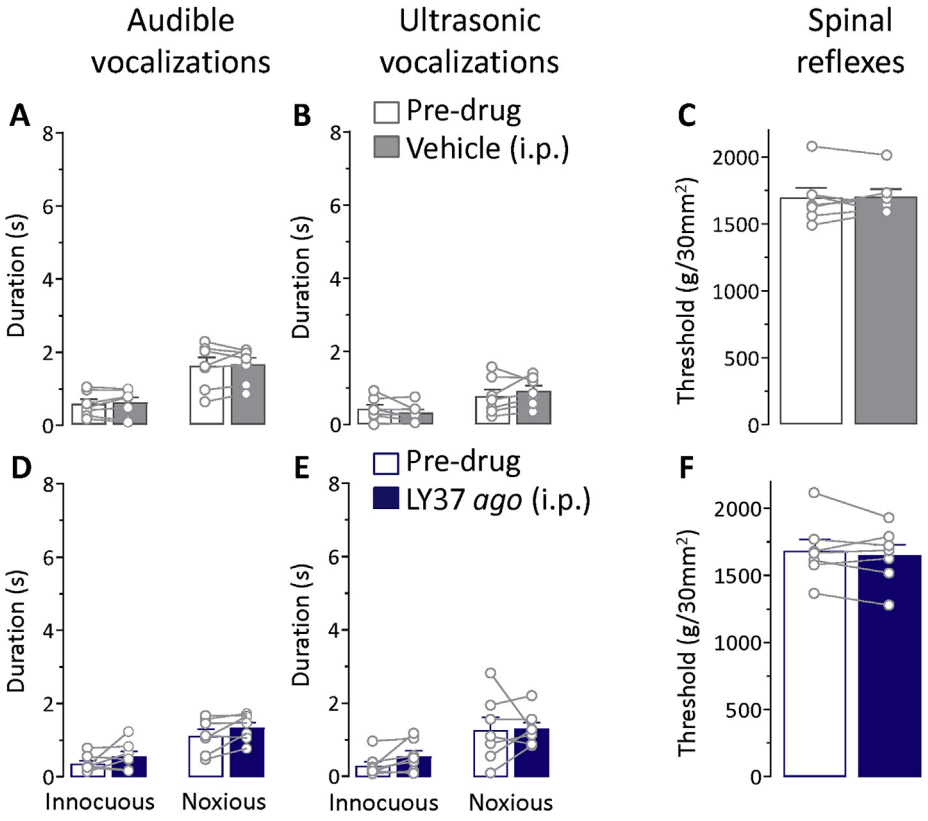

Fig. 1. Under normal conditions, systemically applied LY379268 (LY37 *ago*) had no behavioral effects. In normal naïve rats, a group II mGluR agonist (LY379268, 5 mg/kg, i. p., n = 7 rats) or vehicle (i.p., n = 7 rats) had no significant effect on audible (**A, D**) and ultrasonic (**B, E**) vocalizations evoked by innocuous (500 g/30 mm^2) and noxious (1500 g/30 mm^2) mechanical stimuli, and on hindlimb withdrawal thresholds (**C, F**). Mechanical stimuli were applied to the left knee joint with a calibrated forceps (see Methods). Values 30 min after drug/vehicle application were compared to pre-drug values using a paired *t*-test ($p > 0.05$). Bar histograms show mean ± SEM.

with polyethylene tubing. For drug applications into the CeA of anesthetized animals in the electrophysiology experiments (spinal recording), a microdialysis probe was inserted into the CeA using the same coordinates as in the behavioral experiments (depth of tip was 7.5 mm). Drugs or vehicle (ASCF) were applied for 40 min at 5 µl/min. In experiments where the effect of intra-CeA antagonist administration on systemic agonist application was tested, the stereotaxic administration started 10 min before the systemic injection (see 2.7.1).

2.6. Histological verification of microdialysis probe location

Locations of the tips of the microdialysis probes were verified histologically after experiments. Rats were euthanized with FATAL-plus (125 mg/kg, intravenously) followed by decapitation using a small guillotine (Harvard Apparatus Decapitator). Brains were rapidly removed and submerged in 4% paraformaldehyde and kept at 4 °C overnight. Brain tissues were then transferred to 30% sucrose in 0.1 M phosphate buffer and kept at 4 °C until sectioning. Sections were cut at 30 µm using a cryostat (Vibratome UltraPro 5000), mounted on gel-coated glass slides, and stained with hematoxylin and eosin (H&E), coverslipped, and then analyzed under the microscope. The locations of the microdialysis tips were identified from the slides and plotted on standard diagrams.

2.7. Experimental protocol

2.7.1. Behavioral studies

Behavioral tests (see 2.3) were done in naïve and arthritic rats (5–6 h after the induction, see 2.2). To determine the effect of systemic application (i.p.) of the mGluR2/3 agonist (LY379268) on pain-related behaviors, the tests were performed before and 30 min after drug or vehicle injection. In order to investigate the contribution of the amygdala to the behavioral effects of the systemically applied group II mGluR agonist, a group II mGluR antagonist was administered stereotaxically into the right CeA amygdala by microdialysis (see 2.5) for 40 min, starting 10 min before systemic drug (or vehicle) application. To establish the effect of the mGluR2/3 activation into the amygdala, behavioral assays were performed 20 min after starting mGluR2/3 agonist application by microdialysis, and repeated 15 min after starting the stereotaxically administration of the combination of the group II mGluRs agonist and antagonist. Rats were randomly assigned to a treatment group.

2.7.2. Electrophysiology

Extracellular recordings of spinal dorsal horn WDR neurons (see 2.4) were done in naïve and arthritic rats (5–6 h after the arthritis induction, see 2.2). To determine the effect of systemic application (i.p.) of the group II mGluR agonist (LY379268) on neuronal activity, LY379268 or vehicle was injected after a 30 min baseline recording period (three sets of stimuli consisting of brush, innocuous and noxious stimuli, separated by 10 min each) and the drug effect was evaluated for 90 min. In order to explore the contribution of the amygdala to the spinal effects of a systemically applied group II mGluR agonist effect on the spinal evoked activity, a group II mGluR antagonist was administrated stereotaxically into the CeA by microdialysis (see 2.5) for 40 min, starting 10 min before systemic drug (or vehicle) application. Rats were randomly assigned to receive a given treatment.

2.8. Data and statistical analysis

All averaged values are presented as means ± SEM. GraphPad Prism 7.0 software (Graph-Pad Software, San Diego, CA) was used for all statistical analyses. Statistical significance was accepted at the level $P < 0.05$. Repeated measures one-way ANOVA with Bonferroni post hoc tests was used for multiple comparisons, and paired and unpaired t-tests were used for comparison of two sets of data that had Gaussian distribution and similar variance as indicated.

3. Results

3.1. Systemic LY379268 has no effect on pain-like behaviors in naïve rats

Vocalizations in the audible and ultrasonic range were evoked by the application of innocuous and noxious mechanical stimuli to the left knee for 10 s. In normal naïve rats, intraperitoneal (i.p.) injection of vehicle (n = 7 rats) did not significantly affect audible (Fig. 1A) and ultrasonic (Fig. 1B) vocalizations evoked by brief (10 s) innocuous (Fig. 1A, p = 0.5340, t = 0.6596; Fig. 1B, p = 0.2200, t = 1.369; paired *t*-test) and noxious (Fig. 1A, p = 0.6684, t = 0.4502; Fig. 1B, p = 0.2632, t = 1.234; paired *t*-test) stimuli compared to the pre-drug values. Systemic application of a group II mGluR agonist (LY379268, 5 mg/kg, i. p.; n = 7 rats) had no effect, 30 min after injection, on audible (Fig. 1D) and ultrasonic (Fig. 1E) vocalizations elicited by innocuous (Fig. 1D, p = 0.2146, t = 1.388; Fig. 1E, p = 0.0899, t = 2.02; paired *t*-test) and noxious (Fig. 1D, p = 0.0757, t = 2.145; Fig. 1E, p = 0.9206, t = 0.1039; paired *t*-test) stimuli under normal conditions.

Hindlimb withdrawal thresholds were measured by mechanical compression of the knee joint with a continuously increasing force, using a calibrated forceps, until a withdrawal reflexes was evoked (see 2.3). Systemic application of vehicle (n = 7 rats) or LY379268 (5 mg/kg, i. p.; n = 7 rats) had no significant effect on mechanical reflex thresholds (Fig. 1C and F) compared to pre-drug values (Fig. 1C, p = 0.8637, t = 0.1791; Fig. 1F, p = 0.4043, t = 0.897; paired *t*-test) in normal rats.

3.2. Systemic LY379268 inhibits pain-like behaviors in arthritic rats, and this effect is blocked by intra-amygdalar LY341495

In the arthritis pain model (5 h postinduction), systemic application of vehicle (n = 7 rats) had no significant effect on vocalizations (Fig. 2A and B) evoked by innocuous (Fig. 2A, p = 0.0930, t = 1.996; Fig. 2B, p = 0.7110, t = 0.3886; paired *t*-test) and noxious (Fig. 2A, p = 0.1748, t = 1.539; Fig. 2B, p = 0.2436, t = 1.293; paired *t*-test) stimulation of the arthritic knee joint, compared to pre-drug values. Systemic injection of a group II mGluR agonist (LY379268, 5 mg/kg, i. p.; n = 7 rats), significantly decreased, 30 min after the injection, audible (Fig. 2D) and ultrasonic (Fig. 2E) vocalizations evoked by brief (10 s) innocuous (Fig. 2D, p = 0.0019, t = 5.276; Fig. 2E, p = 0.0222, t = 3.061; paired *t*-test) and noxious (Fig. 2D, p = 0.0397, t = 2.618; Fig. 2E, p = 0.0227, t = 3.043; paired *t*-test) compression of the arthritic knee compared to pre-drug values. Hindlimb withdrawal thresholds evoked by mechanical compression of the arthritic knee with a continuously increasing force were not affected by systemic (i.p.) vehicle application (Fig. 2C; n = 7 rats; p = 0.1792, t = 1.521, paired *t*-test). Systemic application of LY379268 (5 mg/kg, i. p.) increased reflex thresholds in arthritic rats significantly (Fig. 2F; n = 7 rats; p < 0.0001, t = 23.75, paired *t*-test) compared to pre-drug values.

To determine the contribution of group II mGluRs in the amygdala to the inhibitory effect of systemically applied LY379268 in the arthritis pain model (5–6 h postinduction), a group II mGluR antagonist (LY341495, 100 μM, concentration in the microdialysis fiber, 40 min) was administered into the amygdala (right CeA) stereotaxically by microdialysis (Fig. 2G–I; see 2.7.1). LY341495 (n = 7 rats) administered into the CeA blocked the effect of systemically applied LY379268 (5 mg/kg, i. p.) on audible (Fig. 2G) and ultrasonic (Fig. 2H) vocalizations evoked by innocuous (Fig. 2G, p = 0.1949, t = 1.459; Fig. 2H, p = 0.0808, t = 2.097; paired *t*-test) and noxious (Fig. 2G, p = 0.2326, t = 1.328; Fig. 2H, p = 0.6400, t = 0.4922; paired *t*-test) stimulation of the arthritic knee joint compared to pre-drug values measured in the presence of ACSF in the microdialysis fiber. Intra-CeA administration of LY341495 (n = 7 rats) also blocked the effect of systemically applied LY379268 on hindlimb withdrawal thresholds (Fig. 2I, p = 0.9996, t = 0.0004783, paired *t*-test). Positions of the microdialysis probes in the CeA were verified histologically (Fig. S1A). The data suggest an important contribution of group II mGluRs in the CeA to the inhibitory effects of systemic group II agonist application in a pain condition.

3.3. Intra-amygdala LY379268 inhibits pain-like behaviors in arthritic rats

To determine the modulatory function of group II mGluRs in the amygdala on behaviors in the arthritis pain model (5–6 h post-induction), a group II mGluR agonist (LY379268, 10 μM, concentration in the microdialysis fiber, 20 min, n = 9 rats) was administered stereotaxically into the right CeA followed by co-administration of a group II mGluR antagonist (LY341495, 100 μM, concentration in the microdialysis fiber, 15 min) to confirm receptor-mediated effects. Intra-CeA administration of LY379268 decreased audible (Fig. 3A) and ultrasonic (Fig. 3B) vocalizations evoked by innocuous (Fig. 3A, p < 0.0001, $F_{2,16}$ = 26.81; Fig. 3B, p = 0.0165, $F_{2,16}$ = 8.446; repeated measures one-way ANOVA with Bonferroni posthoc tests) and noxious (Fig. 3A, p = 0.00021, $F_{2,16}$ = 17.48; Fig. 3B, p = 0.0095, $F_{2,16}$ = 7.134; repeated measures one-way ANOVA with Bonferroni posthoc tests) mechanical compression of the arthritic knee joint, compared to pre-drug values that were measured in the presence of ACSF in the microdialysis fiber. Intra-CeA administration of LY379268 also increased mechanical reflex thresholds (Fig. 3C, p < 0.0001, $F_{2,16}$ = 31.02; repeated measures one-way ANOVA with Bonferroni posthoc tests) compared to pre-drug ACSF. Co-administration of LY341495 blocked the inhibitory effects of LY379268 on audible (Fig. 3A, innocuous, p < 0.0001; noxious, p = 0.0002) and ultrasonic (Fig. 3B, innocuous, p = 0.0165; noxious p = 0.0024) vocalization, and spinal thresholds (Fig. 3C, p < 0.0001) in the arthritic pain condition. Positions of the microdialysis probes in the CeA were verified histologically (Fig. S1A).

3.4. Systemic LY379268 has no effect on spinal nociceptive activity in naïve rats

Extracellular single unit recordings were made from WDR neurons (n = 10 neurons in n = 10 rats) in the spinal dorsal horn (L2-L4) of normal naïve rats (no arthritis). This population of neurons responded more strongly to noxious than innocuous mechanical stimulation of the knee (brushing the skin or joint compression with a calibrated forceps for 10 s; see 2.4). Intraperitoneal (i.p.) injection of vehicle (n = 5 neurons in 5 rats) did not significantly change the responses of spinal neurons to brush, innocuous and noxious mechanical compression of the knee joint compared to pre-drug values (Fig. 4A, p = 0.8511, $F_{11,44}$ = 0.5587; Fig. 4B, p = 0.8024, $F_{11,44}$ = 0.6193; Fig. 4C, p = 0.6119, $F_{11,44}$ = 0.8296; repeated measures one-way ANOVA with Bonferroni posthoc tests). For the analysis, three responses before drug/vehicle application were averaged and set to 100%. Responses (spikes/s) were then expressed as percent of pre-drug values. Fig. 4D shows an individual example of the responses of a WDR neuron before and 30 min after vehicle. Systemic application of LY379268 (5 mg/kg, i. p.; n = 5 neurons in 5 rats) also had no significant effect on the neurons' responses to mechanical stimuli compared to the pre-drug baseline (Fig. 4A, p = 0.2624, $F_{11,44}$ = 1.289; Fig. 4B, p = 0.5475, $F_{11,44}$ = 0.9004; Fig. 4C, p = 0.8249, $F_{11,44}$ = 0.592; repeated measures one-way ANOVA with Bonferroni posthoc tests). Fig. 4E shows an individual example of the responses of a WDR neuron before and 30 min after LY379268 application. Recording depths in the spinal cord are shown in Fig. S1C.

3.5. Systemic LY379268 inhibits spinal nociceptive activity in arthritic rats, and this effect is blocked by intra-amygdalar LY341495

In arthritic rats (5–6 h postinduction), the responses of WDR neurons (n = 23 neurons in n = 23 rats) to brushing the skin and to innocuous and noxious compression of the arthritic knee with a calibrated forceps (for 10 s; see 2.4) were significantly increased compared to those (n = 10 neurons) recorded in normal naïve rats (Fig. 5A,

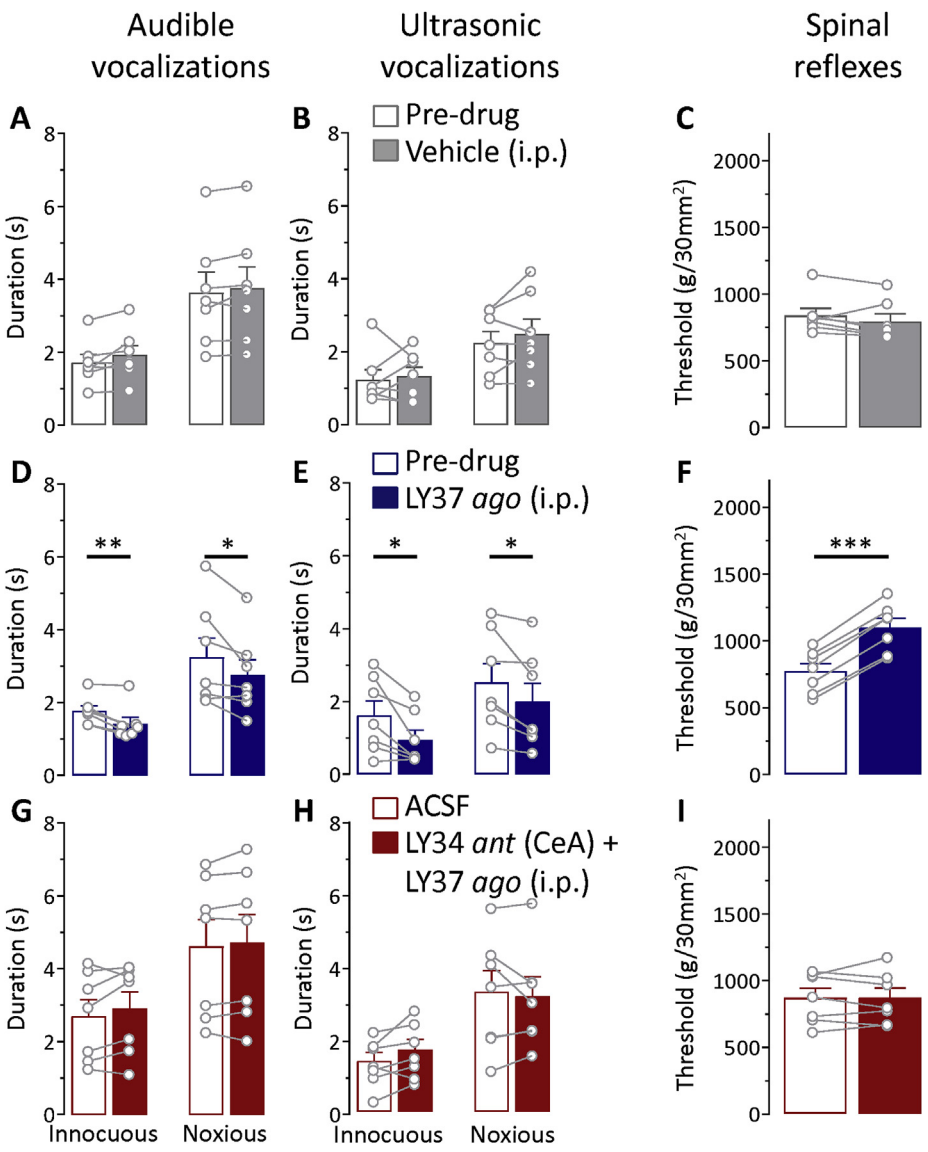

Fig. 2. In the arthritis pain model, systemically applied LY379268 (LY37 *ago*) had inhibitory behavioral effects that were blocked by intra-amygdalar administration of LY341495 (LY34 *ant*). In arthritic rats (5 h post-induction), a group II mGluR agonist (LY379268, 5 mg/kg, i. p., n = 7 rats; **D-F**), but not vehicle (i.p., n = 7 rats; **A-C**), decreased audible (**A**, **D**) and ultrasonic (**B**, **E**) vocalizations evoked by innocuous and noxious stimuli, and hindlimb withdrawal thresholds (**C**, **F**). Stereotaxic administration of a group II mGluR antagonist (LY341495, 100 μM, concentration in microdialysis probe, 40 min, n = 7 rats) into the CeA 10 min before systemic application of LY379268, blocked the inhibitory effect on audible (**G**) and ultrasonic (**H**) vocalizations and on reflex thresholds (**I**). Mechanical stimuli of different intensities were applied to the arthritic knee joint with a calibrated forceps (see Methods). Bar histograms show mean ± SEM; *, ***p < 0.05, 0.001, compared to pre-drug, paired *t*-test.

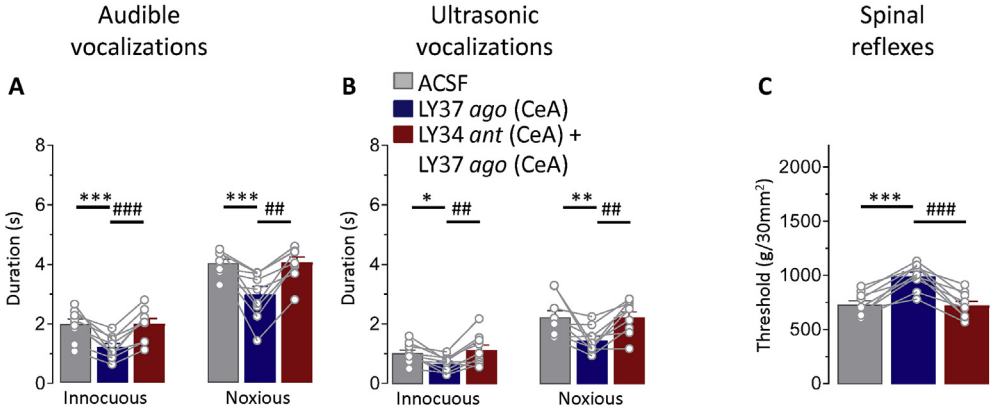

Fig. 3. In the arthritis pain model, intra-amygdalar administration of LY379268 (LY37 *ago*) had inhibitory behavioral effects that were blocked by intra-amygdalar co-administration of LY341495 (LY34 *ant*). In arthritic rats (5 h post-induction), stereotaxic administration of a group II mGluR agonist (LY379268, 10 μM, concentration in microdialysis probe, 20 min, n = 9 rats) into the CeA decreased audible (**A**) and ultrasonic (**B**) vocalizations evoked by innocuous and noxious stimuli, and hindlimb withdrawal thresholds (**C**). Stereotaxic administration of a group II mGluR antagonist (LY341495, 100 μM) together with LY379268 (10 μM) (15 min, n = 9 rats) blocked the inhibitory effect on audible (**A**) and ultrasonic (**B**) vocalizations and on reflex thresholds (**C**). Mechanical stimuli of different intensities were applied to the arthritic knee joint with a calibrated forceps (see Methods). Bar histograms show mean ± SEM; *, ***p < 0.05, 0.001, compared to pre-drug values (in ACSF), repeated measures one-way ANOVA with Bonferroni posthoc tests.

p = 0.0017, t = 3.435; Fig. 5B, p = 0.0017, t = 3.444; Fig. 5C, p = 0.0008, t = 3.714; unpaired *t*-test). Systemic (i.p.) application of vehicle (n = 5 neurons in 5 rats) had no significant effect on the responses to mechanical stimulation of the knee compared to pre-drug values (Fig. 5D, p = 0.0651, $F_{11,44}$ = 1.904; Fig. 5E, p = 0.8235, $F_{11,44}$ = 0.5938; Fig. 5F, p = 0.5433, $F_{11,44}$ = 0.9051; repeated measures one-way ANOVA with Bonferroni posthoc tests). Systemic application of LY379268 (5 mg/kg, i. p.; n = 6 neurons in 6 rats)

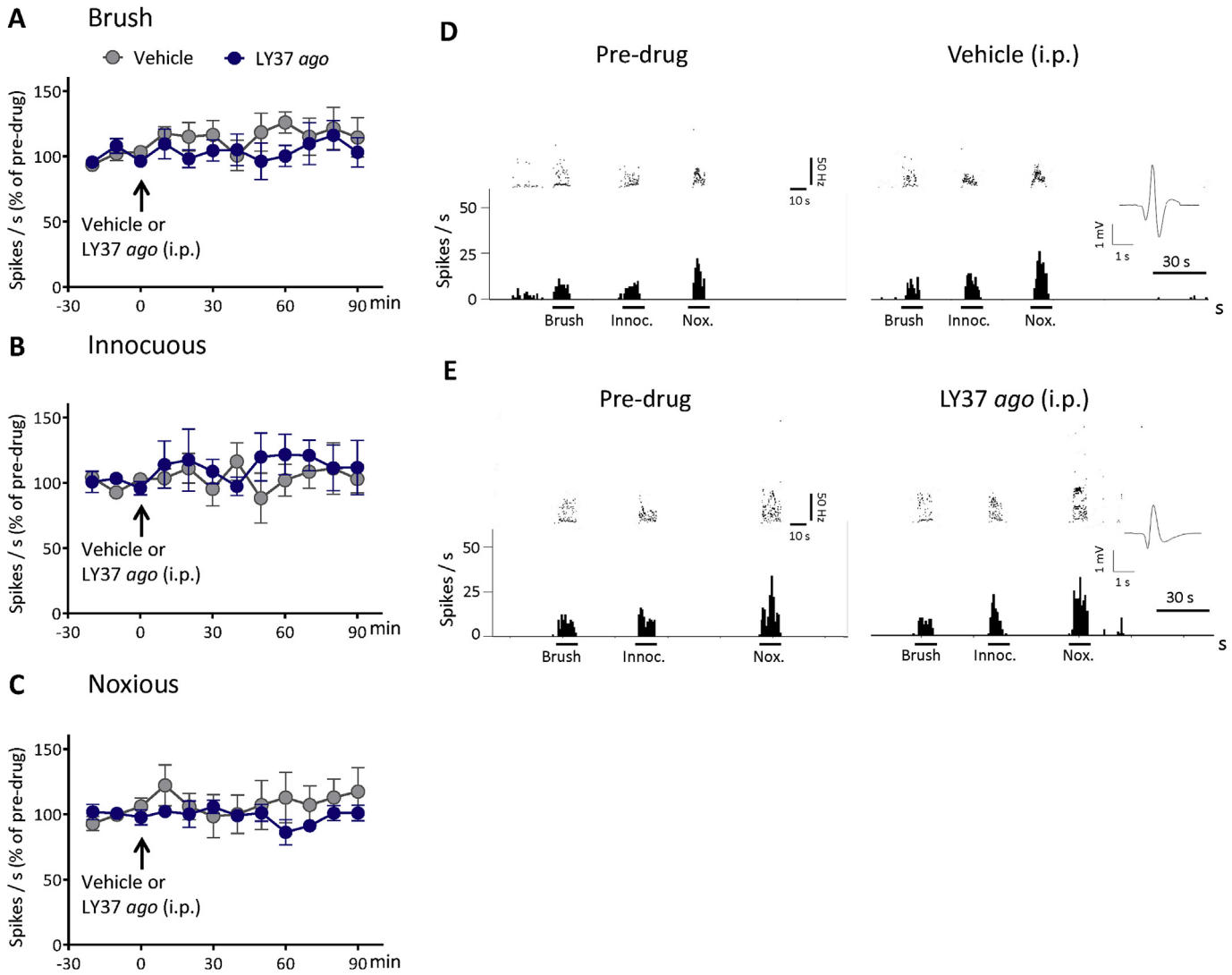

Fig. 4. Under normal conditions, systemically applied LY379268 (LY37 *ago*) had no effect on spinal dorsal horn neuronal activity. Extracellular single-unit recordings were made from WDR neurons in the spinal dorsal horn of anesthetized rats. (**A-C**) Summary and time course. In normal naïve rats, a group II mGluR agonist (LY379268, 5 mg/kg, i. p., n = 5 neurons, one neuron per rat) had no significant effect on the responses to cutaneous brush (**A**), and innocuous (**B**) and noxious (**C**) compression of the knee with a calibrated forceps (see Methods), compared to vehicle (i.p., n = 5 neurons, one neuron per rat). Symbols show means ± SEM. Values after drug/vehicle application were compared to pre-drug values using repeated measures one-way ANOVA with Bonferroni posthoc tests. Three responses before drug/vehicle application were averaged and set to 100%. (**D** and **E**) Peristimulus time histograms and raster plots show individual examples. (**D**) Evoked responses of a WDR neuron before (Pre-drug) and 30 min after systemic vehicle application. (**E**) Evoked responses of another WDR neuron before (Pre-drug) and 30 min after systemic LY379268 application.

significantly decreased the evoked responses compared to pre-drug baseline (Fig. 5D, p = 0.0198, $F_{11,55}$ = 2.398; Fig. 5E, p = 0.0001, $F_{11,55}$ = 4.327; Fig. 5F, p < 0.0001, $F_{11,55}$ = 5.794; repeated measures one-way ANOVA with Bonferroni posthoc tests). Fig. 5D–F shows the summary and time course data (same display as in Fig. 4A–C). Fig. 5G and H shows individual examples of WDR neurons before and 30 min after systemic vehicle or LY379268 application.

To determine the contribution of group II mGluRs in the amygdala to the inhibitory effect of systemically applied LY379268 in the arthritis pain model (5–6 h post-induction), a group II mGluR antagonist (LY341495, 100 μM, concentration in the microdialysis fiber, 40 min) was administered into the amygdala (right CeA) stereotaxically by microdialysis 10 min before the systemic agonist application (see 2.7.2). LY341495 administered into the CeA blocked the effect of systemically applied LY379268 (5 mg/kg, i. p.; n = 5 neurons in 5 rats) so that the agonists had no significant effect on neuronal responses to brush and innocuous and noxious compression of the arthritic knee joint compared to pre-drug values that were measured in the presence of ACSF in the microdialysis fiber (Fig. 5D, p = 0.3220, $F_{10,40}$ = 1.197; Fig. 5E, p = 0.5507, $F_{10,40}$ = 0.8897; Fig. 5F, p = 0.7414, $F_{10,40}$ = 0.674; repeated measures one-way ANOVA with Bonferroni posthoc tests). Fig. 5D–F shows the summary and time course data (same display as in Fig. 4A–C). Fig. 5I shows an individual example of the responses of a WDR neuron before (in ACSF) and after stereotaxic administration of LY341495 into the CeA 30 min after systemic application of LY379268. Positions of the microdialysis probe in the CeA and recording depths in the spinal cord are shown in Figs. S1B and S1C, respectively.

3.6. Intra-amygdala LY379268 inhibits spinal neuronal activity in arthritic rats

To determine the modulatory function of group II mGluRs in the amygdala on spinal nociceptive processing in the arthritis pain model (5–6 h post-induction), a group II mGluR agonist (LY379268, 10 μM, concentration in the microdialysis fiber, 40 min, n = 7 rats) was

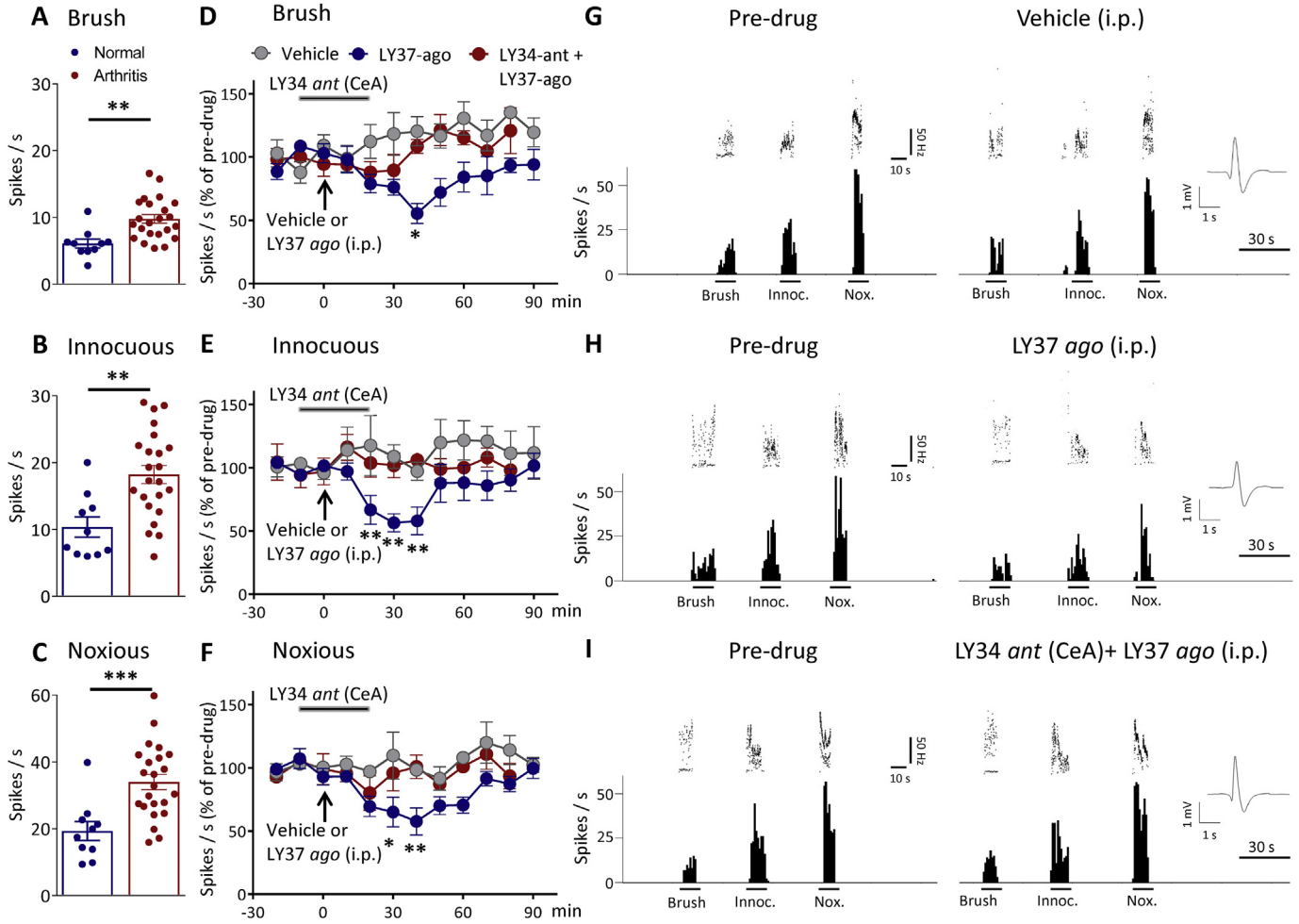

Fig. 5. In the arthritis pain model, systemically applied LY379268 (LY37 *ago*) inhibited spinal neuronal activity, which was blocked by intra-amygdalar administration of LY341495 (LY34 *ant*). Extracellular single-unit recordings were made from WDR neurons in the spinal dorsal horn of anesthetized rats. Neuronal activity evoked by brushing the skin (**A**) and innocuous (**B**) and noxious (**C**) compression of the knee was increased in arthritic rats compared to normal naïve rats. Symbols show net evoked responses (background activity was subtracted from total activity) of individual neurons. Symbols show means ± SEM. **, ***$p < 0.01$, 0.001, compared to normal condition values, unpaired-T test. Bar histograms show mean ± SEM. (**D-F**) Summary and time course data (same display as in Fig. 4). In arthritic rats (5h post-induction), a group II mGluR agonist (LY379268, 5 mg/kg, i. p., n = 6 neurons, one neuron per rat) decreased the responses to cutaneous brush (**D**) and innocuous (**E**) and noxious (**F**) compression of the knee with a calibrated forceps (see Methods), compared to vehicle (i.p., n = 5 neurons, one neuron per rat). Stereotaxic administration a group II mGluR antagonist (LY341495, 100 μM, concentration in microdialysis probe, 40 min, n = 5 neurons) into the CeA 10 min before systemic LY379268 application blocked the inhibitory effect of LY379268 on evoked responses. Symbols show means ± SEM. *, **$p < 0.05$, 0.01, compared to pre-drug values using repeated measures one-way ANOVA with Bonferroni posthoc tests. Three responses before drug/vehicle application were averaged and set to 100%. (**G** and **H**) Peristimulus time histograms and raster plots show individual examples (3 different neurons). (**G**) Evoked responses of a WDR neuron before and 30 min after systemic vehicle. (**H**) Evoked responses of a WDR neuron before and after systemic LY379268 injection. (**I**) Evoked responses of a WDR neuron before (in ACSF) and after systemic LY379268 injection together with intra-CeA administration of LY341495.

administered stereotaxically into the right CeA. Intra-CeA administration of LY379268 decreased responses of WDR neurons (n = 7) to brush, innocuous and noxious stimulations applied to the ipsilateral knee compared to pre-drug that were evaluated in the presence of ACSF in the microdialysis fiber (Fig. 6A, $p = 0.002$, $F_{11,66} = 3.874$; Fig. 6B, $p = 0.0065$, $F_{11,66} = 2.684$; Fig. 6C, $p = 0.0250$, $F_{11,66} = 2.195$; repeated measures one-way ANOVA with Bonferroni posthoc tests). Summary and time course data are shown in Fig. 6A–C (same display as in Fig. 4A–C, and Fig. 5D–F). Fig. 6D shows an individual example of the responses of a WDR neuron before (in ACSF) and 30 min after stereotaxic administration of LY379268 into the CeA. Positions of the microdialysis probe in the CeA and recording depths in the spinal cord are shown in Figs. S1B and S1C, respectively.

4. Discussion

This study shows for the first time that group II mGluRs in the central nucleus of the amygdala (CeA) contribute critically to the inhibitory effects of a systemically applied group II mGluR agonist on pain-related behaviors and spinal nociceptive processing in an arthritis pain model. The data further show inhibitory effects of intra-CeA activation of group II mGluRs on arthritis pain-related behaviors and spinal neuronal activity. This is consistent with evidence for a functional link between the CeA and descending pain modulation from a previous study showing that activation of mGluR8 (group III mGluR) in the CeA inhibits ON-cells and increases OFF-cell activity in the rostral ventromedial medulla (Palazzo et al., 2011). A group II mGluR agonist (LY379268) applied systemically (intraperitoneally) significantly decreased emotional-affective behaviors (audible and ultrasonic vocalizations) and mechanical withdrawal reflexes in a model of knee joint monoarthritis induced by intraarticular injections of kaolin and carrageenan, but not in normal naïve rats. This well-established model of a localized arthritis produces behavioral and neuronal changes with a well-defined time course, where changes reach a peak at 5–6 h

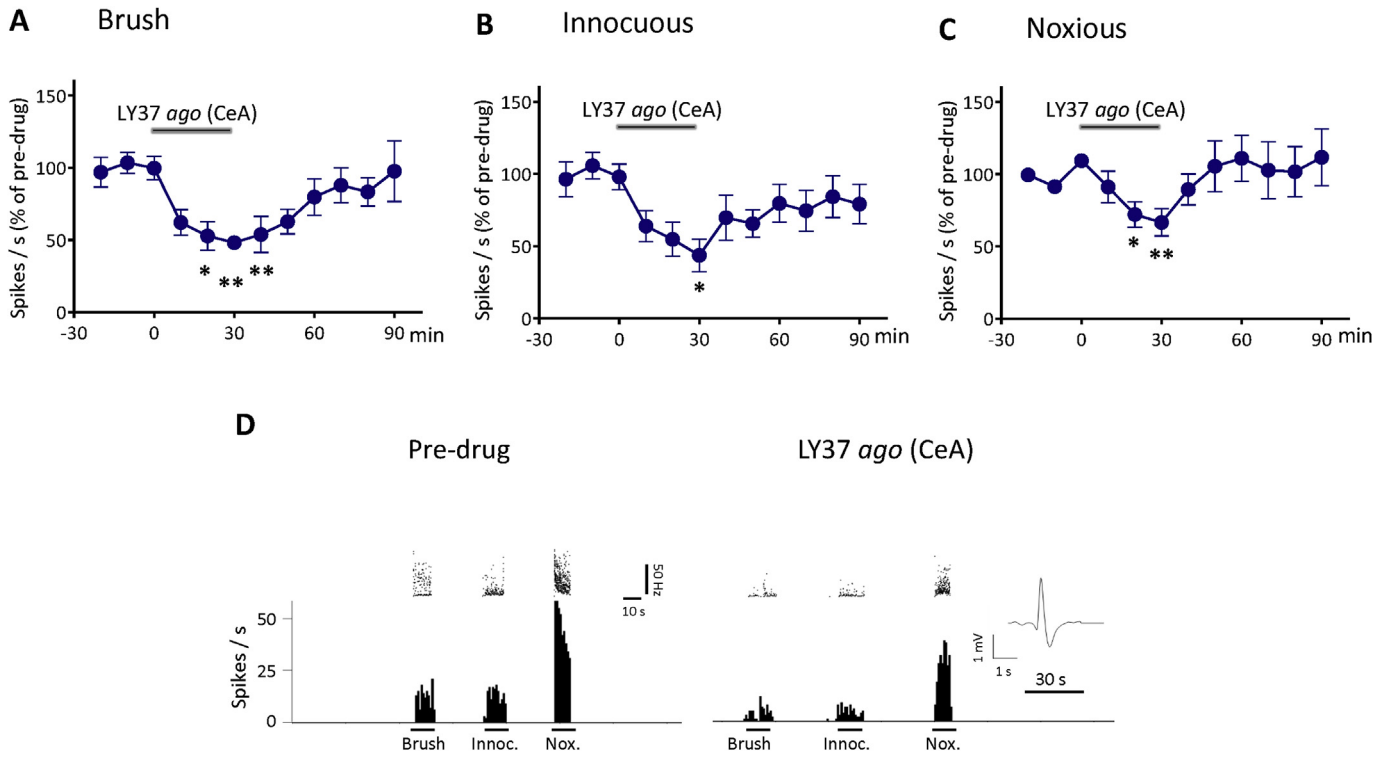

Fig. 6. In the arthritis pain model, intra-amygdalar administration of LY379268 (LY37 *ago*) inhibited activity of the spinal dorsal horn neurons. Extracellular single-unit recordings were made from WDR neurons in the spinal dorsal horn of anesthetized rats. (**A-C**) Summary and time course. In arthritic rats, a group II mGluR agonist (LY379268, 10 μM, concentration in microdialysis probe, 40 min, n = 7 rats, one neuron per rat) significantly decreased the responses to cutaneous brush (**A**), and innocuous (**B**) and noxious (**C**) compression of the knee with a calibrated forceps (see Methods), compared to pre-drug values. Symbols show means ± SEM. *, **p < 0.05, 0.01, compared to pre-drug values using repeated measures one-way ANOVA with Bonferroni posthoc tests. Three responses before drug application were averaged and set to 100%. (**D**) Peristimulus time histograms and raster plots show individual examples. (**D**) Evoked responses of a WDR neuron before (Pre-drug) and 30 min after intra-CeA LY379268 application.

postinduction and persist for a week (Neugebauer et al., 2007). Vocalizations and spinal reflexes were measured before and 30–40 min post systemic injection, because the time course analysis of the effects of LY379268 on spinal dorsal horn neurons in our electrophysiological experiments showed a maximum inhibitory effect about 30 min after the systemic application of LY379268. This finding and our protocol are consistent with previous studies on anxiolytic and antinociceptive effects of LY379268 (Aujla et al., 2008; Jones et al., 2005). Systemic application of a group II mGluR agonist had no significant effect on nociceptive behaviors and on evoked activity of WDR neurons under normal conditions, which is consistent with the findings of previous studies on group II mGluRs (Johnson et al., 2017; Simmons et al., 2002).

LY379268 is a potent and selective agonist, targeting both mGluR2 and mGluR3, and binds to the same site as the endogenous ligand (glutamate). Evidence from previous studies suggested antinociceptive effectiveness of systemically applied LY379268 in decreasing mechanical allodynia in a neuropathic pain model (spinal nerve ligation, SNL) and paw licking in the formalin pain model (Simmons et al., 2002; Zammataro et al., 2011). Antinociceptive effects of a different compound, the oral pro-drug (LY2969822) for the selective mGluR2/3 agonist LY2934747, were also observed in various models of inflammatory (formalin, capsaicin, complete Freund's adjuvant [CFA]), postsurgical (plantar incision), visceral (colorectal distension), and neuropathic (SNL) pain (Johnson et al., 2017).

Electrophysiological in vivo studies showed that spinal administration of LY379268 by microdialysis blocked the central sensitization of primate spinothalamic tract cells in a capsaicin pain model (Neugebauer et al., 2000) and intra-amygdala (CeA) administration of another group II mGluR agonist (LY354740) inhibited hyperexcitability of CeA neurons in the arthritis pain model (Li and Neugebauer, 2006). Spinal dorsal horn neuronal activity evoked by electrical stimulation of C-fibers was reduced by the intrathecal administration of a group II mGluR agonist (ACPD) in the carrageenan-induced hindpaw inflammation model (Stanfa and Dickenson, 1998). Brain slice physiology studies found that inhibitory effects of LY354740 on CeA neurons (Han et al., 2006) and of LY379268 on medial prefrontal cortical pyramidal cells in the arthritis pain model were presynaptic (Kiritoshi and Neugebauer, 2015).

Orthosteric antagonists with high selectivity for mGluR2 and mGluR3 have been developed (Yin and Niswender, 2014). They typically block the beneficial effects of the mGluR2/3 agonists acting in a competitive mode (Jane et al., 1996; Johnson et al., 1999). In fact, intrathecal application of EGLU inhibited the antinociceptive effects of an mGluR2/3 agonist (DCG-IV) on mechanical allodynia and hyperalgesia in the spinal nerve ligation model of neuropathic pain (Zhou et al., 2011), and intraperitoneal injection of LY341495 reversed the antinociceptive behavioral effects of a group II mGluR agonist (LY379268) in the formalin pain test (Simmons et al., 2002). Surprisingly, intrathecal application of LY341495 alone improved mechanical allodynia, but not thermal hyperalgesia, in the complete Freund's adjuvant (CFA)-induced pain model (Zhang et al., 2009).

A number of technical considerations should be noted. We did not test the effect of intra-amygdala group II mGluR antagonist administration on the systemically applied group II mGluR agonist (LY379268) under normal condition but only in the arthritis pain model, because LY379268 had no behavioral and electrophysiological effects under normal conditions. We used normal rats as a control for the arthritis pain condition, because our previous studies found no difference between normal naive animals and animals with needle insertion into the knee joint cavity and subsequent movements of the joint, either with intraarticular saline injection (Neugebauer et al., 2003) or without

injection of any chemicals (Grégoire and Neugebauer, 2013; Kiritoshi and Neugebauer, 2018). The appropriate control for this arthritis pain model is debatable because any intervention targeting the knee joint directly could have irritating confounding effects.

The present study showed the involvement of amygdala group II mGluRs in the systemic antinociceptive effects of a group II mGluR agonist but did not determine the subtype (mGluR2 and mGluR3). The recent availability of novel tools such as selective positive and negative allosteric modulators (PAMs and NAMs) for mGluR2 and mGluR3 (Bollinger et al., 2017; Mazzitelli et al., 2018; Sheffler et al., 2011) and transgenic mGluR2 and mGluR3 knockout mice (Bernabucci et al., 2012; Olszewski et al., 2017; Zammataro et al., 2011) will allow future studies to determine any differential roles of these subtypes in pain modulation. At this point, there is evidence for a contribution of mGluR2 as well as mGluR3 to pain modulation (Mazzitelli et al., 2018), but they may play differential roles in different aspects of pain.

In conclusion, the results of the present study suggest that group II mGluR activation in the amygdala can inhibit arthritis pain-related behaviors and spinal nociceptive processing, and the amygdala group II mGluRs mediate the beneficial antinociceptive effects of a systemic group II mGluR agonist. The data provide insight into the site of drug action and further evidence for the pharmacological activation of group II mGluRs as a potential therapeutic strategy for the relief of pain.

Competing interests

There are no conflicts of interest.

Authors' contributions

MM carried out the behavioral and electrophysiological experiments, analyzed data, created figures and provided a first draft of the manuscript. VN conceived the study, supervised experiments and data analysis, and finalized the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2019.107706.

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