Dynamic modulation of inflammatory pain-related affective and sensory symptoms by optical control of amygdala metabotropic glutamate receptor 4

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Supplementary methods

Behavioral studies

Animals
Animals were housed in groups with food and water ad libitum. They were maintained in a controlled environment (22 ± 1°C, 55 ± 5% humidity) with a 12h:12h light:dark cycle. Experiments were performed on 8- to 12-weeks-old C57BL/6j mice (Charles River) or mGlu4 KO mice on a C57BL/6J background with wild type (WT) littermates used as controls. Genotyping for mGlu4 KO mice was performed as previously described. Only males and a minimum of 5 mice per group were used, and all the experiments were replicated 2 or 3 times. Behavioral testing was performed between 10:00am to 14:00pm. For this study, we chose an inflammatory pain model induced by unilateral intraplantar injection (30 µl) of complete Freund's adjuvant (CFA; Mycobacterium tuberculosis; Sigma) in the left hind paw, while the control received an intraplantar injection of PBS. The animals were treated in accordance with the European Community Council Directive 86/609, modified by the decrees 87/848 and 2001/464 and with the guidelines of the French Agriculture and Forestry Ministry for handling animals (C34-172-13). The regional animal welfare committee (CEEA-LR) approved all protocols and all efforts were made to minimize the number of animals used and potential pain (not induced by the model) and distress.

Experimental design and treatments
After cannula implantation and recovery, animals were subjected to a series of different behavioral tests to assess mechanical sensitivity, fear extinction learning and memory, anxiety-like behavior, locomotor activity, and depressive-like behavior. Following the basal measurement of mechanical sensitivity and the fear conditioning session, the animals were injected with CFA. Mice were submitted to different behavioral tests at least one week after CFA injection. Mice in the behavioral pharmacology and optopharmacology experiments received intra-amygdala injection of mGlu4 ligands (LSP4-2022 at 5 µM or optogluram at 30 µM).
Surgery
Cannula implantations were performed under aseptic conditions using stereotactic guidance. All coordinates were relative to bregma according to the Franklin & Paxinos mouse atlas. Before surgery, mice were anaesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Cannula guides (Gauge 26) were implanted bilaterally over the intermediate capsule (-1.34 mm anteroposterior (AP); ±2.9 mm mediolateral (ML); and -4.25 mm dorsoventral (DV); PlasticsOne, Roanoke, VA). Longer injectors were also used to gain access to ventral part of the amygdala including the ventral ITCs (DV -4.75mm) during drug infusions. A dummy cap was inserted in the guide cannula to avoid blockade at the injection site. For optopharmacology, hybrid cannulae combining fluid tubing (Gauge 25) and an optical fiber (NA=0.63; Doric Lenses, Quebec, Canada) were implanted at -1.34 mm AP; ±2.9 mm ML; -4.65 mm DV. Mice were allowed to recover for 1 week before the behavioral experiments started.

Cannulation site verification
For the verification of the cannulation site, animals were sacrificed by decapitation and brains post-fixed in 4% PFA-PBS for 3 days. Fixed brains were cut using a vibratome in 80 μm-thick serial coronal sections. Cannulation sites were identified under a dissecting microscope and reported manually according to the Franklin & Paxinos mouse atlas.

Mechanical allodynia
Mechanical sensitivity was evaluated using von Frey filament stimulation first on healthy mice (before paw injection, Naive) and subsequently 10 days after CFA or vehicle injection. For all the behavioral experiments, homogenous groups prior to treatments were formed based on the basal mechanical sensitivity of the mice. Mice were placed in individual plastic boxes with grid floors and allowed to adapt for 2h the day before the first test and 2h prior to testing (days 0 & 10). We used three different hair filaments with different bending forces: 0.07 g corresponding to innocuous, 0.6 g to intermediate, and 1.4 g to noxious stimulation. The filaments were pressed to the plantar surface of the hindpaw until obtaining a C-shape curve. For each filament, five stimuli were applied and the number of paw lifts was counted. The effects of compounds on mechanical sensitivity were evaluated 20, 40 and 60 min after intra-amygdala injection.

Pavlovian fear conditioning, extinction, and extinction recall
Fear conditioning and extinction experiments were carried out according to previously
published procedures with minor modifications\textsuperscript{3} and performed in a conditioning box (20 cm width × 20 cm length × 20 cm height) that was placed within a sound proof chamber (Panlab, Harvard Apparatus). Two different contexts were used for fear conditioning and extinction (A: black walls, metal grid on black floor, washed with 1% acetic acid solution; B: white walls, white rubber floor, washed with 1% vanilla-extract solution). Mice were conditioned in context A and received three pairings (60–120 s variable inter-stimulus intervals) of a conditioned stimulus (CS, 3 kHz, 80 dB, 30 s tone) followed by an unconditioned stimulus (US, 2 s, 0.6 mA scrambled footshock) using a Freezing system (Panlab, Barcelona, Spain) after an exploration period of 2 min (baseline). The initial CS-recall and subsequent CS-extinction were performed 8 days later in context B. Mice were allowed to explore for 2 min (baseline) and then received 40 CS presentations (Figure 1) or 36 CS presentations (Figure 2, Extended Data Figure 4) with a 5 s inter-stimulus interval. For clarity, data are shown as 10 or 9 blocks of four CS presentations. The shorter extinction protocol in the pharmacological experiments (Figure 2) was required to stay within the time span of the efficacy of the injected drug. The day after (day 9), context-specific extinction recall was also tested in context B. After 2 min of exploration (baseline), mice received 4 CS presentations (120–180 s variable inter-stimulus intervals), shown as one block of the four CS presentations. Freezing (no visible movement except respiration) was measured automatically using a load cell coupler (Panlab, Barcelona, Spain) and was defined as the lack of activity above a body weight-corrected threshold for a duration of 1 s or more, as analyzed using Freezing software (Panlab, Barcelona, Spain). Intra-amygdala injections of drug or vehicle were performed 20 min before extinction training and before the extinction recall on subsequent days. Groups receiving the vehicle before the training session (Extended Data Fig. 4), were split into two groups for the extinction test, one injected with vehicle and the other one with drug. To evaluate within session extinction, we performed a first analysis of freezing along all blocks of CS presentations, and subsequently focused our main statistical analysis on comparing only the first CS block (Fear recall) versus the last CS block (end of extinction training) in each experiment.

\textit{Elevated plus maze}

Anxiety-like behavior was assessed by analysing the propensity to explore the open arms of an elevated plus maze. The apparatus consisted of a plus-shaped maze with two opposite open (23.5 x 8 cm) and enclosed arms (23.5 x 8 x 20 cm high). The arms extended from a central platform (8 x 8 cm) and the maze was elevated to a height of 50 cm above the floor. Each mouse was placed at the centre of the maze and could freely explore it. The time spent in open arms and the number of entries in both arms were recorded manually over a total period of 10 mins for classical pharmacology and 12 min for optopharmacological
experiments (see details in optopharmacology section). Results were expressed as total time spent in the open arms and the number of entries in closed or open arms.

**Splash test**
Depressive-like behavior was assessed by measuring the grooming behavior after squirting a drop of 10% sucrose solution on the dorsal coat of the mice. The duration that mice spent pursuing grooming behavior was recorded manually over a total period of 5 min for classical pharmacology and 9 min for optopharmacology experiments (see details in optopharmacology section).

**Locomotor activity**
Locomotion was assessed in a circular corridor with four infrared beams placed at 90° angles (Imetronic, Pessac, France). Locomotor activity was measured by counting the consecutive interruption of two adjacent beams (i.e. mice moving through one-quarter of the circular corridor). All mice were habituated to the test apparatus for 2h one day before assessing locomotor activity. For intra-amygdala drug injections, mice were allowed to explore the activity box for 1h prior to and 40 min after injection. Results were analysed with cyclotron software (Pessac, France) and expressed as means of 1/4 turns in the circular cage for 40 min.

**Immunocytochemistry for light and electron microscopy**

**Animals and tissue preparation**
All experiments were carried out on 4-6 adult male C57BL/6J mice (Charles River, Sulzfeld, Germany or Saint Germain Nuelles, France) per experiment, except for Extended Figure 7c where mGlu4 KO mice (n=3) and their WT littermate (n=3) were used. Depending where experiments were performed, experimental protocols were approved by the Austrian Animal Experimentation Ethics Board in compliance with both the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC) or in accordance with the European Community Council Directive 86/609, modified by the decrees 87/848 and 2001/464 and with the guidelines of the French Agriculture and Forestry Ministry for handling animals (C34-172-13) and approved by the regional animal welfare committee (CEEA-LR). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.
For experiments in Fig. 3a-b, animals were deeply anesthetized by intraperitoneal injection of thiopental (100 mg/kg, i.p.) and perfused transcardially with phosphate buffered saline (PBS; 0.9% NaCl, pH 7.4) followed by ice-cold fixative made of 4% w/v paraformaldehyde (PFA) and 15% v/v of a saturated solution of picric acid in phosphate buffer (PB; 0.1 M, pH 7.4) for 10 min, without postfixation. For experiments in Fig. 3c-d, and Supplementary Figs. 5 and 6b, animals were deeply anesthetized by intraperitoneal injection of pentobarbital (300 mg/kg) and perfused transcardially with PBS followed by 4% PFA. To study the neuronal activation of ITCs (Zif268 staining), LSP4-2022 was administered by intraperitoneal injection (10mg/kg) 1h before the intracardiac fixation. Brains were removed and postfixed in 4% PFA for 24 h. For electron microscopy (EM) experiments (Fig. 3e-h), glutaraldehyde (25%) at a final dilution of 0.05% v/v was added to the fixative just before the perfusion. Brains processed for EM were cut in tissue blocks of approximately 5-6 mm. The blocks containing the amygdala were cryo-protected in 20% sucrose in 0.1M PB overnight at 6 °C and freeze-thawed once to allow antibody penetration. Coronal sections were sliced on a Leica VT1000S vibratome (Leica Microsystems, Vienna, Austria) at 40 µm for light microscopy or 70 µm for electron microscopy. Sections were stored in 0.1 M PB containing 0.05% sodium azide at 6°C until immunohistochemical experiments were performed.

**Immunocytochemistry for light and electron microscopy**

Immunocytochemistry experiments in Fig. 3a-b, were carried out according to previously published procedures with minor modifications. Briefly, after blocking unspecific binding sites with a solution containing 20% normal goat serum (NGS) and 0.1% v/v Triton X-100 in TBS (TBS-T) for 2 hours at RT, free floating sections were incubated for 72 hours at 6°C with a rabbit polyclonal antibody raised against the C-terminal domain of the rat mGlu4 (1:500) in TBS-T and containing 2% NGS. In all EM experiments Triton X-100 was omitted. For light microscopy, mGlu4 was visualized using a Cy3-donkey anti-rabbit secondary antibody (1:400, Jackson ImmunoResearch Laboratories). Immunofluorescence was analyzed using either a Zeiss AxioImager M1 microscope with epifluorescence illumination or with a confocal microscope (TCS SP5 confocal, Leica Microsystems, Vienna, Austria) using the LAS-AF software (Leica) for acquisition. Confocal images were deconvolved with the Huygens software (Scientific Volume Imaging, Hilversum, Netherlands), and further processed with Imaris 7.1 (Bitplane, Zürich, Switzerland).

For experiments in Fig. 3c-3d and Supplementary Figs. 5 and 6b, free-floating coronal sections were rinsed in PBS, and then blocked and permeabilised in a solution containing 10% horse serum, 2% BSA and 0.4% triton in PBS for 2h. Afterwards, sections were incubated overnight at room temperature with rabbit anti-mGlu4 (1:200, Invitrogen), mouse anti-VGAT (1:200, Synaptic Systems), guinea-pig anti-VGLUT1 (1:500, Synaptic Systems), mouse anti-VGLUT2 (1:200, Millipore), guinea-pig anti-VGLUT3 (1:5000, Chemicon) or rabbit anti-Zif268
Sections were then incubated for 2 h with the corresponding AlexaFluor secondary antibodies (1:500, Invitrogen). Sections were mounted onto slides and coverslipped with fluorescent mounting media (Prolong Gold, Invitrogen). Images were captured using a Zeiss LSM 780 confocal microscope with ZEN imaging software. In pre-embedding EM experiments (Fig. 3e-h), mGlu4 was visualized either by horseradish peroxidase (HRP) or by nanogold-silver-enhanced reaction using a biotinylated goat anti-rabbit antibody (1:100, Vector Laboratories) or a goat anti-rabbit Fab fragment conjugated to 1.4 nm nanogold (1:100, Nanoprobes, Yaphank, NY, USA), respectively. Sections processed for the HRP reaction were incubated overnight at 6°C in ABC complex (1:100; Vector Laboratories) made up in TB. The antigen/antibody complex was detected with 3,3′-diaminobenzidine (0.5 mg/ml; Sigma, Munich, Germany) using 0.003% H2O2 as the electron donor for 5-6 min. The sections were then washed and incubated in 1% glutaraldehyde and 4% PFA in PB 0.1M for 10 min at RT. After this additional fixation, the sections were rinsed with ultra-pure water. Nanogold particles were amplified with silver using the HQ silver™ Enhancement kit (Nanoprobes) for 8+4 min at RT. Sections were contrast enhanced with 2% OsO4 in 0.1M PB for 40 min at RT and 1% uranyl-acetate in 50% ethanol for 30 min at RT, making sure they were protected from light. After dehydration with graded ethanol and propylene oxide, sections were embedded in epoxy resin (Durcupan ACM-Fluka, Sigma, Gillingham, UK). Serial ultrathin sections (70 nm) were cut using a diamond knife (Diatome, Biel, Switzerland) on an ultramicrotome (EM UC7, Leica, Vienna, Austria), collected on copper slot grids coated with pioloform (Agar, Stansted, England) and analyzed with a transmission electron microscope (Philips CM120) equipped with a Morada CCD camera (Soft Imaging Systems, Münster, Germany). Images were level adjusted and cropped in Photoshop (Adobe) without changing any specific feature within.

**Electrophysiological studies**

*Animals, stereotactic injections, and analysis of injections sites and projections*

For these experiments, we used male decarboxylase 67 (GAD67)–GFP mice on a C57BL/6J background, which allowed us to target ITCs for recordings. Mice were 5- to 7-weeks-old when they received thalamic injections of recombinant adeno-associated virus (rAAV). All procedures were performed in accordance with the EU directive on use of animals in research and approved by the Regierungspraesidium Tuebingen, state of Baden-Wuerttemberg, Germany, and care was taken to minimize the animals suffering. Mice were maintained under isoflurane anesthesia, fixed in a stereotactic frame (Stoelting, USA) and bilateral craniotomies were made with a 0.5 mm microdrill. Coordinates from bregma (in mm) were calculated and adjusted based on the mouse brain atlas. rAAV was injected using pressure injection with a Toohey Spritzer (Toohey Company, USA) using glass
capillaries (borosilicate glass, 1B150F-4, World Precision Instruments, Germany) pulled on a horizontal pipette puller (P-1000, Sutter Instruments). Bilateral injections of 0.5 µl rAAV-CAG-hChR2(H134R)-mCherry (Penn Vector Core, USA) into PIN/MG were performed at the following coordinates from bregma (in mm): posterior -3.0, lateral ±1.8, ventral 3.8. For light activation of sensory inputs and recordings from ITCs and LA principal neurons, acute amygdala slices were prepared for recording 4 weeks after viral injection.

To confirm the location of viral injection sites in PIN/MG, brain slices caudal to the amygdala were cut. To image axon trajectories, acute amygdala slices were used after recording. Slices were sandwiched between filter papers, fixed with 4% PFA in 0.01M PBS, and resectioned at 70 µm. Injection sites were imaged on a laser-scanning microscope (LSM 710, Carl Zeiss, Germany). Some sections were counterstained with NeuroTrace435 (1:400, Life Technologies, Germany) to reveal the cytoarchitecture of the amygdala. Electrophysiological data were analyzed only from animals with confirmed injection sites in PIN/MG (n=10 mice).

Slice recordings
To obtain acute brain slices, mice were deeply anesthetized with 3-5% Isofluran in oxygen, decapitated and the brains were rapidly removed. Coronal amygdala brain slices (320 µm) were prepared in ice cold artificial CSF (ACSF) supplemented with an additional 8.7 mM MgSO4 on a vibratome (HM650V, Microm, Germany). Slices were recovered in a custom-built interface chamber at 37°C for 35-45 min and subsequently stored at room temperature in ACSF containing (in mM): 124 NaCl 1.25 NaH2PO4, 1.3 MgSO4, 2.7 KCl, 26 NaHCO3, 2 CaCl2, 18 D-glucose, 4 L-Ascorbic Acid and oxygenated with 95% O2, 5% CO2 until recording. Slices were transferred to a submerged recording chamber and superfused with ACSF (1–2 ml/min perfusion rate) at 30°C. Oblique infrared and fluorescence illumination were used to target cells for recording. Whole-cell recordings were performed on identified LA principal neurons or mITCd cells (based on location and absence or presence of green fluorescence, respectively) with pipettes pulled on a horizontal pipette puller (P-1000, Sutter Instruments, USA) from borosilicate glass (GB150F-8P, Science Products, Germany) and had resistances of 3–5 MΩ (LA neurons) 6–8 MΩ (mITCd cells) when filled with internal solution. The intracellular solution was Cs-based and contained (in mM): 115 Cs-Methlysulphonate, 20 CsCl, 4 Mg-ATP, 0.4 Na-GTP, 10 Na2-phosphocreatine, 10 HEPES, 0.6 EGTA, (290–295 mOsm, pH 7.2-7.3). Data were acquired with a Multiclamp 700B amplifier, Digidata 1440, and Clampex software (all from MDS, USA). Signals were filtered at 2 kHz and digitized at 5 kHz. Series resistance was monitored and data were excluded if it changed >20%. Excitatory postsynaptic currents (EPSCs) were isolated in the presence of 100 µM Picrotoxin to block inhibitory transmission and evoked either electrically using bipolar tungsten stimulation electrodes (Science Products, Germany), or optically using 470 nm light
pulses (0.2–1 ms, 0.5–2 mW/mm²) from a light emitting diode (LED, CoolLed, UK) delivered through a 60x 1.0 NA objective of an upright microscope (BX51, Olympus, Japan). All data were analyzed using the NeuroMatic suite of macros (www.neuromatic.thinkrandom.com) and additional custom-written macros in IgorPro Software (Wavemetrics, USA). EPSC amplitudes were measured as average negative peak in a 1 ms window. Paired pulses were delivered at 50 ms interval and the paired pulse ratio (PPR) as an indicator of presynaptic release probability was measured by dividing the second EPSC amplitude by the first EPSC amplitude. All chemicals were reagent grade (from Roth, Merck, or Sigma, Germany). Picrotoxin was obtained from Sigma (Germany), stored as stock solution at –20°C and diluted into ACSF prior to use.


