



Supporting Online Material for

Induction of Synaptic Long-Term Potentiation After Opioid Withdrawal

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Published 10 July 2009, *Science* **325**, 207 (2009)
DOI: 10.1126/science.1171759

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Materials and Methods

All experiments were performed in accordance with the European Community's Council directives (86/609/ECC) and were approved by the Austrian Federal Ministry for Education, Science and Culture.

Spinal cord slice preparation

Under deep isoflurane anesthesia, the lumbar spinal cord was removed from young rats (aged 17 to 25 days). Transverse slices (about 500 μm thick) with long dorsal roots (10-15 mm) attached were cut using a vibrating microslicer. Slices were incubated in a solution kept at 33°C containing (in mM): NaCl, 95; KCl, 1.8; KH_2PO_4 , 1.2; CaCl_2 , 0.5; MgSO_4 , 7; NaHCO_3 , 26; glucose, 15; sucrose, 50; pH was 7.4, osmolarity 310-320 $\text{mosmol}\cdot\text{l}^{-1}$, and oxygenated with 95% O_2 and 5% CO_2 . A single slice was then placed in the recording chamber and continuously superfused with oxygenated recording solution at a rate of 3-4 $\text{ml}\cdot\text{min}^{-1}$. The recording solution was identical to the incubation solution except for (in mM): NaCl, 127; CaCl_2 , 2.4; MgSO_4 , 1.3 and sucrose 0, and was kept at 33 °C with an inline solution heater.

Electrophysiological recordings in vitro

Neurons were visualized with infrared optics (SI). Only neurons lying within a distance of maximally 20 μm from the dorsal white/grey matter border were considered as being lamina I neurons and used for the experiments. All recordings were made in the whole-cell patch-clamp configuration at a holding potential of -70 mV using an Axopatch 200B patch-clamp amplifier and the pCLAMP 9 acquisition software. Signals were low-pass filtered at 2-10 kHz, sampled at 10 kHz. Patch pipettes (impedance 2-5 M Ω) from borosilicate glass were pulled on a horizontal puller and filled with

internal solution (mM): potassium gluconate 120, KCl 20, MgCl_2 2, Na_2ATP 2, NaGTP 0.5, HEPES 20, EGTA 0.5, pH 7.28 with KOH, measured osmolarity 300 $\text{mosm}\cdot\text{l}^{-1}$. For some experiments, patch pipettes were filled with a K^+ free solution composed of (in mM): CsMeSO_3 , 120; TEA-Cl, 20; MgCl_2 , 2; HEPES, 10; EGTA, 10; Na_2ATP , 2; and GDP βS , 500 μM . The potassium free GDP βS -solution was used to prevent postsynaptic opioid receptor mediated effects (S2, S3). For blocking intracellular Ca^{2+} the Ca^{2+} -chelator BAPTA (20 mM) was added to the pipette solution consisting of (in mM): potassium gluconate 100, KCl 20, MgCl_2 4, Na_2ATP 2, NaGTP 0.5, HEPES 10, pH 7.28 with KOH, measured osmolarity 300 $\text{mosm}\cdot\text{l}^{-1}$. For blocking postsynaptic NMDA-receptors MK-801 (10 μM) was added to the standard internal solution.

Dorsal root afferents were stimulated through a suction electrode with an isolated current stimulator. After determination of the threshold to elicit an EPSC, test pulses of 0.1 ms were given at intervals of 30 s. Intensity of test stimulation ranged from 3 to 5 mA. Evoked EPSCs were classified as C-fiber-evoked when the calculated conduction velocity was below 0.5 $\text{m}\cdot\text{s}^{-1}$. Monosynaptic C-fiber input was identified by the absence of failures in response to 10 stimuli applied at 2 Hz to the dorsal roots and a jitter in response latencies of less than 2 ms.

In some experiments electrophysiology was combined with UV-flash photolysis. For flash photolysis Diazo-2 tetrapotassium salt (2 mM) was added to the pipette solution composed of (in mM): potassium gluconate 120, KCl 20, MgCl_2 2, Na_2ATP 2, NaGTP 0.5, HEPES 20, pH 7.28 with KOH, measured osmolarity 300 $\text{mosm}\cdot\text{l}^{-1}$. Uncaging was performed with a UV-

flash (Mercury Lamp 100 W) at 360 nm for 1 sec.

In vitro Ca²⁺ imaging of spinal cord neurons

Fluorometric measurements of free cytosolic Ca²⁺ concentrations of single lamina I dorsal horn neurons were performed by loading the cells in the recording chamber via a patch pipette by replacing EGTA in the standard pipette solution with the cell-impermeant dye fura-2 pentapotassium salt (250 μM). Slices were continuously superfused with recording solution containing bicuculline and strychnine. Experiments were performed at 33°C. Neurons were clamped at -70 mV in the whole cell patch-clamp configuration. For Ca²⁺ imaging, cells were illuminated with a monochromator and images were taken with a cooled CCD camera. Paired exposures to 340 and 380 nm were obtained at a frame rate of 0.2 Hz. In all experiments, one drug effect was tested per slice.

Animal surgery for in vivo experiments

Experiments were performed on adult male Sprague Dawley rats (200-250 g for electrophysiology and 90-120 g body weight for imaging). Isoflurane (4 vol %) in two thirds N₂O and one third O₂ was initially applied through an anesthesia mask to induce anesthesia. Animals were then intubated using a 16 G venous cannula and mechanically ventilated at a rate of 75 strokes·min⁻¹ and a tidal volume of 4-6 ml. Anesthesia was maintained using 1.5 vol % isoflurane.

Core temperature was kept at 37.5 ± 2°C with a feedback controlled heating blanket. Deep surgical level of anesthesia was verified by stable mean arterial blood pressure. The right femoral vein and artery were cannulated to allow i.v. infusions and for monitoring arterial blood pressure, respectively. Muscle relaxation was achieved by 2 μg·kg⁻¹·h⁻¹ pancuronium bromide i.v.. For cardiopulmonary bypass (see below) in- and outflow a carotid artery and a jugular vein were cannulated. During anesthesia, animals continuously received i.v. solution (58% Ringer solution, 30% HAES, 8% glucose and 4% sodium bicarbonate, 2 ml·h⁻¹) for stabilization of blood pressure (mean arterial blood pressure 120 ± 10 mmHg), blood-glucose (mean 100 ± 10 mg·dl⁻¹) and base excess (-1.5 ± 0.8 mmol·l⁻¹). Following cannulation, the left sciatic nerve was dissected free for bipolar electrical stimulation with a silver hook electrode. The lumbar segments L4 and L5 were exposed by

laminectomy. The dura mater was incised and reflected.

A portable stereotactic frame with two adjustable brackets for vertebral column fixation was custom made to fit on the microscope stage for imaging experiments (see below). The stereotactic frame permitted a prone position of the animal for cardiopulmonary bypass and for electrophysiological recordings in vivo. An agarose pool was formed around the exposed spinal segments and filled with artificial cerebrospinal fluid. A bipolar hook stimulation electrode was fixed directly onto the steel base plate for stimulation of sciatic nerve fibers. At the end of each electrophysiological experiment animals were decapitated in deep anesthesia, the spinal cord was removed and cryo-fixed for detection of a rhodamine B spot at the recording site under a fluorescence microscope. Only those experiments were taken into further analysis where the recording site was in laminae I or II.

Electrophysiological recordings in vivo

Electrophysiological recordings were performed as described elsewhere (S4). Briefly, C-fiber-evoked field potentials were recorded with glass electrodes (impedance 2-3 MΩ) from laminae I and II of the spinal cord dorsal horn in response to stimulation of sciatic nerve fibers. The pipette solution consisted of (in mM) NaCl 135, KCl 5.4, CaCl₂ 1.8, HEPES 10 and MgCl₂ 1, and in addition 0.2 % rhodamine B. At the end of each electrophysiological experiment, pressure was applied to the electrode (300 mbar, 1 min) for marking the recording site with rhodamine B. Electrodes were driven by a microstepping motor. Recordings were made with an ISO-DAM-amplifier using a band width filter of 0.1 – 1000 Hz. Signals were monitored on a digital oscilloscope and digitized by an A/D converter. Afferent input from the hindpaw was identified by mechanical stimulation of the foot while acoustically evaluating the evoked responses with an audio monitor. Test stimuli were delivered to the sciatic nerve and consisted of single pulses of 0.5 ms at intensity of 25 V given every 5 minutes using an electrical stimulator. To explore the signal transduction pathways involved, specific blockers were topically applied to the spinal cord dorsum (S5).

Cardiopulmonary bypass for in vivo Ca²⁺ imaging experiments

The extracorporeal circuit consisted of a venous reservoir, a membrane oxygenator and a heat

exchanger. Blood was drained from the right atrium via the jugular vein catheter into a 5 ml reservoir, which was closed for generation of a slight vacuum to support venous outflow. The venous reservoir was connected to a roller pump equipped with a 12 cm long silicone tube with a 2 mm internal diameter. A heat exchanger was located between the roller pump and the oxygenator. The membrane oxygenator made of micro porous polypropylene fibers was custom built. Cardiopulmonary bypass was conducted during imaging at a flow rate of $150 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, which is similar to normal cardiac output in the rat (S6). Thereby sufficient blood gas exchange was provided throughout the whole cardiopulmonary bypass period. Blood pressure and arterial blood gas analyses were thereby kept within physiological limits.

In vivo dye loading of spinal cord neurons

Freshly made 250 μM Oregon Green BAPTA-1 AM (solved in 0.5 % Pluronic 127 in buffer) was pressure injected into superficial laminae of the exposed spinal cord. The proper injection area was identified by field potential recordings which were evoked by electrical stimulation of sciatic nerve fibers (see below). Following 30 min de-esterification of the dye, the animal was placed under the 2-photon laser-scanning microscope for imaging. To eliminate movement artefacts from respiration, a cardiopulmonary bypass was used during imaging.

In vivo Ca^{2+} imaging of spinal cord neurons

Imaging was performed on a 2-photon laser scanning microscope consisting of a DM LFS A microscope and a femtosecond Ti-sapphire laser operating at 90 MHz repeat frequency, 140 fs pulse width and a wavelength range of 705 - 980 nm. Excitation light was focused by an x40 water immersion objective (0.8 NA). The excitation wavelength was set to 750 nm for Oregon Green BAPTA. The average power delivered to the spinal cord surface was less than 200 mW. Scanning and imaging acquisition were controlled with confocal software. Emitted light was collected by a non-descanned detector for monitoring the calcium dye Oregon Green BAPTA (525/50). Long-term fluorescent measurements were achieved by repeatedly bidirectional scanning of a region of 512×128 pixels at a time interval of 300 ms – 6 s and heart beat triggered. Fast fluorescent scanning at a region of 128×64 or 256×128 pixels was done at a time interval 92 – 184 ms.

Drugs

For in vitro electrophysiology and in vitro Ca^{2+} imaging experiments, drugs were applied to the bath solution at known concentrations. Drugs used were bicuculline (10 μM), strychnine (10 μM), the selective μ -opioid receptor agonist [D-Ala², N-Me-Phe⁴, Gly-ol]-enkephalin (DAMGO, 500 nM) and β -funaltrexamine (25 μM).

For in vivo recording pancuronium bromide was given as an i.v. infusion ($2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). Remifentanyl was dissolved in sterile NaCl. For a tapered withdrawal remifentanyl infusion was reduced from $450 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ to zero in 15 equal steps at 2 min intervals beginning 60 min after onset of infusion (Fig. 3C). β -Funaltrexamine was applied topically onto the spinal cord surface (250 μM). The competitive N-methyl D-aspartate (NMDA) receptor antagonist D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 100 μM) was dissolved in 0.9% NaCl. The protein kinase C (PKC) blocker chelerythrine chloride (800 μM) was dissolved in ddH₂O. The Ca^{2+} -calmodulin dependent kinase II (CaMKII) blocker 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93, 400 μM) and the ryanodine receptor blocker dantrolene (500 μM) were dissolved in DMSO. All drugs were further dissolved in artificial cerebrospinal fluid to obtain the desired concentrations as indicated in the figure legend and applied directly onto the spinal cord dorsum (S5).

Data analysis and statistics

C-fiber-evoked EPSCs were analyzed offline using Clampfit 9. Synaptic strength was quantified by measuring the peak amplitude of monosynaptic EPSCs. Amplitudes were used to quantify synaptic strength as they were not affected by occasional action potential discharges. The mean amplitude of 6 EPSCs evoked by test stimuli prior to opioid application served as control. Statistical comparison was assessed using SigmaStat 3.1. Data were tested for normality (Kolmogorov-Smirnov Test), and then a one way repeated measures analysis of variance (One Way RM-ANOVA) was performed for each neuron to test for potentiation. Effects of drugs were tested using the Fisher Exact test.

The area under the curve of C-fiber-evoked field potentials was determined offline using the software Clampfit 9. Amplitudes and areas under the curve of C-fiber-evoked field

potentials changed in parallel in all experiments. For data analysis the area under the curve was used as amplitudes displayed higher variability (coefficients of variation of up to 200%; on average 25%). The mean area under the curve of 18 consecutive field potentials prior opioid application served as control. Responses were normalized for each rat. Data were tested for normality, and then a One Way RM-ANOVA was performed to compare the different experimental protocols and treatments. ANOVA was corrected by the Bonferroni adjustment. Effects of drugs were tested using the Fisher Exact test. A p-value < 0.05 was considered statistically significant. Values are expressed as mean \pm standard error of mean (SEM).

In vivo Ca^{2+} transients were measured using 512 x 128 pixels scans at a time interval of 6 s. Images were processed and quantified with ImageJ 1.37c. Fluorescence was averaged over cell body areas and expressed as relative

fluorescence changes (F/F_0) after subtraction of background fluorescence.

The 10 min periods prior to drug application or washout of the opioid served as controls. Data were tested for normality and changes in fluorescence intensity were assessed using the Rank-Sum test. Values are plotted as running averages with a sampling proportion of 0.05.

For in vitro Ca^{2+} imaging, the ratio of exposures to 340 and 380 nm light (F340/F380) was calculated off-line to assess the relative change in Ca^{2+} -concentration without conversion to absolute concentration values. For single cell measuring, a region of interest was placed in the centre of the cell soma. Data were normalized to the period 1 min prior drug application. To assess changes in fluorescence intensity due to drug application a One Way RM-ANOVA was performed. ANOVA was corrected by the Bonferroni adjustment. Data are expressed as mean \pm standard error of mean (SEM).

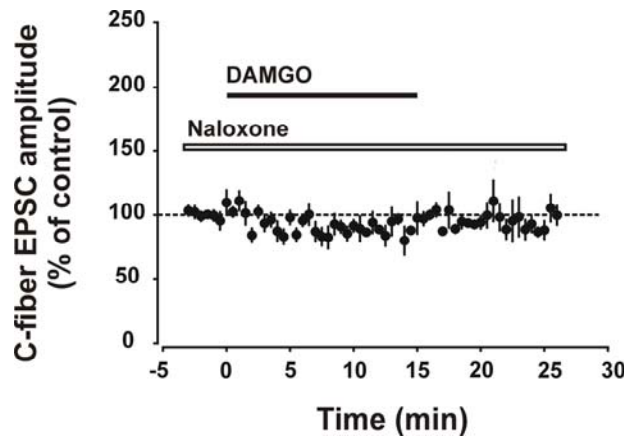


Fig S1. Opioid-induced acute depression and opioid withdrawal LTP are blocked by the opioid-receptor antagonist naloxone. The average time course of C-fiber evoked EPSCs in lamina I neurons is shown. Bath application of naloxone (10 μM) fully blocked both, acute depression and LTP induction in all 5 neurons tested ($p = 0.011$).

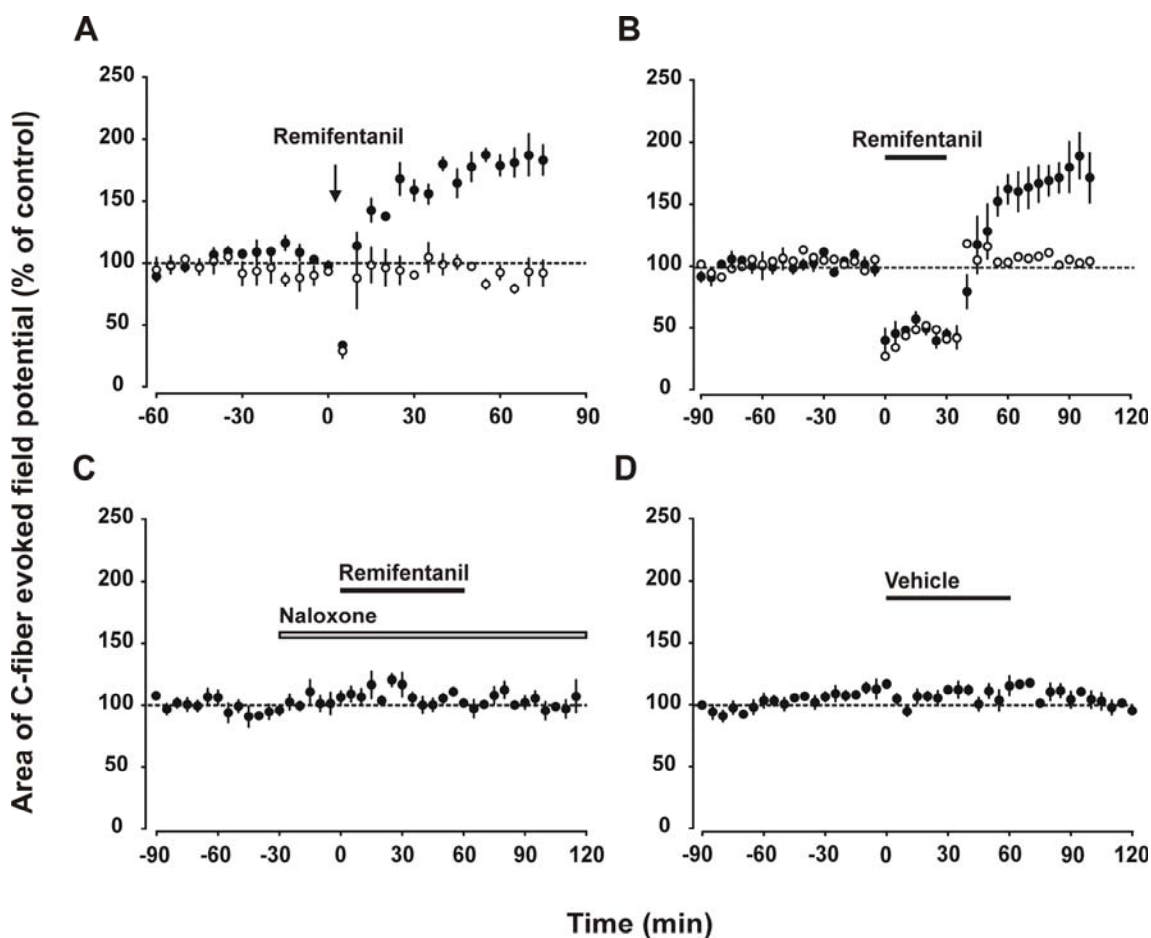


Fig S2. Intravenous infusion of remifentanyl induces LTP at C-fiber synapses *in vivo*. In all graphs, mean time course of area under the curve of C-fiber evoked field potentials are shown. Values were normalized to pre-drug values (dotted line) and plotted against time (min). **(A)** Bolus injection ($30 \mu\text{g}\cdot\text{kg}^{-1}$, downward arrow) of remifentanyl led to potentiation of C-fiber evoked field potentials upon wash-out of the opioid in 3 animals (closed circles, $p = 0.001$) whereas it had no effect in 2 other animals tested (open circles, $p = 0.375$). **(B)** Remifentanyl injected as a bolus followed by an infusion ($450 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, black horizontal bar) for 30 min induced LTP in 4 animals tested (closed circles, $p = 0.004$). No effect was observed in one animal (open circles, $p = 0.266$). **(C)** Spinal superfusion with naloxone ($10 \mu\text{M}$, open horizontal bar) abolished acute depression and opioid withdrawal LTP in all 5 animals that received a one hour remifentanyl infusion ($p = 0.001$). **(D)** The commercially available formula of remifentanyl contains glycine as a vehicle. Intravenous injection of glycine in the appropriate concentration (S7, S8) for one hour (closed bar) had no effect in any of the 4 animals tested ($p = 0.001$).

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