Synaptic Plasticity in Spinal Lamina I Projection Neurons that Mediate Hyperalgesia
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Material and Methods

Young (18-24 day-old) Sprague-Dawley rats were anesthetized with a mixture of ketamine and xylazine and placed in a stereotaxic apparatus. A hole was drilled through the skull in order to allow insertion of a 500-nl Hamilton syringe needle. The animals received a single injection of 200 nl of 2.5 % DiI or 200 nl of 1 % Fluorogold into the right parabrachial area according to the atlas of Paxinos and Watson. After a 2- to 3-day survival period, transverse 400 – 500 µm thick spinal cord slices with dorsal roots attached were obtained. Slices were incubated in a solution that consisted of (in mM): NaCl 95, KCI 1.8, KH2PO4 1.2, CaCl2 0.5, MgSO4 7, NaHCO3 26, glucose 15, sucrose 50 and was oxygenated with 95% O2, 5% CO2; pH 7.4, measured osmolarity 310-320 mOsm. For HFS experiments, slices were incubated in 10 µM remifentanil in the incubation solution for 1 hour before recording after at least 1 hour wash-out. A single slice was then transferred to a recording chamber and perfused with oxygenated recording solution at 3 ml/min at room temperature. The recording solution was identical to the incubation solution except for (in mM): NaCl 127, CaCl2 2.4, MgSO4 1.3 and sucrose 0.

To detect the labeled and non-labeled cells, slices were illuminated with a monochromator, and visualized with an upright fluorescence Olympus BX50WI microscope (Olympus, Japan), equipped with Dodt-infrared optics (S1) using a x 40, 0.80 NA water-immersion objective and a cooled CCD camera (PCO, Kelheim, Germany). Standard whole-cell patch clamp recordings were performed either in current- and voltage-clamp mode from labeled and non-labeled cells in lamina I of spinal dorsal horn as described (S2). The pipette solution consisted of (in mM): potassium gluconate 120, KCl 20, MgCl2 2, Na2ATP 2, NaGTP 0.5, HEPES 20, EGTA 0.5, pH 7.28 with KOH, measured osmolarity 300 mOsm. For HFS experiments QX-314 (5 mM) was added to the pipette solution to prevent discharges of APs.

Membrane properties were recorded, monitored and analyzed as described (S2) using an Axopatch 200B patch-clamp amplifier and the software package pCLAMP 8 (Axon Instruments, Union City, CA, USA). If not stated otherwise membrane potential was hold at –70 mV. To measure excitatory postsynaptic currents (EPSCs) from neurons in lamina I, dorsal root was stimulated through a suction electrode with an isolated current stimulator (A320, World Precision Instruments, Sarasota, FL, USA). Test pulses of 0.1 ms were given at 15 - 30 sec intervals. Intensity of test stimulation was 3 – 5 mA. Afferent input was classified as Aδ-fiber-evoked when calculated conduction velocity ranged between 2.5 and 15 m/s and as C-fiber-evoked for conduction velocities below 2 m/s. Length of the dorsal roots stimulated were between 8 and 15 mm leading to latencies between onset of stimulation artifact and of C-fiber-evoked EPSCs of up to 25 ms. Monosynaptic input was identified by the absence of failures in response to 10 stimuli given at 10 Hz (for Ad-fiber input) or at 1 Hz (for C-fiber input) stimulation of dorsal roots and low jitter in response latencies (S3). To induce synaptic LTP, high frequency stimulation (HFS, 100 pulses at 100 Hz repeated 3 times at a 10-s interval) was applied to the dorsal root with same intensity as test stimulation but at a pulse width of 0.5 ms within 2 - 4 min after establishing whole-cell configuration. HFS applied later than 5 min was ineffective. Synaptic strength was quantified by the peak amplitudes of EPSCs. The mean amplitude of test responses recorded prior to the HFS served as control. Measuring the amplitudes of five consecutive responses at 10 min and 30 min after HFS assessed significant changes from control.

To isolate Ca2+ currents, in the recording solution 30 mM TEA-Cl were substituted for equimolar NaCl, and 4-aminopyridine (4-AP, 5 mM), CsCl (2 mM) and tetrodotoxin (TTX, 0.5 µM) were added for blocking voltage dependent K+ and Na+ currents, respectively. For the same purpose, pipettes were filled with a solution containing (in mM): CsMeSO4 120, TEA-Cl 20, MgCl2 2, Na2ATP 2, NaGTP 0.5, HEPES 10, EGTA 10, pH 7.28 with CsOH, measured osmolarity 300 mOsm. Liquid junction potential was measured (8 mV) and left uncorrected. To differentiate between LVA and HVA currents, activation thresholds were measured by applying increasing depolarizing voltage steps after a prepulse potential of –90 mV and –50 mV, respectively.

Fluorometric measurements of free cytosolic Ca2+ concentrations were performed with 0.1 mM Fura-2 pentapotassium salt (Molecular Probes, USA) instead of EGTA in the pipette solution. Cells were loaded for at least 10 min via the patch pipette before measurements were started. Cells were illuminated with a monochromator, images were captured at 5 Hz with a cooled CCD camera and analyzed off-line (TILLvisION imaging system, T.I.L.L. Photonics, Gräfelfing, Germany). Consecutive paired exposures to 340 and 380 nm were used to achieve digital fluorescence images. Ca2+ influx was induced by depolarizing (300 pA) current injections of 10 - 15 ms duration at a frequency of 40 Hz for 1s in current-clamp mode. Calculations of intracellular Ca2+ concentrations were made off-line. To convert the fluorescence signals into Ca2+ concentrations, we used the isosbestic rationing method. For Ca2+ measurements...
during HFS, cells were preloaded additionally with the acetoxymethyl ester of Fura-2 (Fura-2 AM, Molecular Probes). For this purpose slices were incubated for 40 min in the oxygenated incubating solution containing 10 µM Fura-2 AM at room temperature. In order to assure dye deesterification, slices were kept in the same but Fura-free solution for 30 min thereafter. Cytosolic Ca^{2+} was measured by ratiometric (F_{340}/F_{380}) fluorescence and no conversion to concentrations was made. The drugs used were tetrodotoxin (0.5 µM, Tocris, Bristol, UK), CdCl_2 (200 µM), NiCl_2 (100 µM), verapamil (50 µM), QX-314 (5 mM), BAPTA (20 mM, all from Sigma, Deisenhofen, Germany), U73122 (10 µM), NMDA (50 µM), D-AP5 (50 µM), L703,606 (10 µM), Substance P (2 µM, all from Alexis, Grünstadt, Germany) and 2-APB (100 µM, Calbiochem, Bad Soden, Germany). Significant differences were calculated by t-test, z-test or Wilcoxon Signed Rank test (* P < 0.05, ** P < 0.01).

Supporting Online Text

Spinal lamina I neurons with a projection to the parabrachial area that do not express the NK1 receptor possess high levels of the glycine receptor-associated protein gephyrin and receive dense inhibitory innervations (S4). This specific inhibitory control may well contribute to the absence of LTP induction in these neurons, as activity in (descending) inhibitory pathways was shown to control LTP induction in superficial spinal dorsal horn in vivo (S5).

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![Diagram](image-url)

**Fig. S1.** Substance P facilitates inward currents through NMDA receptor channels. Substance P (SP) supra-additively increased inward currents induced by bath application of NMDA in PNs but not UNs. (A) Original traces showing inward currents induced by application of NMDA (100 µM) for 10 s at 4 min-intervals in a PN (a) and an UN (b). Co-application of SP (2 µM for 10 s) with NMDA at time point 4 min significantly increased the NMDA-induced current in PNs but not UNs (P < 0.01). (c) shows the mean peak inward currents evoked by NMDA in PNs (filled circles) and UNs (open circles) using the protocol described in (a) and (b). (B) The potentiation of the NMDA-evoked inward current by co-application of SP in PNs was prevented in the presence of the PLC inhibitor U73122 (10 µM). The inward currents were normalized to the values obtained at the first NMDA application (0 min). Application of NMDA alone for 4 times at 4 min intervals yielded inward currents of constant amplitude (mean current at 4 min 397 ± 43 pA, n = 5, open triangles). Co-application of SP potentiated the NMDA-evoked inward current (mean at 4 min 612 ± 102 pA, n = 6, filled circles). The presence of U73122 prevented the potentiation of NMDA-evoked inward currents by SP (filled squares). Inward currents induced by substance P alone had mean amplitudes of 27 ± 6 pA, n = 17.
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<th>Unidentified neurons</th>
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<td>Afterhyperpolarizing depth (mV)</td>
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Tab. S1. Membrane properties of spinal lamina I projection neurons retrogradely labeled from the parabrachial area with DiI or with Fluorogold (FG) as compared to unidentified neurons from S2. Significantly different from unidentified neurons: * P < 0.05, ** P < 0.01.

References