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Supporting Online Material for

Spinal Endocannabinoids and CB_1 Receptors Mediate C-Fiber–Induced Heterosynaptic Pain Sensitization

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Materials and Methods

Electrophysiological recordings in spinal cord slices. Transverse 250 – 300 μm thick slices of the lumbar spinal cord were prepared from 10 – 20 day old C57BL/6J mice as described previously (*S1*). Whole-cell patch-clamp recordings were made at room temperature from dorsal horn neurons visually identified with infrared gradient contrast equipment (*S2*). Slices were continuously superfused with extracellular solution containing (in mM) 120 NaCl, 5 Na-HEPES, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose (pH 7.30) and bubbled with 95% O_2 / 5% CO_2. Recording pipettes were filled with intracellular solution containing (in mM): 130 K^+-gluconate, 20 KCl, 2 $MgCl_2$, 0.05 EGTA, 3 Na^+-ATP, 0.1 Na^+-GTP, 10 Na^+-HEPES (pH 7.30) and 5 QX-314. Permission for tissue preparation has been obtained from the Veterinäramt des Kantons Zurich (ref. no. 37/2007).

Electron microscopy. Preparation of spinal cord sections of four adult wild-type C57BL/6-J mice for morphological experiments (peroxidase-based immunocytochemistry and pre-embedding immunogold labeling) and electron microscopy were performed as described previously (*S3*). CB_1 receptors were labeled with one of two polyclonal guinea pig anti-CB_1 antisera (1:150 - 1:200 and 1:530; ~1 μg/ml, *S4*). Vesicular inhibitory aminoacid transporter (VIAAT) protein was labeled with a polyclonal affinity-purified rabbit anti-VIAAT antibody (1:1000 - 1:2000; 1 - 0.5 μg/ml, respectively). The specificity of the anti-CB_1 antibodies was confirmed by the lack of immunostaining in $CB_1^{-/-}$ mice (S*5*). For immunoperoxidase stainings, biotinylated goat anti-guinea pig IgG or biotinylated goat anti-rabbit IgG (both 1:300) were used. In the immunogold staining procedure, sections were incubated in 0.8 nm gold conjugated goat anti-guinea pig or goat anti-rabbit antibodies (both 1:50). Sections were then silver intensified. In the double immunostaining experiments, sections were either developed first for immunogold and then for immunoperoxidase staining or vice versa. Tissue preparation and killing of the mice were carried out according to the guidelines of the institutional ethical code and the Hungarian Act of Animal Care and Experimentation (1998. XXVIII. Section 243/1998.).

***In vivo* electrophysiology.** Adult (250 - 350 g) Sprague Dawley rats were prepared for *in vivo* extracellular single-unit recordings as described previously (*S6, S7*). Action potential firing of individual neurons with receptive fields in the hindpaw was recorded in the deep dorsal horn (600 - 1100 μm) of the lumbar enlargement of the spinal cord (L5/6). Individual neurons were identified by the shape of the recorded action potentials elicited by innocuous and noxious mechanical stimulation of the skin. Neurons were characterized by their responses to the following stimuli applied to the receptive field: innocuous brush (brushing the skin), innocuous pressure (100 g / 6 mm^2) and noxious pinch (400 g / 6 mm^2). Pressure and pinch were applied by means of forceps, which was connected to a force transducer allowing the recording and tight control of the actually applied force. All cells included in this study were wide-dynamic-range (WDR) neurons responding consistently to innocuous and noxious stimulation. After initial characterization, capsaicin (200 μg in 20 μl saline and Tween 80) was injected intracutaneously into the receptive field. Responses of individual dorsal horn neurons were recorded before and for 2 h after capsaicin injection. Each mechanical stimulus was applied for 10 s followed by a 10 s pause. The entire sequence of mechanical stimuli (brush, pressure, and pinch) was repeated three times before capsaicin injection and then every 30 min. Care was taken that the mechanical test stimuli were applied to areas away from the capsaicin injection site, i.e., in the zone of secondary hyperalgesia. All drugs (the CB_1 receptor antagonist AM 251 and the mGluR1 antagonist LY 367,385) were administered 30 min after the capsaicin injection for 30 min directly to the dorsal surface of the spinal cord into a trough,

which was continuously perfused with oxygenated (95% O_2, 5% CO_2) extracellular solution at a rate of 2 µl/min.

Behavioral testing. Behavioral testing was done in wild-type mice (C57BL/6J), $CB_1^{-/-}$ mice (genetic background C57BL/6N, *S7*), *sns*-$CB_1^{-/-}$ mice (mixed genetic background C57BL/6-J x C57BL/6N; *S8*) and *pft1a*-$CB_1^{-/-}$ mice (mixed genetic background C57BL/6 x DBA/2J; *S9*) of either sex. *sns*-$CB_1^{-/-}$ and *pft1a*-$CB_1^{-/-}$ mice were obtained by crossing mice carrying two alleles of CB_1 receptor gene flanked by loxP sites ($CB_1^{fl/fl}$ mice) with $CB_1^{fl/fl}$ mice expressing in addition the *cre* recombinase either in primary nociceptors (*sns*-cre mice; *S10*) or in dorsal horn inhibitory interneurons (*ptf1a*-cre mice; *S9, S11*).
Adult (7 – 8 week old) mice were kept in the test cages for one day before the experiment started to allow accommodation. On day 2, each mouse was tested several times to obtain baseline paw withdrawal thresholds to stimulation with electronically controlled von-Frey filaments. At least three independent measurements were made for each time point. In all behavioral experiments, the observer was blind to the treatment or genotype of the mice. For intrathecal injections AM 251, LY 367,385, URB 597 and UCM 707 were dissolved in 10% DMSO, 90% artificial cerebrospinal fluid (ACSF) and injected in a total volume of 5 µl. MPEP and CP 55,940 were dissolved in 20% DMSO / 80% ACSF and 50% DMSO / 50% ACSF, respectively. Intrathecal DMSO up to 5 µl, 50% had no effects on mechanical responses in naïve mice or in mice with inflamed or neuropathic paws. Intrathecal injections were made into the lower lumbar spinal canal under light anesthesia using a Hamilton syringe (for details see *S12*). All behavioral experiments were performed in an air-conditioned room (22°C) between 9 am and 6 pm. Capsaicin was dissolved in 10% ethanol, 10% Tween 80 and 80% ACSF and injected at a dose of 30 µg in 10 µl subcutaneously into the plantar side of the left hindpaw. Zymosan A (0.06 mg in 20 µl 0.9% NaCl) was injected subcutaneously into the plantar side of the left hindpaw (*S12*). Chronic constriction of the left sciatic nerve was performed as described previously (*S13*). Allodynia in mice following intrathecal CP 55,940 injection was tested by gently stroking the mouse flanks with a paintbrush. Reactions of mice were scored as 0, no reaction, 1, moderate effort to escape, 2, vigorous effort to escape, episodic jumping during stroking. Six measurement per mouse were averaged for each time point
All behavioral testing in genetically modified mice were done with wild-type or $CB_1^{fl/fl}$ littermates as controls. Observers were blind to the genotype or treatment. After the tests the mice were killed by CO_2 inhalation. Permission for behavioral testing has been obtained from the Veterinäramt des Kantons Zurich (ref. no. 92/2007).

Human volunteer study. Permission was obtained from the Ethics Committee of the Medical Faculty of the University of Erlangen-Nürnberg (ref. no. 3583). The effects of the CB_1 receptor antagonist rimonabant on secondary hyperalgesia were tested in a human pain model (*S14*). Intracutaneous electrical stimuli were applied to the left forearms of healthy human volunteers at C-fiber intensity (15 – 100 mA, 500 µs) and at a frequency of 2 Hz for 115 min. This stimulation paradigm induces acute pain at the site of electrical stimulation, a flare response in the skin territories innervated by the activated C-fibers and hyperalgesia and allodynia in skin areas surrounding the stimulation site. Pain intensities were measured on a numeric rating scale (NRS) ranging from 0, no pain, to 10, maximum imaginable pain. Pin-prick hyperalgesia and allodynia (touch-evoked pain) were assessed using a 256 mN von-Frey filament and a cotton wool tip, respectively. Statistical analyses were performed on the integral (area under the curve) of pain ratings and hyperalgesic and allodynic area sizes (cm^2) over time (min).
16 healthy volunteers (11 males and 5 females) were randomized into two age- and sex-matched groups. Basic

demographic parameters of the volunteers are given in Tab. S1. Each volunteer was tested in two sessions 28 day apart: session 1, before treatment and session 2, after a 10 days treatment with rimonabant or placebo. During the first 15 min of the first session, the intensity of electrical stimulation was adjusted in each volunteer individually to yield a pain rating of 6 on the NRS. Stimulation was then continued for another 100 min. Pain ratings and the sizes of hyperalgesic and allodynic skin areas were determined during the entire experiment at regular intervals. Typically, pain ratings declined by about 20% during the session, while the sizes of hyperalgesic and allodynic skin areas remained virtually constant. Differences between the two groups in current intensities required for a pain rating of 6 (on a numeric rating scale from 0 to 10) and the sizes of hyperalgesic or allodynic skin areas were statistically not significant. On day 19 after the first session, volunteers were either treated with rimonabant at an oral dose of 20 mg once per day, or with placebo. Because of the long half-life of rimonabant treatment was performed on 10 consecutive days, which should be sufficient to reach > 90% of the steady state plasma level (*S15*). The second session started 5 hours after of the last rimonabant or placebo administration and pain ratings and the sizes of hyperalgesic and allodynic skin areas were again measured. Of the 8 volunteers treated with rimonabant, 3 complained about nausea, 1 about fatigue and 1 about depression. In the placebo treated group one volunteer complained about reduced appetite.

Supplementary Figures

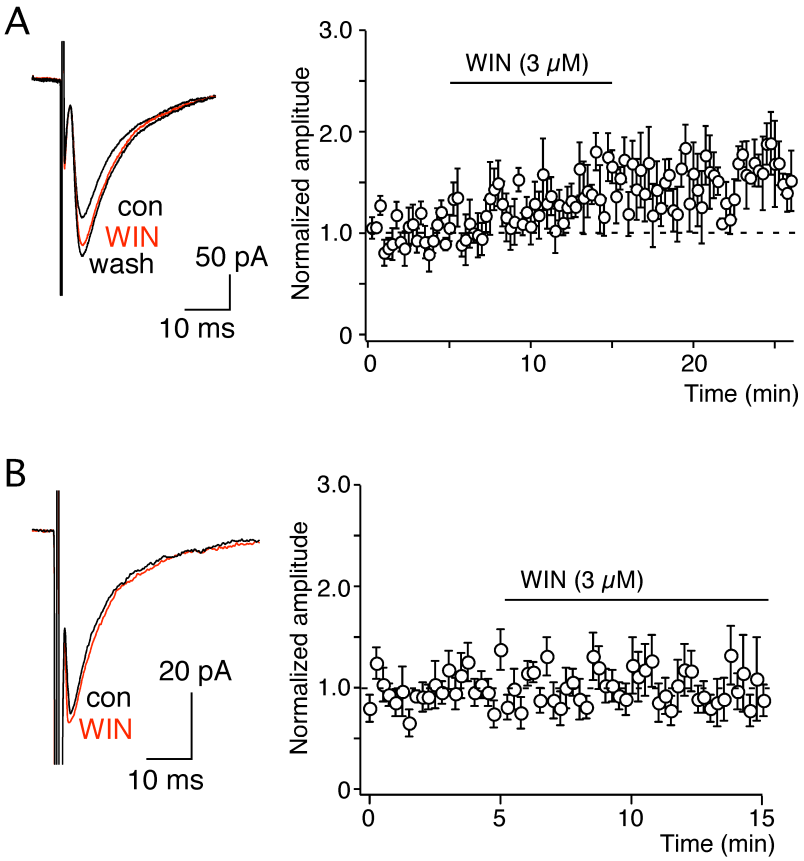

Fig. S1. No inhibition of gly-IPSCs by WIN 55,212-2 (3 μM) in global $CB_1^{-/-}$ mice (A, n = 6) and in dorsal horn inhibitory interneuron-specific *ptf1a*-$CB_1^{-/-}$ mice (B, n = 8). Left, gly-IPSCs recorded in the superficial dorsal horn averaged from 10 consecutive current traces. Right, time course (mean ± sem).

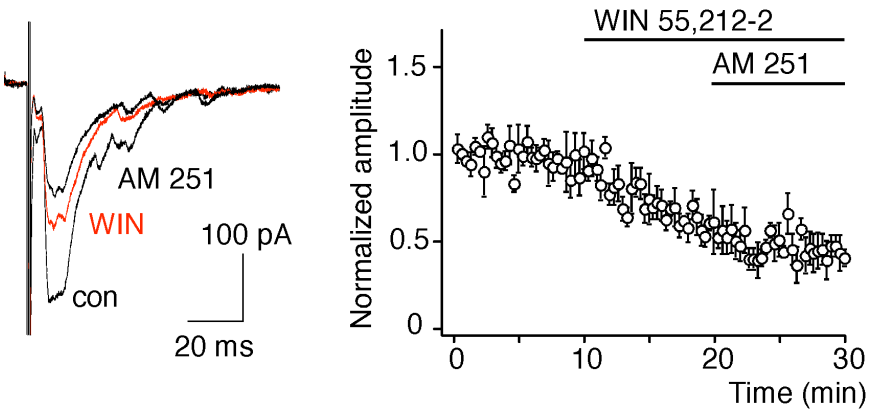

Fig. S2. Inhibition by WIN 55,212-2 (3 μM) of primary afferent EPSCs. Dorsal rootlets were stimulated electrically at C-fiber intensity (>15 V, 500 μs). Left: averaged EPSC trace, right: time course (mean ± sem, n = 4). WIN 55,212-2-induced inhibition of dorsal root stimulation evoked EPSCs was not reversed by AM 251 (5 μM).

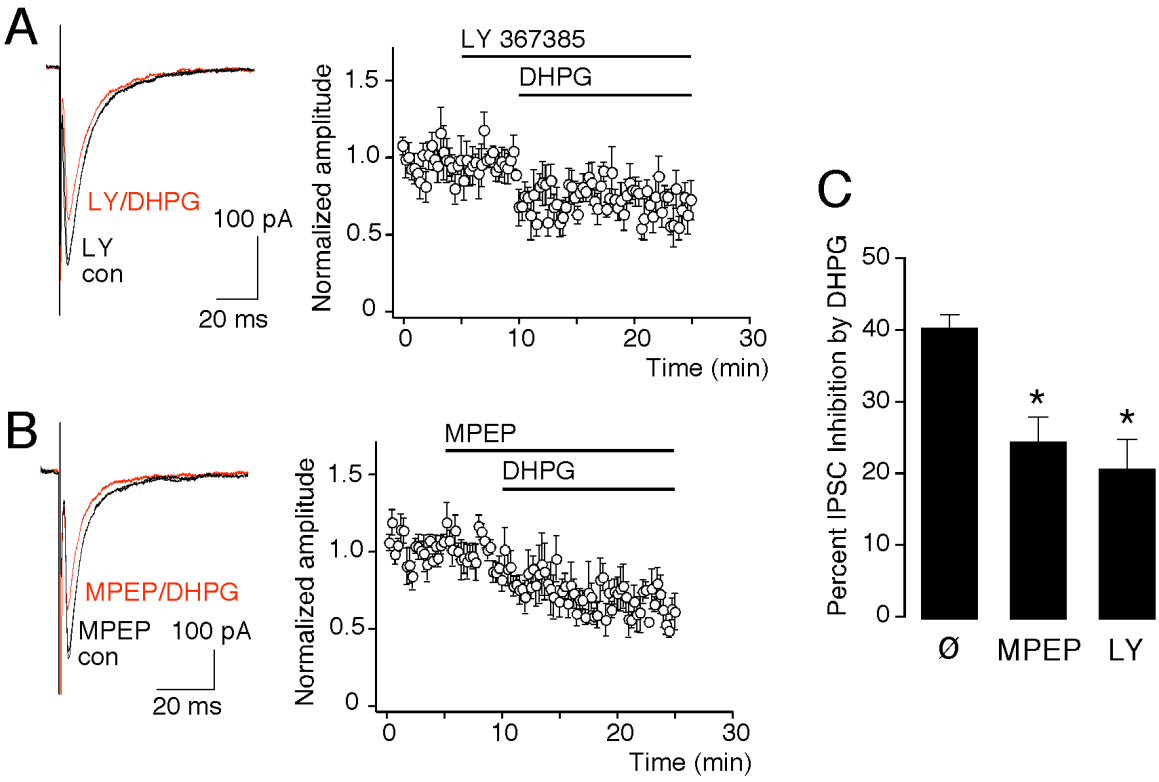

Fig. S3. The mGluR1 antagonist LY 367,385 (100 μM, n = 5) and the mGluR5 antagonist MPEP (10 μM, n = 5) both partially prevented DHPG-induced inhibition of glycinergic IPSCs. *, $P < 0.05$, ANOVA followed by Bonferoni post hoc test.

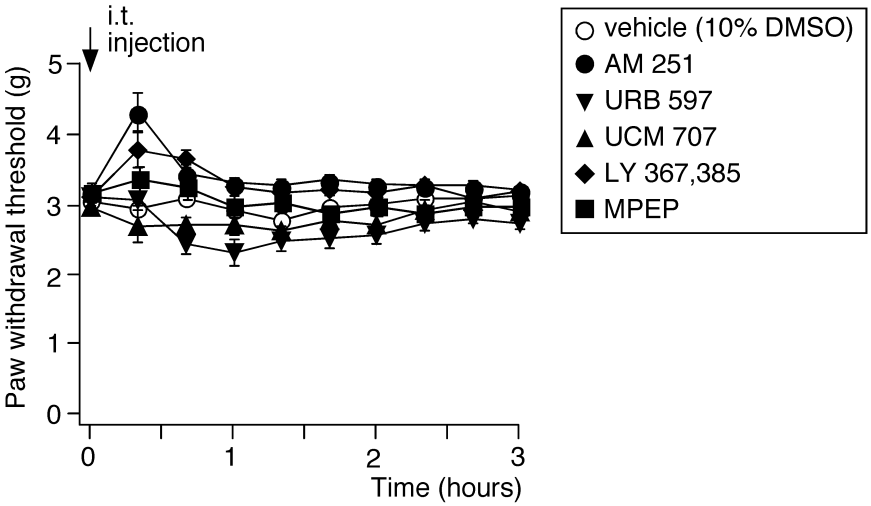

Fig. S4. Effects of AM 251 (0.5 nmoles / mouse i.t.), URB 597 (1.0 nmole), UCM 707 (1.0 nmole), LY 367385 (1.0 nmole) and MPEP (150 nmoles) on mechanical paw withdrawal thresholds (mean ± sem) in naïve mice, n = 6 / group.

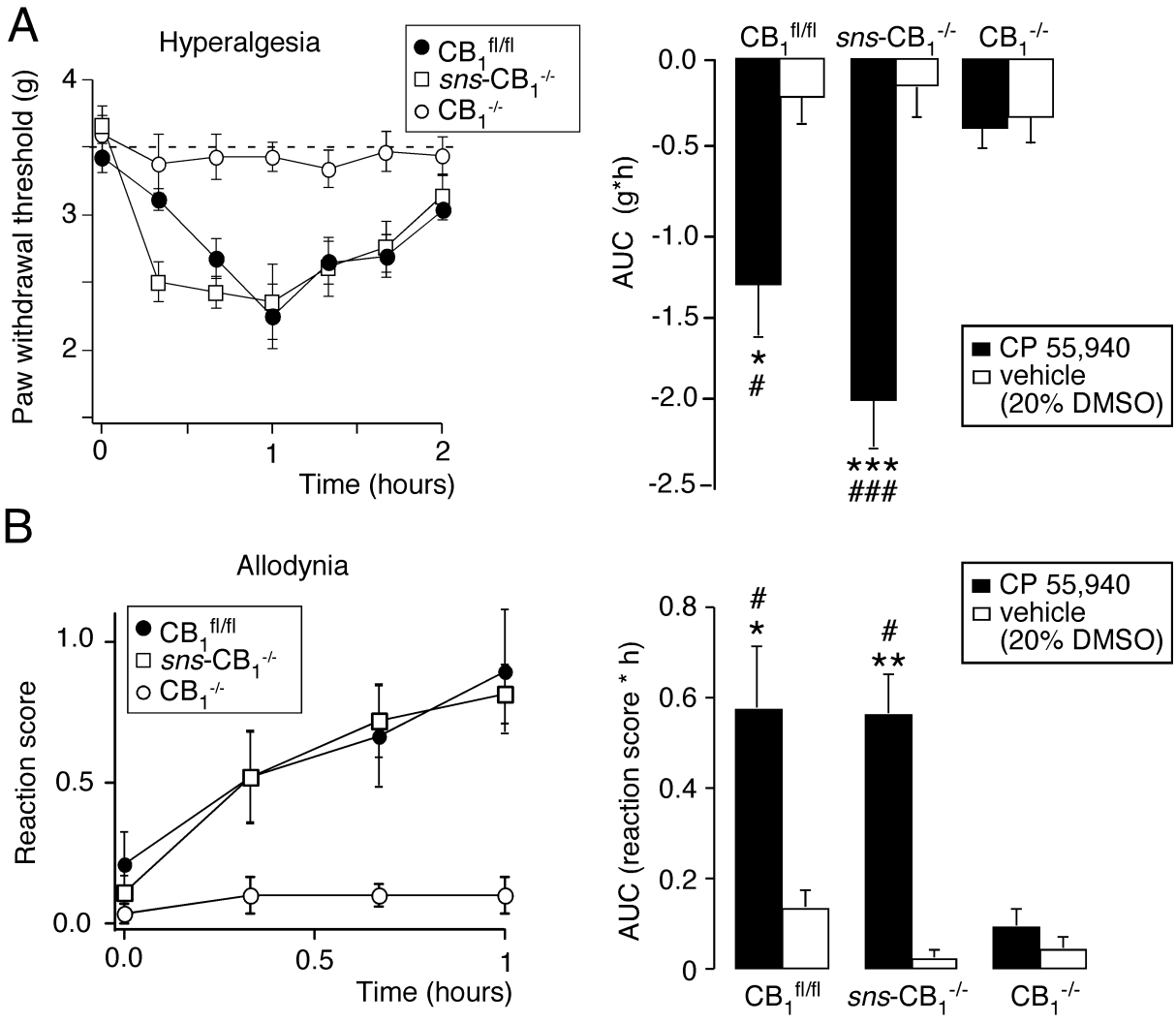

Fig. S5. Mechanical sensitization after intrathecal injection of CP 55,940 (10 nmoles / mouse) in wild-type ($CB_1^{fl/fl}$), *sns*-$CB_1^{-/-}$ and $CB_1^{-/-}$ mice (n = 5 – 8 mice / group). (**A**) Mechanical paw withdrawal thresholds to von Frey filament stimulation. (**B**) Reaction scores (0 – 2) determined upon light mechanical stimulation of mouse' flank with a paintbrush. Right panels in A and B are areas under the curve (AUC, mean ± sem) integrated for 2 hour (A) or 1 hour (B) after i.t. injection of CP 55,940. 10-fold and 100-fold lower doses of CP 55,940 (0.1 and 1 nmol) were without effect (data not shown). ANOVA followed by Bonferroni post-hoc test F (5,37) = 13.5. *, $P = 0.05$; **, $P = 0.001$ significant versus vehicle; #, $P = 0.05$; ###, $P = 0.001$ significant versus $CB_1^{-/-}$.

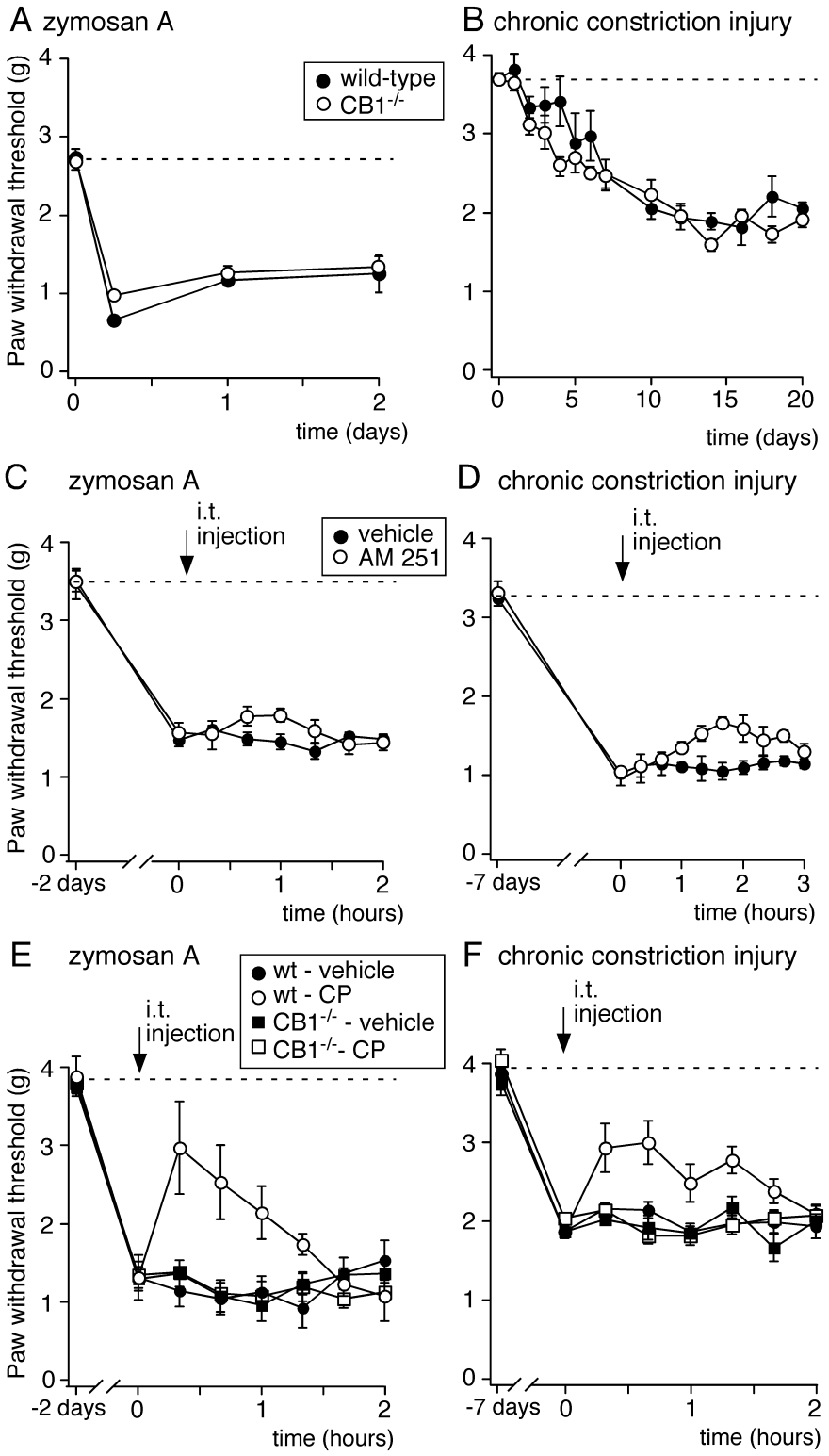

Fig. S6. Global CB_1 receptor gene deletion (**A,B**) and CB_1 receptor blockade with intrathecal AM 251 (0.5 nmol; **C,D**) had only little effects on mechanical sensitization in an inflammatory pain model (subcutaneous zymosan A injection, **A,C**) and in a neuropathic pain model (chronic constriction of the sciatic nerve, **B,D**). (**E,F**) Activation of spinal CB_1 receptors with CP 55,940 (10 nmol) partially reverses inflammatory and neuropathic mechanical sensitization. n = 6 mice / group. Vehicle contained 10% and 20% DMSO in A,B and E,F, respectively.

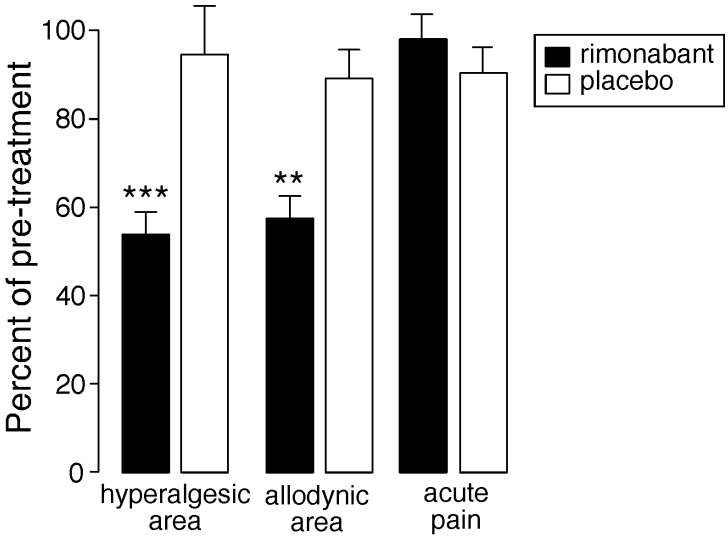

Fig. S7. Effects of CB_1 receptor blockade with rimonabant on C-fiber-induced pin-prick hyperalgesia, touch-evoked allodynia and acute pain ratings in human volunteers. Allodynic and hyperalgesic skin areas and pain ratings were integrated over time for 100 min starting from the beginning of the electrical C-fiber stimulation and compared to pre-treatment conditions (n = 8 volunteers / group). *, $P < 0.05$, **, $P < 0.01$, against pre-treatment, paired Student t-test, corrected for 3 independent comparisons. Effects of placebo were statistically not significant ($P = 0.17$, 0.40 and 0.26) for pain, hyperalgesia and allodynia, respectively.

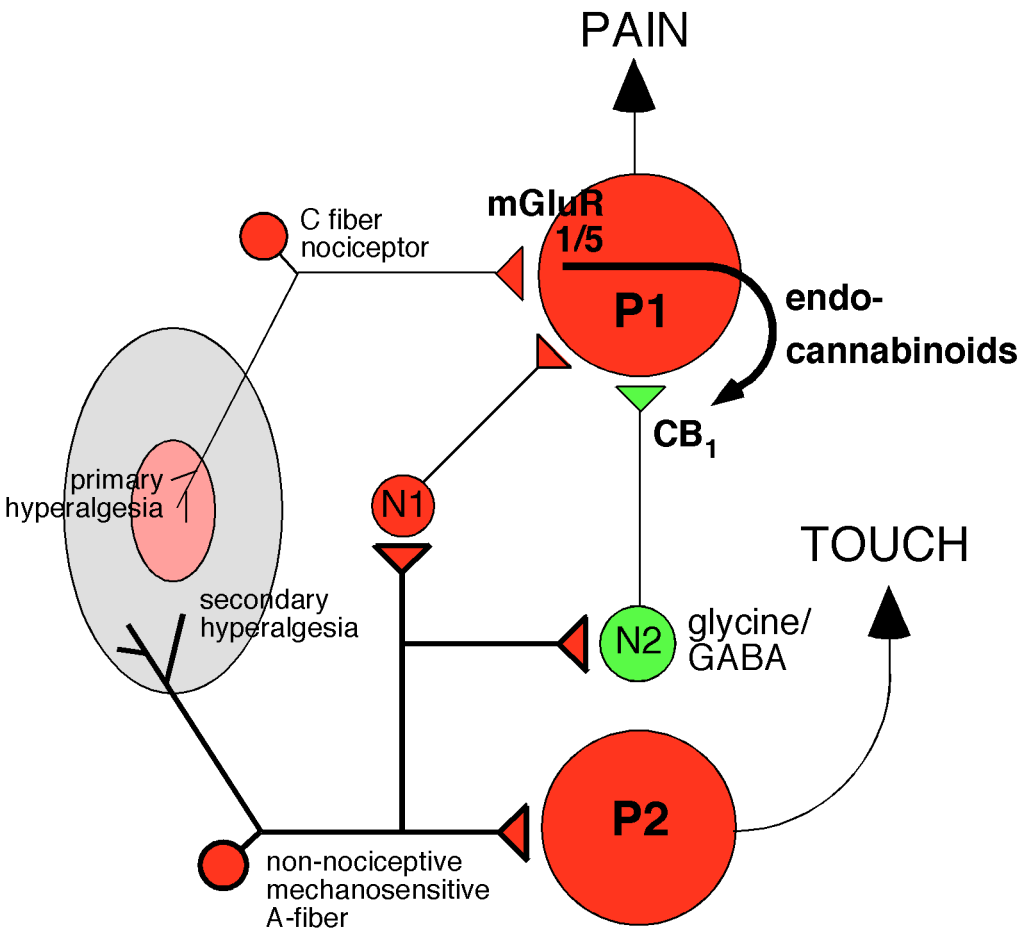

Fig. S8. Schematic representation of a neuronal model circuit of the spinal dorsal horn possibly underlying activity-dependent and endocannabinoid-mediated secondary hyperalgesia. Excitatory and inhibitory neurons are shown in red and green, respectively. Output neurons of the spinal dorsal horn located in lamina I (P1) are normally excited above threshold only by input from nociceptive fibers, while non-nociceptive mechanosensitive A-fibers activate primarily output neurons located in the deep dorsal horn (P2) and excitatory (N1) and inhibitory (N2) interneurons. Although neurons in lamina I are not directly targeted by non-nociceptive A-fibers, they receive polysynaptic input from them. Suprathreshold excitation is normally prevented by simultaneous inhibitory input from local GABAergic and glycinergic interneurons (N2), which are also activated by non-nociceptive mechanosensitive A fibers.

Under conditions of strong nociceptive stimulation, intense C-fiber input activates postsynaptic group I metabotropic glutamate receptors (mGluR1/5) located at dorsal horn neurons and induces production and release of endocannabinoids. These endocannabinoids subsequently activate CB_1 receptors located at the presynaptic terminals of inhibitory GABAergic and glycinergic neurons, where they inhibit transmitter release. This reduced inhibitory control renders normally pain-specific lamina I (P1) neurons susceptible to activation by non-nociceptive A-fibers and leads to secondary hyperalgesia and touch-evoked pain.

	rimonabant	placebo	Statistical significance (t-test) *P*
Size (cm)	171 ± 2.7	174 ± 2.2	0.36
Weight (kg)	69.5 ± 4.0	68.9 ± 4.0	0.91
BMI (kg/m^2)	22.5 ± 0.8	23.7 ± 1.2	0.43
Age (years)	25.3 ± 1.3	25.7 ± 1.8	0.83
Current intensity (mA) required to yield a NRS value of 6 in the first session	77.5 ± 7.0	65.9 ± 12	0.42

Tab. S1. Basic demographic parameters and electrical stimulation strengths of the volunteers participating in the rimonabant study.

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