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Spinal Endocannabinoids and CB₁ Receptors Mediate C-Fiber-Induced Heterosynaptic Pain Plasticity

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Abstract

Diminished synaptic inhibition in the spinal dorsal horn is a major contributor to chronic pain. Pathways, which reduce synaptic inhibition in inflammatory and neuropathic pain states, have been identified, but central hyperalgesia and diminished dorsal horn synaptic inhibition also occur in the absence of inflammation or neuropathy, solely triggered by intense nociceptive (C-fiber) input to the spinal dorsal horn. We found that endocannabinoids produced upon strong nociceptive stimulation activated CB₁ receptors on inhibitory dorsal horn neurons to reduce the synaptic release of GABA and glycine and thus rendered nociceptive neurons excitable by non-painful stimuli. Spinal endocannabinoids and CB₁ receptors on inhibitory dorsal horn interneurons act as mediators of heterosynaptic pain sensitization and play an unexpected role in dorsal horn pain controlling circuits.

Activity-dependent central hyperalgesia can be induced in the absence of any inflammation or nerve damage by selective activation of glutamatergic C-fiber nociceptors, e.g. with the specific transient receptor potential channel (TRP) V1 agonist capsaicin. Local subcutaneous injection of capsaicin induces primary hyperalgesia at the site of injection and a purely mechanical secondary hyperalgesia in the surrounding healthy skin (1). This secondary hyperalgesia originates from changes in the central processing of input from mechanosensitive A-fibers and is characterized by an exaggerated sensitivity to painful stimuli and by pain evoked by light tactile stimulation (allodynia or touch-evoked pain). These symptoms are mimicked by the blockade of inhibitory GABAergic and glycinergic neurotransmission in the spinal dorsal horn (2,3) suggesting that a loss of synaptic inhibition also accounts for C-fiber-induced secondary hyperalgesia. Activity-dependent hyperalgesia can thus be regarded as a correlate of heterosynaptic depression of inhibition (4). In many neuronal circuits of the CNS,

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endocannabinoids (2-arachidonoyl glycerol [2-AG] and anandamide [AEA]) are released upon intense activation of metabotropic glutamate receptors and serve as retrograde messengers mediating either homosynaptic feedback inhibition or heterosynaptic depression of (GABAergic) inhibition (5,6). CB₁ receptors are densely expressed in the superficial dorsal horn of the spinal cord (7), where they exert antihyperalgesia in different inflammatory or neuropathic diseases states (8,9).

To define the role of CB₁ receptors in dorsal horn neuronal circuits, we first characterized the effects of CB₁ receptor activation on neurotransmission in mouse transverse spinal cord slices (Fig. 1). Excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were evoked by extracellular electrical field stimulation at a frequency of 4 / min and recorded from visually identified neurons in the superficial spinal dorsal horn (laminae I and II) (10). The mixed CB₁/CB₂ receptor agonist WIN 55,212-2 (3 μ M) reversibly reduced the amplitudes of glycine receptor IPSCs to $64.3 \pm 3.5\%$ of control amplitudes (mean $\pm$ sem, $n = 13$ neurons, $P < 0.001$, paired Student t-test) (Fig. 1A). Similarly, GABA_A receptor IPSCs were reduced to $64.7 \pm 3.0\%$ ($P < 0.001$, $n = 8$, paired Student t-test) (Fig. 1B). Inhibition of IPSCs by WIN 55,212-2 was confined to the superficial dorsal horn, reversed by the CB₁ receptor antagonist/inverse agonist AM 251 (5 μ M) (Fig. 1A,B) and absent in global CB₁ receptor-deficient mice (CB₁^{-/-} mice; 11) and in mice lacking CB₁ receptors specifically in dorsal horn inhibitory interneurons (*ptf1a*-CB₁^{-/-} mice; 12) (Fig. S1). WIN 55,212-2 had virtually no effect on EPSCs mediated by glutamate receptors of the α -amino-3-hydroxy-5-methylisoxazole (AMPA) subtype (AMPA-EPSCs) (Fig. 1C, see also 13). It did, however, reduce the amplitudes of monosynaptic AMPA-EPSCs evoked by stimulation of dorsal root primary afferent nerve fibers at C-fiber intensity by $34.5 \pm 3.3\%$ ($n = 9$). This inhibition was not reversed by AM 251 (Fig. S2). We next tested whether stimulation of endogenous endocannabinoid production through activation of group I metabotropic glutamate receptors (mGluR1/5) would have a similar effect on inhibitory synaptic transmission. These experiments were carried out in GlyT2-EGFP transgenic mice, which allowed targeted recordings from glycinergic (EGFP-positive) and non-glycinergic (EGFP-negative), presumed excitatory, interneurons (14). (S)-3,5-dihydroxyphenylglycine (DHPG, 10 μ M), an agonist at mGluR1/5, reduced IPSC amplitudes in non-glycinergic presumed excitatory superficial dorsal horn neurons by $40.6 \pm 4.5\%$ ($n = 8$) (Fig. 1D). This inhibition was reversed by AM 251 (5 μ M) (Fig. 1D) and partially prevented by mGluR1 and mGluR5 antagonists (LY 367385, 100 μ M, remaining inhibition: $21.0 \pm 3.9\%$, $n = 5$, and MPEP, 10 μ M, $25.0 \pm 3.4\%$, $n = 5$) (Fig. S3). Glycinergic input to EGFP-positive (glycinergic) neurons was less sensitive to DHPG with an average reduction of only $10.3 \pm 3.6\%$ ($n = 8$) (Fig. 1D). Depolarization-induced suppression of inhibition (DSI) could be induced in 6 out of 8 non-glycinergic neurons, but was not seen in glycinergic neurons ($n = 5$) (Fig. 1E).

Reduction of inhibitory synaptic transmission by endocannabinoids was due to diminished release of GABA and glycine from inhibitory nerve terminals. In paired pulse experiments, WIN 55,212-2 (3 μ M) increased the amplitude ratio (P2/P1) of two consecutive IPSCs, 70 ms apart, from 1.14 ± 0.07 to 1.61 ± 0.15 ($n = 5$, $P < 0.05$, paired Student t-test) (Fig. 2A). Accordingly, the coefficient of variation ($CV = (SD^2/\text{mean}^2)^{1/2}$) of IPSC amplitudes (15) increased from 0.190 ± 0.012 under control condition to 0.306 ± 0.031 in the presence of WIN 55-212-2 again indicative of a presynaptic action ($n = 13$, $P < 0.01$, paired Student t-test) (Fig. 2B). We directly demonstrated the presence of CB₁ receptors on the presynaptic terminals of inhibitory mouse superficial dorsal horn neurons by electron microscopy (Fig. 2C–F). Peroxidase-based and immunogold labeling of CB₁ receptors and high-resolution electron microscopy unequivocally showed the presence of CB₁ receptors on presynaptic terminals of symmetrical (inhibitory) synapses (Fig. 2C–D) and the colocalization of CB₁ with the vesicular inhibitory amino acid transporter (VIAAT, Fig. 2E–F), a marker of inhibitory axon terminals (16).

We next studied the role of endocannabinoids in secondary hyperalgesia in intact rats and performed *in vivo* extracellular single unit recordings (10) from wide dynamic range neurons (i.e. neurons responding to both noxious and innocuous stimulation) with receptive fields in the hindpaw and located in the deep lumbar dorsal horn (Fig. 3). Intracutaneous injection of capsaicin (200 μ g in 20 μ l) into the receptive field of the recorded neuron led to a robust increase in action potential firing in response to mechanical stimulation in an area surrounding the capsaicin injection site, akin to secondary hyperalgesia and allodynia. This increase was reversed not only by local spinal application of the mGluR1 antagonist LY 367,385 (10 μ M, $n = 5$), but also of different concentrations of the CB₁ receptor blocker AM 251 (5 μ M, $n = 6$ neurons).

In mice, we tested the effects of pharmacological and genetic manipulation of the endocannabinoid system on capsaicin-induced secondary hyperalgesia (Fig. 4). Subcutaneous injection of capsaicin (30 μ g in 10 μ l) into one hindpaw of wild-type mice led to a reduction in paw withdrawal thresholds in response to mechanical stimulation with dynamic von-Frey filaments from 2.85 ± 0.04 g under control conditions to 0.53 ± 0.10 g (mean $\pm$ sem, $n = 6$ mice) at 2 hours after capsaicin injection (10). Intrathecal injection (i.e. injection into the lumbar spinal canal, i.t.) of the mGluR1 antagonist, LY 367,385 (1.0 nmol / mouse) two hours after capsaicin reduced mechanical sensitization by $64.9 \pm 2.9\%$ ($n = 6$) (17). Consistent with the role of CB₁ receptors in synaptic disinhibition, intrathecal AM 251 (0.5 nmol) reversed mechanical sensitization by $71.2 \pm 9.0\%$ ($n = 6$). Accordingly, inhibition of endocannabinoid degradation with URB 597 or of endocannabinoid re-uptake with UCM 707 (each 1.0 nmol) (18) prolonged secondary hyperalgesia (Fig. 4A). In naive mice, all five compounds exerted only minor effects on mechanical sensitivity (Fig. S4).

Global CB₁^{-/-} mice and *ptfla*-CB₁^{-/-} mice were protected from capsaicin-induced mechanical sensitization. By contrast, mice devoid of CB₁ receptors only in primary afferent nociceptors (*sns*-CB₁^{-/-} mice; 19) developed normal secondary hyperalgesia (Fig. 4B) indicating that the CB₁ receptors on inhibitory dorsal horn neurons and not those on primary nociceptors mediated capsaicin-induced secondary hyperalgesia. Unchanged responses of *sns*-CB₁^{-/-} mice also indicate that possible direct interactions of CB₁ receptors with TRPV1 channels (20,21) expressed on the spinal terminal of primary nociceptors were not involved.

Mechanical sensitization could also be evoked by intrathecal injection of the CB₁/CB₂ agonist CP 55,940 (Fig. S5). Intrathecal CP 55,940 (10 nmol) significantly decreased the thresholds of mechanical stimulation with von Frey filaments in wild-type (CB₁^{fl/fl}) and *sns*-CB₁^{-/-} mice and rendered both types of mice extremely sensitive to touch. In both tests, mechanical sensitization by CP 55,940 was absent in global CB₁^{-/-} mice. The pronociceptive effects of endocannabinoids suggested here are specific for C-fiber-mediated, activity-dependent hyperalgesia. In models of mild inflammatory pain (subcutaneous zymosan A injection; 10) and of neuropathic pain (chronic constriction injury; 10) CB₁^{-/-} mice behaved normally (Fig. S6 A,B). AM 251 had only negligible effects (Fig. S6 C,D), while CP 55,940 exerted antihyperalgesic actions in these models (Fig. S6 E,F). Both of these models also involve spinal disinhibitory processes but the underlying mechanisms are most likely different and involve the spinal release of pronociceptive prostaglandin E₂ (22) and changes in the transmembrane chloride gradient (23).

Finally, we tested the effect of CB₁ receptor blockade on C-fiber-induced secondary hyperalgesia and allodynia in human volunteers (Fig. S7). Secondary hyperalgesia was induced by intracutaneous electrical stimulation at C-fiber strength (2 Hz, 15 – 100 mA) of a small skin area of the left forearm, 10). In the first session, the intensity of electrical stimulation was adjusted to yield a value of 6 on a numeric rating scale ranging from 0, no pain, to 10, maximum imaginable pain, and pain ratings and the sizes of hyperalgesic skin areas surrounding the site

of electrical stimulation were determined for 100 min at regular intervals. In a second session, 28 days later, the volunteers were tested again after a 10-day treatment with either placebo or rimonabant (20 mg / day, p.o.), a CB₁ receptor antagonist/inverse agonist closely related to AM 251. Rimonabant treatment had no effect on acute pain ratings induced by electrical stimulation ($-2.0 \pm 5.7\%$, $n = 8$ volunteers / group), but decreased the sizes of hyperalgesic and allodynic skin areas to $53.7 \pm 5.2\%$ and to $57.4 \pm 5.0\%$, respectively.

The contribution of endocannabinoids to activity-dependent pain sensitization, which we propose here, builds upon a model of secondary hyperalgesia and allodynia (Fig. S8), in which normally “pain-specific” dorsal horn neurons receive not only monosynaptic input from C-fiber nociceptors but also polysynaptic input from non-nociceptive fibers (24). The suprathreshold activation of these neurons by such non-nociceptive input is normally prevented by the activity of dorsal horn inhibitory interneurons. The present study shows that intense glutamatergic input from C-fiber nociceptors diminishes this inhibitory control through endocannabinoids acting at CB₁ receptors located on dorsal horn inhibitory interneurons. Our findings thus attribute to endocannabinoids an unexpected role in dorsal horn neuronal circuits as mediators of spinal activity-dependent pain sensitization. They are also an example of a pronounced phenotype of mice lacking CB₁ receptors specifically in inhibitory interneurons, while previously reported phenotypes of global CB₁ receptor deficient mice could be ascribed to the lack of CB₁ receptors on glutamatergic neurons (25).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Synaptic effects of CB₁ receptor activation in dorsal horn neuronal circuits. (A–C) Effects of the mixed CB₁/CB₂ receptor agonist WIN 55,212-2 (3 μM) on glycinergic IPSCs (A), GABAergic IPSCs (B) and AMPA-EPSCs (C). Left: current traces averaged from 10 consecutive stimulations under control conditions, after addition of WIN 55,212-2 and after the additional application of AM 251 (5 μM). Right: time course. Mean ± sem, n = 7 – 13 neurons. (D) Inhibition of glycinergic IPSCs in non-glycinergic (EGFP-negative) neurons (n = 8) by the mGluR1/5 agonist DHPG (10 μM) and its reversal by AM 251 (5 μM). Only a minor inhibition was observed in glycinergic (EGFP-positive) neurons (n = 8). (E) DSI (1 s depolarization of the postsynaptic neuron to 0 mV) in non-glycinergic neurons (6 out of 8 neurons) and its prevention by AM 251 (5 μM). No DSI occurred in glycinergic neurons (n = 5). Glycinergic IPSCs were evoked at a frequency of 0.2 Hz.

Fig. 2.

Inhibition of glycinergic and GABAergic synaptic transmission via presynaptic CB₁ receptors. **(A)** Paired pulse experiments. Current traces of two consecutive glycinergic IPSCs (P1 and P2) under control conditions (black) and in the presence of 3 μ M WIN 55,212-2 (red). **(B)** Variation analysis. Top: individual traces of glycinergic IPSCs recorded under control conditions and in the presence of WIN 55,212-2 (3 μ M). Bottom: changes in the coefficient of variation in 13 cells are plotted versus changes in the mean amplitude induced by WIN 55,212-2. **(C–F)** Electron microscopic analysis (**a–c** and **a–b**, serial sections) of CB₁ receptor localization in the superficial spinal dorsal horn. Arrowheads, symmetrical synapses. Arrows, immunogold labeling. **(Ca–Cc)** CB₁-immunostaining coupled to immunoperoxidase reaction (DAB). CB₁ receptors are present in an axon terminal (t) forming a symmetric (inhibitory) synapse on an immuno-negative dendritic shaft (d) in lamina II. Asterisk labels a CB₁-negative bouton of another symmetric synapse on the same dendrite. **(Da–Db)** High-resolution pre-embedding immunogold staining for CB₁. CB₁ receptor located presynaptically on the plasma membrane of an inhibitory axon terminal (t). **(Ea–Eb)** DAB staining for vesicular inhibitory aminoacid transporter (VIAAT) and pre-embedding immunogold labeling for CB₁. CB₁ cannabinoid receptors (indicated by arrows) on an inhibitory (VIAAT-positive) axon terminal (t). Note that in this reaction, silver intensification results in weaker electron density of the DAB precipitate. **(Fa–Fb)** Immunoperoxidase staining for CB₁ combined with pre-embedding

immunogold labeling for VIAAT demonstrates co-localization of the two proteins. Similar results were obtained in 4 animals. Scale bar 0.1 μm .

Fig. 3.

Extracellular single unit recordings from deep dorsal horn neurons in intact rats. Frequency histograms of action potentials evoked by mechanical stimulation within the receptive field of one hindlimb with brush (br), pressure (pr) and pinch (pi) but outside the capsaicin-injected area. (A) responses (spikes / s) of a representative neuron. (B) statistical analysis of background-corrected action potential activity (mean ± sem). (C) dose response analyses. Repeated measures ANOVA followed by Newman-Keuls multiple comparison post-hoc tests. $n = 5 - 6$ neurons per group. *, $P \leq 0.05$; **, $P < 0.01$, ***, $P < 0.001$, against control (pre-capsaicin). +, $P \leq 0.05$; ++, $P < 0.01$, +++, $P < 0.001$, against capsaicin.

Fig. 4.

Effects of pharmacological and genetic manipulations of the endocannabinoid system on capsaicin-induced mechanical hyperalgesia in mice. **(A)** Mechanical paw withdrawal thresholds (mean $\pm$ sem) were determined with electronically controlled von-Frey filaments at 20 min intervals for 2 hours following capsaicin injection into the left hindpaw and for another 2 hours after i.p. injections (vehicle [10% DMSO], AM 251 [0.5 nmol / mouse], URB 597 [1.0 nmol], UCM 707 [1.0 nmol], LY 367,385 [1.0 nmol], MPEP [150 nmol]). Left: time course (mean $\pm$ sem). Right: treatment-induced changes in hyperalgesia, areas under the curve (AUC) integrated over 2 – 4 h after capsaicin injection. Note that the time course of sensitization in wild-type mice treated with i.p. vehicle is the same as in wild-type mice, which did not receive i.p. injections **(B)**. $n = 5 - 6$ mice per group, for statistical analyses three groups of vehicle-injected mice were pooled. One-way ANOVA followed by Dunnett's post-hoc test $F(11,74) = 21.18$ *, $P \leq 0.05$ **, $P < 0.01$ ***, $P < 0.001$. **(B)** Capsaicin-induced secondary hyperalgesia in wild-type mice versus $CB_1^{-/-}$ mice ($n = 9$ mice / group), and in $ptf1a-CB_1^{-/-}$ ($n = 7$ and 11 mice / group) and $sns-CB_1^{-/-}$ mice versus mice carrying a CB_1 receptor gene flanked by two loxP sites ($CB_1^{fl/fl}$ mice) ($n = 5$ mice / group). Left: time course. Right: AUC (0 – 4 hours after capsaicin injection). ***, $P < 0.001$ unpaired Student t-test.