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Cannabinoid CB1 receptor facilitation of substance P release in the rat spinal cord, measured as neurokinin 1 receptor internalization

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Abstract

The contribution of CB1 receptors in the spinal cord to cannabinoid analgesia is still unclear. The objective of this study was to investigate the effect of CB1 receptors on substance P release from primary afferent terminals in the spinal cord. Substance P release was measured as NK1 receptor internalization in lamina I neurons. It was induced in spinal cord slices by dorsal root stimulation and in live rats by a noxious stimulus. In spinal cord slices, the CB1 receptor antagonists AM251, AM281 and rimonabant partially but potently inhibited NK1 receptor internalization induced by electrical stimulation of the dorsal root. This was due to an inhibition of substance P release and not of NK1 receptor internalization itself, because AM251 and AM281 did not inhibit NK1 receptor internalization induced by exogenous substance P. The CB1 receptor agonist ACEA increased NK1 receptor internalization evoked by dorsal root stimulation. The effects of AM251 and ACEA cancelled each other. In vivo, AM251 injected intrathecally decreased NK1 receptor internalization in spinal segments L5 and L6 induced by noxious hind paw clamp. Intrathecal AM251 also produced analgesia to radiant heat stimulation of the paw. The inhibition by AM251 of NK1 receptor internalization was reversed by antagonists of μ-opioid and $GABA_B$ receptors. This indicates that CB1 receptors facilitate substance P release by inhibiting the release of GABA and opioids next to primary afferent terminals, producing disinhibition. This results in a pronociceptive effect of CB1 receptors in the spinal cord.

Keywords

C-fiber; dorsal horn; $GABA_B$ receptor; μ-opioid receptor; primary afferent

The psychotropic and therapeutic properties of cannabis have been known since antiquity. Its active compound, Δ^9-tetrahydrocannabinol, activates three G protein-coupled receptors (GPCRs): CB1, CB2 and GPR55 and receptors (Kano *et al.*, 2009; Ross, 2009). Several endogenous ligands (endocannabinoids) for these receptors have been identified, mainly anandamide and 2-arachidonylglycerol. Endocannabinoids act primarily as retrograde messengers: they are generated postsynaptically and activate presynaptic CB1 receptors to inhibit GABA and glutamate release (Wilson & Nicoll, 2001; 2002).

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Cannabinoids produce antinociception in animals and humans, and are comparable to opiates in potency and efficacy (Pertwee, 2001; Karst *et al.*, 2003; Hohmann & Suplita, 2006; Mackie, 2006; Jhaveri *et al.*, 2007a; Ashton & Milligan, 2008). Cannabinoid analgesia involves effects at the supraspinal (Wilson & Nicoll, 2002; Hohmann *et al.*, 2005; Hohmann & Suplita, 2006), spinal (Richardson *et al.*, 1998) and peripheral levels (Ibrahim *et al.*, 2005; Agarwal *et al.*, 2007).

One way by which cannabinoids could produce analgesia is by inhibiting the release of glutamate, substance P and calcitonin gene-related peptide (CGRP) from primary afferent terminals. The presence of cannabinoid receptors in the central terminals of primary afferent was suggested by a decrease in binding sites in the dorsal horn for the artificial cannabinoid [^{3}H]CP55940 after rhizotomy (Hohmann *et al.*, 1999) and by the presence of CB1 receptor mRNA and immunoreactivity in some DRG neurons (Hohmann & Herkenham, 1999; Bridges *et al.*, 2003; Binzen *et al.*, 2006; Agarwal *et al.*, 2007). Moreover, cannabinoid agonists decreased EPSCs in dorsal horn neurons evoked by dorsal root stimulation (Morisset & Urban, 2001), and inhibited substance P release in the spinal cord (Lever & Malcangio, 2002). However, other studies indicated that CB1 receptors are not transported to the central terminals of nociceptive afferents (Farquhar-Smith *et al.*, 2000; Khasabova *et al.*, 2004; Agarwal *et al.*, 2007), while they are abundant in dorsal horn interneurons (Farquhar-Smith *et al.*, 2000; Salio *et al.*, 2002; Pernia-Andrade *et al.*, 2009).

Importantly, cannabinoids still produced analgesia in CB1 receptor knockout mice (CB1 −/−), showing that other cannabinoids receptors contribute to cannabinoid antinociception. These receptors include CB2 receptors and TRPV1 channels in primary afferents (Smart & Jerman, 2000; Jhaveri *et al.*, 2007b; Anand *et al.*, 2009). Intriguingly, CB1 −/− mice were also hypoalgesic compared with wild-type mice (Zimmer *et al.*, 1999), suggesting that CB1 receptors have some pronociceptive effects. Importantly, a recent report (Pernia-Andrade *et al.*, 2009) demonstrated that CB1 receptors decrease GABA release from inhibitory interneurons in the dorsal horn. The resulting decrease in inhibitory tone in the dorsal horn leads to pronociceptive actions of CB1 receptors.

The objective of this study was to investigate the modulation of substance P release in the spinal cord by cannabinoid receptors. We used NK1R internalization in spinal cord slices and in vivo to measure substance P release in terms of the activation of its receptor (Mantyh *et al.*, 1995; Abbadie *et al.*, 1997; Allen *et al.*, 1997; Marvizon *et al.*, 2003a; Adelson *et al.*, 2009). These data were previously presented as a meeting abstract (Zhang *et al.*, 2008).

Materials and methods

Experimental Animals

Animals used in this study were male, Sprague-Dawley rats purchased from Harlan (Indianapolis, IND). A total of 107 rats were used in the study. Spinal cord slices were prepared from 78 juvenile rats (3-5 weeks old). Intrathecal catheters were implanted in 29 adult rats (2-4 months old), of which 16 rats were used to induce NK1R internalization with noxious stimulation and 13 rats were used to measure paw withdrawal responses to radiant heat. The anesthetic used and other procedural details are given below. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used and their suffering.

Chemicals

ACEA (arachidonyl-2-chloroethylamide), AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide), AM281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide), CGP-55845 ((2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid) and Tocrisolve (20% soya oil emulsified in water with Pluronic F68) were purchased from Tocris (Ellisville, MO). Rimonabant (SR141716A) was from the National Institute of Drug Abuse. Isoflurane was from Halocarbon Laboratories (River Edge, NJ). Prolong Gold was from Invitrogen (Eugene, OR). Capsaicin, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2,) and other chemicals were from Sigma.

Compounds were dissolved in water except for the following. Capsaicin and ACEA were dissolved in ethanol. For experiments in slices, AM251, AM281 and CGP-55845 were dissolved at 10 mM in dimethyl-sulfoxide (DMSO) and then diluted to their desired concentrations. For the intrathecal injection of 1 nmol AM251 (in 10 μl), a stock solution of 10 mM AM251 was prepared in 100% DMSO and then diluted to 0.1 mM in saline. For the intrathecal injection of 10 nmol AM251 (in 10 μl), AM251 was diluted from 10 mM to 1 mM in 1% Tocrisolve in saline.

Media

Artificial cerebrospinal fluid (aCSF) contained (in mM) 124 NaCl, 1.9 KCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$ and 10 glucose; K^+-aCSF contained 5 mM of KCl, and sucrose-aCSF contained 5 mM KCl and 215 mM sucrose instead of NaCl (iso-osmotic replacement). All these media were constantly bubbled with 95% O_2 / 5% CO_2.

Spinal cord slices

Spinal cords were obtained from 3-5 weeks old male Sprague-Dawley rats by dorsal laminectomy. The rats were anesthetized with 3% isoflurane in an induction box and kept under isoflurane anesthesia during the extraction of the spinal cord, which took less than 2 min and included euthanasia by bilateral thoracotomy. Coronal slices (400 μm) were cut with a vibratome (Integraslice 7550PSDS, Campden Instruments USA, Lafayette, IN) from a lumbar spinal cord segment (L2-L4), as described (Marvizon *et al.*, 2003a; Lao & Marvizon, 2005; Adelson *et al.*, 2009). The spinal cord segment was glued vertically to a block of agar on the stage of the vibratome and immersed in ice-cold sucrose-aCSF. Slices were cut using minimum forward speed and maximum vibration while observing them with a stereo microscope mounted over the vibratome. Slices were prepared either without roots or with one dorsal root, which was used for electrical stimulation. In the later case, fiber continuity between the dorsal root and the dorsal horn was assessed by examining the dorsal root and the dorsal surface of the slice with the stereo microscope. Slices were discarded if they did not meet the following criteria: 1) at least 80% of the dorsal funiculus had to be continuous with the dorsal root, and 2) the dorsal root had no cuts or compression damage. Slices were kept for one hour in K^+-aCSF at 35 °C, and then in regular aCSF at 35 °C.

Dorsal root stimulation of slices

The dorsal root attached to the slice was electrically stimulated using a custom-made chamber, as previously described (Marvizon *et al.*, 2003b; Adelson *et al.*, 2009). The root was placed on a bipolar stimulation electrode (platinum wire of 0.5 mm diameter, 1 mm pole separation) in a compartment separated from the superfusion chamber by a grease bridge. The root and the electrodes were covered with mineral oil, and any excess aCSF was suctioned away. This ensured that electrical current circulated through the root and that the stimulus was consistent between preparations. Electrical stimulation was provided by a

Master-8 stimulator and SIU5A stimulus isolating unit (A.M.P. Instruments, Jerusalem, Israel), and consisted of 1,000 square pulses of 20 V and 0.4 ms (C-fiber intensity) delivered at 1 Hz or 100 Hz. In some experiments, the root was chemically stimulated by incubating it for 10 min with 1 μM capsaicin in aCSF in the side compartment of the chamber, as described (Lao *et al.*, 2003). Slices were superfused at 3-6 ml/min with aCSF at 35 °C. Drugs were present in the superfusate continuously starting 5 or 10 min before root stimulation. Ten minutes after the stimulus slices were fixed by immersion in ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in 0.1 M sodium phosphate buffer). A round hole was punched in the ventral horn of the slice ipsilateral to the stimulus in order to identify it in the histological sections after immunohistochemistry.

Incubation of slices with capsaicin or substance P

To induce NK1R internalization, some slices were incubated with 1 μM capsaicin or 1 μM substance P in aCSF at 35 °C for 10 min. The slices were placed on a nylon net glued to a plastic ring inserted halfway down a plastic tube containing 5 ml aCSF. The aCSF was superficially gassed with 95% O_2/5% CO_2 delivered through a needle inserted through the cap of the tube. To change solutions, the ring and net with the slice was transferred to another tube. At the end of the incubations, slices were fixed as describe above.

Intrathecal injections

Chronic intrathecal catheters were implanted from the lumbar vertebrae, as described (Storkson *et al.*, 1996). Rats (2-4 months old rats) were anesthetized with isoflurane (2–4% in oxygen) and kept under anesthesia on a metal platform kept at 35 °C by a feedback device. The skin and muscle were cut to expose vertebrae L5 and L6. A blunted 20G needle was inserted between the L5 and L6 vertebrae to puncture the dura mater, which was inferred from a flick of the tail or paw and the backflow of spinal fluid. The needle was removed and the catheter (20 mm of PE-5 tube heat-fused to 150 mm of PE-10 tube) was inserted into the subdural space and pushed rostrally to terminate over L5-L6. The PE-10 catheter was then tunneled under the skin and externalized over the head. The skin was sutured, and the catheter was flushed with 10 μl saline and closed with an electrical cauterizer. Rats were housed separately and allowed to recover for 5-7 days. They were given an antibiotic (enrofloxacin) and an analgesic (carprofen) for 5 days. A criterion for immediate euthanasia of the rat was the presence of motor weakness or signs of paresis, but this did not occur in any of the rats in this study.

Intrathecal injection volume was 10 μl of injectate plus 10 μl saline flush (Zorman *et al.*, 1982; Jensen & Yaksh, 1984; Aimone *et al.*, 1987; Kondo *et al.*, 2005). This volume leads to the distribution of the injectate over most of the spinal cord, but not into the brain (Yaksh & Rudy, 1976; Chen *et al.*, 2007). Solutions are preloaded, in reverse order of administration, into a tube (PE-10), and delivered with a 50 μl Hamilton syringe within 1 min. The position of the catheter was examined postmortem. We established as a criteria for exclusion of the animal from the study 1) termination of the catheter inside the spinal cord, and 2) any signs of occlusion of its tip. However, it was not necessary to exclude any rats from the study according to these criteria.

Noxious mechanical stimulation

A noxious mechanical stimulus was used to induce NK1R internalization in vivo, and was given 5-7 days after implanting the intrathecal catheters. Rats were anesthetized with isoflurane (2-3%) in an induction box and kept under isoflurane anesthesia until they were euthanized. Rats were given an intrathecal injection of 10 μl saline or drug plus a 10 μl catheter flush. After 10 minutes, one hind paw was clamped with a hemostat (closed to the first notch) for 30 sec (Le Bars et al., 1987a). Ten minutes later, rats were euthanized with

pentobarbital (100 mg/Kg). Rats were fixed immediately by aortic perfusion of 100 ml phosphate buffer (0.1 M sodium phosphate, pH 7.4) containing 0.01% heparin, followed by 400 ml of ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in phosphate buffer).

Paw withdrawal responses to radiant heat

Paw withdrawal latencies were measured using a "Plantar Analgesia Meter" model 390G (IITC Life Sciences, Woodland Hills, CA), consisting of an acrylic enclosure on an elevated warm glass surface (Cheppudira, 2006). Rats implanted with intrathecal catheters were acclimated to the instrument for 30 min for 3 days. The test consisted in heating the plantar surface of the hind paw from below with a radiant heat source. The intensity of the lamp was set at 30% of maximal power. Cut-off time was 25 s to prevent tissue damage. Baseline paw withdrawal latencies were measured three times at 5 min intervals. Within 2 min of establishing the baseline, drugs were injected intrathecally. Ten minutes after the injection, paw withdrawal latencies were measured again, four times at 5 min intervals. Results were calculated as percentage of the maximum possible response (%MPE) (Paronis & Holtzman, 1991):

$$\%\text{MPE} = 100 \times (\text{latency} - \text{baseline}) / (\text{cut-off} - \text{baseline})$$

Characterization of the NK1R antiserum

The NK1R antibody was rabbit antiserum # 94168, made at CURE: Digestive Diseases Research Center, UCLA, under the sponsorship of Dr. Nigel Bunnett, UCSF. It was generated in rabbits using a peptide corresponding to the C-terminus of the rat NK1R (amino acids 393-407, KTMTESSSFYSNMLA) coupled to KLH (Grady *et al.*, 1996). It labeled by immunofluorescence cells transfected with rat NK1R, and it did not label nontransfected cells. Staining of the transfected cells was eliminated by preadsorption with its immunizing peptide. In Western blots from cells transfected with the NK1R, the antiserum produced a single band corresponding to a molecular weight of 100 kDa (Grady *et al.*, 1996).

Immunohistochemistry

Spinal cord slices were be fixed, cryoprotected, frozen and re-sectioned at 25 μm in a cryostat as described (Marvizon *et al.*, 2003a; Adelson *et al.*, 2009). Rats were fixed by aortic perfusion as described above, and lumbar spinal cord segments were similarly processed and sectioned at 25 μm in the coronal plane (Chen *et al.*, 2007; Lao *et al.*, 2008). Sections were washed four times and then incubated overnight with the NK1R antiserum diluted 1:3000 in phosphate-buffered saline containing 0.3% Triton X-100, 0.001% thimerosal and 10% normal goat serum. After three washes, the secondary antibody was applied at for 2 hours at 1:2000 dilution. The secondary antibody was goat anti-rabbit IgG coupled to Alexa Fluor 488 (Invitrogen). Sections were washed four more times, mounted on glass slides, and coverslipped with Prolong Gold (Invitrogen). All incubations were done at room temperature.

Quantification of NK1R internalization

The amount of NK1R internalization was quantified using a standard method (Mantyh *et al.*, 1995; Marvizon *et al.*, 2003a). NK1R neurons were visually counted while classifying them as with or without internalization, using a Zeiss Axio-Imager A1 microscope with a 63x oil (NA 1.40) objective. The criterion for having internalization was the presence in the neuronal soma of ten or more NK1R endosomes, defined as a small region of bright staining separated from the cell surface. The person counting the neurons was blinded to the

treatment. All NK1R neurons in lamina I were counted in each histological section. In experiments in slices, at least three sections per slice were counted. In experiments in vivo, four sections were counted per spinal segment.

Confocal microscopy

Confocal images were acquired using a Leica TCS-SP confocal microscope, using objectives of 20x (numerical aperture 0.70) and 100x (numerical aperture 1.40). One set of images (Fig. 1D) was acquired with a Zeiss LSM-710 confocal microscope using similar objectives. Excitation light for the Alexa Fluor 488 fluorophore was provided by the 488 nm line of argon lasers. The emission window was 500-570 nm (emission peak for Alexa Fluor 488 is 519 nm). The pinhole was 1.0 Airy unit corresponding to the objective used. Images were acquired in grayscale as confocal stacks of sections of 1024×1024 pixels. Photomultiplier gain and offset was individually adjusted for each image to avoid pixel saturation and loss of background detail. Each section was averaged 2-4 times to reduce noise.

Image processing

Images of the medial and central parts of the dorsal horn obtained with the 20x objective were used to show the location of the neurons imaged with the 100x objective (Fig. 1). Confocal stacks acquired with the 20x objective were processed using adaptive point spread function ('blind') deconvolution to reduce blur (Wallace *et al.*, 2001;Cannell *et al.*, 2006;Holmes *et al.*, 2006), using the program AutoQuant X 2.0.1 (Media Cybernetics, Inc., Bethesda, MD). Images taken with the 100x objective were not deconvolved because their native low blur made this unnecessary. The program Imaris 6.1.5 (Bitplane AG, Zurich, Switzerland) was used to crop the confocal stacks in three dimensions. Images at 20x were cropped only in the z dimension to choose the five brightest optical sections. Images at 100x were cropped in x-y to show the soma and proximal dendrites of the target neurons, and in the z dimension into three optical sections through the middle of the soma. Occasionally, several neurons were cropped from the same confocal stack. Image resolution was preserved in the cropping, so that pixels in Fig. 1 correspond to the pixels acquired by the confocal microscope. Voxel dimensions were 488 × 488 × 1180 nm with the 20x objective and 98 × 98 × 285 nm with the 100x objective. After cropping, a two-dimension projection picture was generated in Imaris and imported into Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA), which was used to make slight adjustments in the gamma of the images so that important details are clearly visible in Fig. 1. Adobe Photoshop was also used to compose the multi-panel figures and to add text and arrows.

Data analysis

Prism 5 (GraphPad Software, San Diego, CA) was used to analyze data and make the graphs. Error bars represent the standard error of the mean (SEM). Statistical analyses usually consisted of one or two-way ANOVA and Bonferroni's post-hoc tests. Statistical significance was set at 0.05. In two-way ANOVA, the two variables typically were "drugs" (drug combinations or concentrations) and "stimulus" (by comparing the side of the slice ipsilateral or contralateral to the stimulus). The Bonferroni's post-hoc test was applied to the variable "drugs" to compare effects on the ipsilateral side. NK1R internalization in the contralateral side was consistently low and unaffected by the drugs used in this study.

Concentration-response data were fitted using non-linear regression by a sigmoidal dose-response function:

$$Y=\text{bottom}+(\text{top}-\text{bottom})/(1+10\hat{}(\text{LogIC}_{50}-\text{LogX}))$$

, where the IC_{50} is the concentration of drug that produces half of the inhibition. Baseline measures (zero concentration of drug) were included in the non-linear regression by assigning them a concentration value three log units lower than the estimated IC_{50}. Parameter constraints were: 0% < top < 100%, 0% < bottom. Statistical errors of the EC_{50} or IC_{50} were expressed as 95% confidence intervals (CI). Prism was set to detect and exclude outliers by using the "robust regression and outlier removal" (ROUT) algorithm with Q = 1% (Motulsky & Brown, 2006). An F-test (Motulsky & Christopoulos, 2003) was used to compare alternative non-linear regression fittings with different number of parameters, i.e., when one parameter was constrained to a fixed value.

Results

CB1 antagonists decrease and a CB1 agonist increases NK1R internalization evoked by electrical stimulation of the dorsal root

First, we studied the effect of CB1 receptors on substance P release in rat spinal cord slices. Using an approach developed in our laboratory (Marvizon *et al.*, 1997; Adelson *et al.*, 2009), we prepared spinal cord slices with one contiguous dorsal root that was electrically stimulated to induce substance P release, which was measured as NK1R internalization. As we previously reported, neurons showing NK1R internalization were virtually absent in the contralateral dorsal horn (Fig. 1 A) but numerous in the ipsilateral dorsal horn, particularly in its central part (Fig. 1 B). Two electrical stimulation protocols were used, low (1 Hz) and high (100 Hz) frequency, because we previously found that the stimulation frequency influences substance P release and its modulation by GABA and other neurotransmitters (Marvizon *et al.*, 1999; Lao & Marvizon, 2005; Adelson *et al.*, 2009). The electrical pulses used were of enough amplitude (20 V) and duration (0.4 ms) to recruit C-fibers (Adelson *et al.*, 2009).

Dorsal root stimulation at 1 Hz induced NK1R internalization in nearly half of the NK1R neurons in lamina I (Fig. 2 A). The number of NK1R neurons with internalization was increased by the selective CB1 receptor agonist ACEA (100 nM) and decreased by the selective CB1 antagonist AM251 (100 nM, Fig. 2 A). Combining ACEA with AM251 cancelled their effects and brought NK1R internalization back to control levels. Two-way ANOVA revealed significant effects of the two variables 'drugs' (degrees of freedom [*Df*]=3, F=9.1, p=0.0001), 'stimulus' (*Df*=1, F=336, p<0.0001) and their interaction (*Df*=3, F=12, p<0.0001). Bonferroni's post-hoc test showed that the effects of ACEA and AM251 were significant and significantly reversed when combined (Fig. 2 A).

Dorsal root stimulation at 100 Hz produced higher NK1R internalization (Fig 2 B). The increase produced by ACEA was less pronounced and the inhibition by AM251 more pronounced than with 1 Hz stimulation. Combining ACEA and AM251 cancelled their effects, but this time the inhibition by AM251 predominated. Other CB1 antagonists, AM281 (100 nM) and rimonabant (SR141716A, 100 nM), also decreased the evoked NK1R internalization. However, the inhibition by rimonabant was less pronounced than the inhibition by AM251 and AM281 (p<0.001). Two-way ANOVA of the data in Fig. 2 B yielded significant effects of the two variables 'drugs' (*Df*=7, F=524, p<0.0001), 'stimulus' (*Df*=1, F=25749, p<0.0001) and their interaction (*Df*=7, F=455, p<0.0001). The decrease in the number of lamina I neurons with NK1R internalization produced by AM281 is illustrated in Fig. 1 C, corresponding to the dorsal horn ipsilateral to the stimulated root.

Since AM251 is also an agonist of the putative new cannabinoid receptor GPR55 (Lauckner *et al.*, 2008; Kano *et al.*, 2009; Ross, 2009), it is possible that its inhibition of NK1R internalization was mediated by GPR55 and not CB1 receptors. To explore this possibility, we determined whether the selective GPR55 agonist O-1640 (Johns *et al.*, 2007; Oka *et al.*,

2007; Waldeck-Weiermair *et al.*, 2008) inhibited the evoked NK1R internalization. O-1640 produced no effect (Fig. 2 B, $p>0.05$, Bonferroni's post-hoc test) consistent with the idea that the inhibition produced by AM251 was caused by blockade of CB1 receptors.

To confirm that AM251 inhibited substance P release and not NK1R internalization itself, we determined whether 100 nM AM251 inhibited NK1R internalization induced by incubating spinal cord slices with substance P (1 μM). AM251 produced no effect in this case (Fig. 3, one-way ANOVA: $Df=2$, $F=1.65$, $p=0.27$).

Concentration-responses of the CB1 antagonists AM251 and AM281

To further characterize the inhibition of substance P release by CB1 receptor antagonists, we obtained concentration-response curves of the CB1 antagonists AM251 (Fig. 4 A) and AM281 (Fig. 4 B). NK1R internalization was evoked by stimulating the dorsal root at 100 Hz. AM251 and AM281 dose-dependently inhibited the evoked NK1R internalization, except that an outlier was found with the highest concentration of AM281, 1 μM. This data point was excluded by the outlier detection feature of the non-linear regression program (see Data Analysis in Methods) (Motulsky & Brown, 2006). We attributed this outlier to the interaction of AM281 at high concentrations with receptors other than CB1. For example, rimonabant and AM251, which are structurally similar to AM281, inhibit adenosine A1 receptors at micromolar concentrations (Savinainen *et al.*, 2003).

Non-linear regression analysis of these data yielded IC_{50} values of 13 nM (96% CI = 2-73 nM) for AM251 and 6 nM (96% CI = 2-16 nM) for AM281, corresponding to the curves shown in Fig. 4. Therefore, both antagonists potently inhibited substance P release. Two-way ANOVA for AM251: significant effects of 'concentration' ($Df=7$, $F=4.8$, $p=0.0004$), stimulus ($Df=1$, $F=148$, $p<0.0001$) and their interaction ($Df=7$, $F=4.1$, $p=0.0014$). Two-way ANOVA for AM281: significant effects of concentration ($Df=5$, $F=18$, $p<0.0001$), stimulus ($Df=1$, $F=518$, $p<0.0001$) and their interaction ($Df=5$, $F=17$, $p<0.0001$).

AM251 and AM281 produced a partial inhibition of the evoked NK1R internalization, with their effects reaching plateaus at 21±5% and 27±3%, respectively, as determined by non-linear regression (Fig. 4). To confirm that the inhibition was indeed partial, we used an F-test (Motulsky & Christopoulos, 2003) to compare two alternative non-linear regression fittings: one with the 'bottom' parameter unconstrained (i.e., partial inhibition) and the other with 'bottom' constrained to the value obtained in the contralateral dorsal horn (i.e., complete inhibition). The null hypothesis was that the value of 'bottom' was equal to the averaged contralateral values: 4.0% for AM251 (Fig. 4 A), 7.4% for AM281 (Fig. 4 B). The statistically preferred model in the F-test was partial inhibition for both AM251 ($F_{1,28}=7.47$, $p=0.0107$) and AM281 ($F_{1,17}=28.69$, $p<0.0001$). Therefore, these CB1 receptor antagonists decreased substance P release with high potencies, but did not completely abolish it.

We did not obtain concentration-response curves for rimonabant because at 100 nM its inhibition was smaller than the inhibition produced by AM251 and AM281 (Fig. 2), and at higher doses it became even less clear. Thus, rimonabant at 10 μM produced a marginal, not significant, decrease in NK1R internalization induced by root stimulation at 1 Hz (control, 44±4%, $N=6$; rimonabant 10 μM, 27±11, $N=3$; two-way ANOVA, 'rimonabant', $Df=1$, F=4.2, $p=0.059$, 'stimulus', $Df=1$, F=56, $p<0.0001$, interaction, $Df=1$, F=3.3, $p=0.09$). Likewise, rimonabant at 5 μM did not significantly decrease NK1R internalization induced by root stimulation at 100 Hz (control, 60±3%, $N=5$; rimonabant 5 μM, 43±17%, $N=6$; two-way ANOVA: 'rimonabant', $Df=1$, F=0.70, $p=0.42$, 'stimulus', $Df=1$, F=27, $p<0.0001$, interaction, $Df=1$, F=0.86, $p=0.37$).

Concentration-response of the CB1 agonist ACEA

Similarly, we studied the concentration-response of the facilitatory effect of the CB1 agonist ACEA. Since facilitation by ACEA was more pronounced when stimulating the dorsal root at 1 Hz (Fig. 2), we used this stimulation frequency. ACEA failed to increase the evoked NK1R internalization at 3, 10, 30 nM (Fig. 5). It produced a significant effect at 100 nM, but NK1R internalization was back at control levels at 300 nM ACEA. This was attributed to the interaction of ACEA at this concentration with CB2 receptors, which bind ACEA with a K_i of 3±1 μM (Hillard *et al.*, 1999). This biphasic effect prevented the determination of the EC_{50} for ACEA. Still, a two-way ANOVA revealed significant effects of the variables 'ACEA concentration' (*Df*=5, *F*=5.9, *p*=0.0005), 'stimulus' (*Df*=1, *F*=799, *p*<0.0001) and their interaction (*Df*=5, *F*=9.1, *p*<0.0001).

Effect of AM251 on NK1R internalization evoked by capsaicin

The electrical pulses used here (20 V, 0.4 ms) to stimulate the dorsal root recruits both A and C fibers. It is possible to selectively stimulate C fibers in the dorsal root by immersing it in capsaicin (Lao *et al.*, 2003), because A fibers lack the TRPV1 channels activated by capsaicin. As in our previous study (Lao *et al.*, 2003), capsaicin applied to the root induced NK1R internalization in about half the NK1R neurons in the ipsilateral dorsal horn (Fig. 6 A). Absence of NK1R internalization contralaterally confirms that capsaicin did not reach the slice. In these conditions, AM251 (1 μM) also inhibited the evoked NK1R internalization. Two-way ANOVA of results in Fig. 6 A revealed significant effects of the variables 'AM251' (*Df*=1, *F*=29, *p*<0.0001), 'stimulus' (i.e. ipsilateral vs. contralateral to capsaicin on the root, *Df*=1, *F*=82, *p*<0.0001) and their interaction (*Df*=1, *F*=18.5, *p*=0.0004). This result indicates that AM251 inhibits substance P release from C fibers.

Incubating spinal cord slices with capsaicin is a powerful stimulus to induce substance P release and subsequent NK1R internalization (Marvizon *et al.*, 2003a; Nazarian *et al.*, 2007). We have shown, however, that this stimulus bypasses the physiological control mechanisms of substance P release (Lao *et al.*, 2003). Thus, capsaicin causes Ca^{2+} entry through TRPV1 channels located in primary afferent terminals, so that inactivation of voltage-gated Ca^{2+} channels by $GABA_B$ receptors (Strock & Diverse-Pierluissi, 2004; Raingo *et al.*, 2007) becomes ineffective to induce substance P release (Lao *et al.*, 2003). Fig. 6 B shows that this applies also to the facilitation of substance P release by CB1 receptors. Incubating spinal cord slices with 0.3 μM capsaicin induced a large amount of NK1R internalization in lamina I neurons, which was not inhibited by 1 μM AM251 (Student's t-test, non-directional, *p*=0.92).

NK1R internalization induced by noxious stimulation was inhibited by intrathecal AM251

Next, we determined whether facilitation of substance P release by CB1 receptors could also be observed in vivo. Substance P release and subsequent NK1R internalization can be induced by applying a noxious stimulus to the hind paw of a rat (Abbadie *et al.*, 1997; Allen *et al.*, 1997; Honore *et al.*, 1999; Kondo *et al.*, 2005; Chen & Marvizon, 2009). In this experiment we anaesthetized rats with isoflurane and then clamped their hind paw with a hemostat for 30 s. This evoked a large amount of NK1R internalization in the ipsilateral dorsal horn, which was maximal in the L5 spinal segment (Fig. 7) receiving abundant innervation from the paw through the sciatic nerve. An intrathecal injection of AM251 (10 nmol) 10 min prior to paw clamp significantly reduced the evoked NK1R internalization in segments L5 and L6 (Fig. 7). AM251 had no effect contralaterally, where NK1R internalization was negligible. Two-way ANOVA revealed significant effects of the variables 'AM251' (*Df*=1, *F*=11.5, *p*=0.0014), 'spinal region' (defined by combining the four spinal segments with the two sides, *Df*=7, *F*=35, *p*<0.0001) and their interaction (*Df*=7, *F*=2.5, *p*=0.028).

AM251 is insoluble in water. To maintain it in solution in the injectate while keeping the concentration of DMSO low enough to avoid unwanted effects, we used Tocrisolve as an emulsifier, so that AM251 was administered in 10% DMSO, 1% Tocrisolve (see 'Chemicals' in Material and Methods). Control rats were injected intrathecally with the same vehicle (10% DMSO, 1% Tocrisolve in saline). NK1R internalization evoked by hind paw clamp in these control rats was similar to that reported previously (Trafton *et al.*, 1999; Kondo *et al.*, 2005; Lao *et al.*, 2008; Chen & Marvizon, 2009), showing that it was not affected by the vehicle.

Analgesia produced by intrathecal AM251

Substance P release is an indicator of the activity of nociceptors (Hua & Yaksh, 2009). Therefore, their facilitation of substance P release suggests that CB1 receptors increase synaptic transmission between primary afferents and dorsal horn neurons, which would lead to a pro-nociceptive effect. Since inhibition of substance P release by CB1 antagonists was more pronounced than its increase by the CB1 agonist ACEA, we predicted that this pro-nociceptive effect of CB1 receptors could be observed as antinociception produced by a CB1 antagonist. To investigate this possibility, we injected intrathecally AM251 at two doses: 1 nmol (in 1% DMSO) and 10 nmol (in 10% DMSO with 1% Tocrisolve). Control rats received intrathecal vehicle: 3 rats received 1% DMSO and 4 rats received 10% DMSO, 1% Tocrisolve. We measured paw withdrawal responses to radiant heat. Control responses with the two vehicles were almost identical, so they were pooled in Fig. 8. Both doses of AM251 produced statistically significant increases in the latency of the paw withdrawal responses (Fig. 8). Two-way ANOVA revealed a significant effect of the variable 'AM251' (Df=2, F=57, p<0.0001) but not of the variable 'time after injection' (Df=4, F=1.6, p=0.19) or their interaction (Df=8, F=0.77, p=0.63). Bonferroni's post-hoc tests (Fig. 8) revealed significant differences between control and either dose of AM-251 at most time points, but no significant differences were found between the effects of the 1 nmol and 10 nmol doses of AM251, suggesting that the effect of AM251 was maximal at these doses. The effect of 10 nmol AM251 was already present 10 min after the injection and lasted at least 30 min. These results demonstrate that intrathecal AM251 produces antinociception to acute thermal stimuli.

Mechanism of the facilitation of substance P release by CB1 receptors

CB1 receptors usually couple to inhibitory G proteins (α_i or α_o) and inhibit neurotransmitter release (Kano *et al.*, 2009). For this reason, we hypothesized that their facilitation of substance P release was caused by disinhibition, that is, that CB1 receptors inhibit the release of neurotransmitters that decrease substance P release. Two important inhibitors of substance P release are GABA, acting on $GABA_B$ receptors (Malcangio & Bowery, 1993; Marvizon *et al.*, 1999; Riley *et al.*, 2001; Lao *et al.*, 2003), and opioids, acting on μ-opioid receptors (Yaksh *et al.*, 1980; Kondo *et al.*, 2005). CB1 receptors could inhibit GABA or opioid release in the dorsal horn. In this case, and given that endocannabinoids are released during dorsal root stimulation, CB1 antagonists would increase GABA or opioid release, resulting in an inhibition of substance P release mediated by $GABA_B$ or μ-opioid receptors, respectively. This hypothesis predicts that the inhibition produced by AM251 would be reversed by $GABA_B$ or μ-opioid receptor antagonists.

This prediction was tested in the experiment in Fig. 9, in which we used the selective μ-opioid receptor antagonist CTAP (10 μM) and the $GABA_B$ receptor antagonist CGP55845 (100 nM). In previous studies in spinal cord slices we determined that these concentrations of CTAP and CGP55845 produce a complete blockade of μ-opioid receptors (Song & Marvizon, 2003) and $GABA_B$ receptors (Lao & Marvizon, 2005), respectively. Spinal cord slices were electrically stimulated at the dorsal root at 100 Hz or 1 Hz, because different

frequencies of root stimulation evoke different patterns of neurotransmitter release in the dorsal horn (Marvizon *et al.*, 1999;Lever *et al.*, 2001;Lao & Marvizon, 2005).

When the dorsal root was stimulated at 100 Hz (Fig. 9 A), the inhibition produced by AM251 (100 nM) was reversed by CTAP but not by CGP55845. This suggests that during high frequency stimulation AM251 increases opioid release, leading to inhibition of substance P release mediated by μ-opioid receptors. Two-way ANOVA for the data in Fig. 9 A revealed significant effects of the variables 'drugs' (Df=5, F=21, p<0.0001), 'stimulus' (Df=1, F=1352, p<0.0001) and their interaction (Df=5, F=20, p<0.0001).

When the dorsal root was stimulated at 1 Hz (Fig. 9 B), the inhibition produced by AM251 (100 nM) was reversed by both CTAP and CGP55845 (100 nM). This suggests that during low frequency stimulation AM251 increases both opioid and GABA release, leading to inhibition of substance P release mediated by μ-opioid receptors and $GABA_B$ receptors. Two-way ANOVA for the data in Fig. 9 B revealed significant effects of the variables 'drugs' (Df=5, F=2.5, p=0.041), 'stimulus' (Df=1, F=581, p<0.0001) and their interaction (Df=5, F=3.3, p=0.012). Neither CTAP nor CGP55845 alone affected NK1R internalization evoked with either 100 Hz or 1 Hz stimulation (Fig. 9), indicating that the stimulus elicited little opioid or GABA release in these conditions.

Discussion

This study shows that cannabinoid CB1 receptors facilitate substance P release from primary afferent terminals. The mechanism involved in this facilitation appears to be the inhibition of the release of GABA and opioids from dorsal horn neurons, leading to disinhibition of the effect of $GABA_B$ receptors and μ-opioid receptors on substance P release.

CB1 receptors facilitate substance P release

Our results indicate that CB1 receptors facilitate substance P release from primary afferent terminals. This facilitation was observed primarily as an inhibition of evoked NK1R internalization produced by the CB1 receptor antagonists AM251, AM281 and rimonabant (Kano *et al.*, 2009). AM251 and AM281 inhibited substance P release and not the NK1R internalization mechanism itself, since they did not decrease NK1R internalization induced by exogenous substance P.

The fact that AM251 inhibited substance P release evoked by stimulating the dorsal root with capsaicin indicates that CB1 receptors facilitate substance P release from nociceptors. Although a few A-fibers contain substance P (Lawson *et al.*, 1993), they do not have TRPV1 receptors, so this experiment shows that AM251 is able to inhibit substance P release from C-fibers. Importantly, intrathecal AM251 inhibited NK1R internalization evoked by a noxious stimulus in vivo, showing that facilitation of substance P release by CB1 receptors takes place in physiological conditions.

The effect of AM251 and AM281 was dose-dependent, with IC_{50} values (13 nM and 6 nM, respectively) consistent with the affinity of these compounds for CB1 receptors (Gatley *et al.*, 1997; Gatley *et al.*, 1998; Lan *et al.*, 1999a; Lan *et al.*, 1999b). The inhibition that they produced was partial, leveling off at about 50% of the NK1R internalization found in control slices. This partial inhibition was found independently of the stimulus used to evoke substance P release: electrical stimulation at low (1 Hz) and high frequency (100 Hz) (Marvizon *et al.*, 1997; Lao & Marvizon, 2005; Adelson *et al.*, 2009) or capsaicin applied to the root (Lao *et al.*, 2003). One possible explanation for this partial inhibition is that CB1 receptors facilitate substance P release from a subset of the substance P-containing

terminals. Alternatively, the effect of CB1 receptors may consist in disinhibition of mechanisms that only partially decrease substance P release (see below).

The facilitatory effect of CB1 receptors was also detected as an increase in the evoked NK1R internalization by the selective CB1 receptor agonist ACEA (Hillard *et al.*, 1999; Pertwee, 1999). The decrease in NK1R internalization produced by the antagonist AM251 and the increase produced by the agonist ACEA cancelled each other, supporting the idea that these effects were mediated by opposing actions at CB1 receptors. However, the increase produced by ACEA was small compared with the inhibition produced by the antagonists. This was probably because the effect of ACEA was masked by the release of endocannabinoids. The increase in NK1R internalization produced by ACEA disappeared at concentrations higher than 100 nM, preventing us from obtaining a concentration-response curve. It is possible that this is caused by the binding of ACEA to CB2 receptors at micromolar concentrations (K_i of 3±1 μM) (Hillard *et al.*, 1999). There is evidence for the expression of CB2 receptors in neurons and glia throughout the CNS (Gong *et al.*, 2006), including in the spinal cord and primary afferents (Beltramo *et al.*, 2006). ACEA is also a TRPV1 agonist at micromolar concentrations (Price *et al.*, 2004). However, opening of TRPV1 channels by ACEA would further increase substance P release (Marvizon *et al.*, 2003a), so this could not explain the reversal of the increase in NK1R internalization at high concentrations of ACEA.

Our results are at variance with those of Lever & Malcangio (Lever & Malcangio, 2002), who found that capsaicin-induced substance P release from mouse spinal cord slices was considerably increased by the CB1 antagonist rimonabant and inhibited by the endocannabinoid anandamide. However, they used rimonabant at a dose, 5 μM, at which it may activate other receptors like adenosine A1 receptors (Savinainen *et al.*, 2003). We found that the inhibition of NK1R internalization produced by rimonabant and AM281 disappeared at micromolar doses. As for anandamide, its inhibition could have been mediated by receptors other than CB1 that also bind anandamide, such as CB2 receptors (Devane *et al.*, 1992; Kano *et al.*, 2009) and TRPV1 (Zygmunt *et al.*, 1999; Starowicz *et al.*, 2007).

Facilitation of substance P release is not mediated by GPR55 receptors or TRPV1 channels

AM251 is also an agonist of the novel cannabinoid receptor GPR55 (Ryberg *et al.*, 2007; Kano *et al.*, 2009). However, its inhibition of substance P release cannot be attributed to this receptor for various reasons. First, unlike AM251, the GPR55 agonist O-1640 (Johns *et al.*, 2007; Oka *et al.*, 2007; Waldeck-Weiermair *et al.*, 2008) did not inhibit NK1R internalization evoked by dorsal root stimulation (Fig. 2 B). Second, rimonabant, which acts as an antagonist of GPR55 (Ross, 2009), inhibited NK1R internalization like AM251. Third, AM281, which is ineffective at GPR55 (Ross, 2009), also inhibited NK1R internalization.

TRPV1 channels are activated by some endocannabinoids (Kano *et al.*, 2009). However, the effects of the synthetic cannabinoids used in this study cannot be attributed to TRVP1, either. Thus, NK1R internalization induced by direct application of capsaicin to the slices was not inhibited by AM251 (Fig. 6 B). We have previously shown (Lao *et al.*, 2003) that capsaicin-induced substance P release bypasses the inhibition produced by $GABA_B$ receptors and probably other GPCRs. This is because GPCRs inhibit substance P release by inactivating voltage-dependent Ca^{2+} channels (Strock & Diverse-Pierluissi, 2004; Raingo *et al.*, 2007), whereas TRPV1 channels provide an alternative route for Ca^{2+} entry into the terminal that bypasses the voltage-dependent Ca^{2+} channels.

Inhibition by the CB1 receptor antagonists indicates endocannabinoid release

The inhibition of substance P release by the CB1 antagonists AM251, AM281and rimonabant is likely caused by blockade of the effect of endocannabinoids released in the dorsal horn. This idea is supported by the relative small increases in evoked NK1R internalization produced by the CB1 agonist ACEA, which suggests that the CB1 receptors are partially occupied by endogenous agonists. For some time it was though that some CB1 antagonists act as inverse agonists (i.e., by blocking a constitutive activity of the CB1 receptors), but the current consensus is that the effects of CB1 antagonists can be attributed solely to blockade of the effects of endocannabinoids (Savinainen *et al.*, 2003; Kano *et al.*, 2009). For example, the basal activity of CB1 receptors was decreased by inhibition of diacylglycerol lipase (DGL), the enzyme that synthesizes the endocannabinoid 2-archidonyl-glycerol (Turu *et al.*, 2007). Accordingly, our results indicate that endocannabinoids are present in the dorsal horn, possibly because their synthesis is triggered by the stimulus used to evoked substance P release.

CB1 receptor facilitation is caused by disinhibition of the effect of $GABA_B$ receptors and μ-opioid receptors

The most likely explanation for the facilitation of substance P release by CB1 receptors is the disinhibition mechanism depicted in Fig. 10. According to this model, the CB1 receptors producing this effect are located in the presynaptic terminals of GABAergic and opioidergic interneurons in the dorsal horn, where they inhibit neurotransmitter release. Since substance P release from primary afferent terminals is inhibited by μ-opioid receptors (Yaksh *et al.*, 1980;Aimone & Yaksh, 1989;Kondo *et al.*, 2005) and $GABA_B$ receptors (Malcangio & Bowery, 1993;Marvizon *et al.*, 1999;Riley *et al.*, 2001), reduced agonist binding to these receptors results in a facilitation of substance P release. Several lines of evidence support this model.

First, it is unlikely that the facilitation of substance P release is mediated by CB1 receptors located in the substance P-containing terminals themselves. While CB1 receptors frequently inhibit neurotransmitter release, no instances of direct facilitation of neurotransmitter release by this receptor has been found (Kano *et al.*, 2009). Whether CB1 receptors are present in the central terminals of primary afferent terminals has been controversial until recently. Initially, CB1 receptor mRNA and immunoreactivity was detected in some DRG neurons (Hohmann & Herkenham, 1999; Bridges *et al.*, 2003; Binzen *et al.*, 2006; Agarwal *et al.*, 2007). However, other studies found that CB1 receptor immunoreactivity in the dorsal horn was unaffected by rhizotomy (Farquhar-Smith *et al.*, 2000) or by selective CB1 receptor knockout in DRG neurons (Agarwal *et al.*, 2007), suggesting that CB1 receptors may not be transported centrally from the DRG. Yet, a recent studied (Nyilas *et al.*, 2009) provided solid evidence for the presence of CB1 receptors in C-fiber and Aδ-fiber terminals in the dorsal horn. It remains to be clarified whether CB1 receptors are present in C-fiber terminals that contain substance P (Farquhar-Smith *et al.*, 2000; Khasabova *et al.*, 2004). If they are, they may affect substance P release only weakly, or their inhibition of substance P release may be masked by the indirect facilitation described here.

Second, strong support for this model was provided by a recent study by Pernia-Andrade et al. (Pernia-Andrade *et al.*, 2009) showing that CB1 receptors decrease GABA release from inhibitory interneurons in the dorsal horn, measured as inhibitory postsynaptic currents. The same study, using electron microscopic immunohistochemistry, found CB1 receptors in axon terminals forming inhibitory synapses in the superficial dorsal horn.

Third, the experiment shown in Fig. 9 confirmed our prediction that the inhibition produced by AM251 was caused by an increase in GABA and opioid release. Thus, inhibition by

AM251 was reversed by $GABA_B$ and μ-opioid receptors antagonists. Interestingly, the $GABA_B$ antagonist CGP55845 reversed the inhibition by AM251 when the dorsal root was stimulated at 1 Hz but not at 100 Hz. This is consistent with our previous studies (Marvizon *et al.*, 1999;Lao & Marvizon, 2005) showing that root stimulation at 1 Hz, but at 100 Hz, induces the activation of $GABA_B$ receptors.

Physiological relevance of CB1 receptor facilitation of substance P release

The fact that CB1 receptors facilitate substance P release reveals an unexpected pronociceptive role of cannabinoids in the spinal cord. Because of the prominent role that substance P and NK1Rs play in the induction of central sensitization (Traub, 1996; Mantyh *et al.*, 1997; De Felipe *et al.*, 1998; Laird *et al.*, 2000), an increase in substance P release would lead to sustained hyperalgesia. Furthermore, inasmuch as substance P release is an indicator nociceptor activity (Hua & Yaksh, 2009), its facilitation could signal an increase in acute nociception. Indeed, we show that CB1 receptors in the spinal cord increase acute thermal nociception (Fig. 8).

Our findings are consistent with the study by Pernia-Andrade et al. (Pernia-Andrade *et al.*, 2009) showing pronociceptive effects of spinal CB1 receptors during hyperalgesia induced by cutaneous capsaicin injection. They found that spinal application of AM251 decreased neuronal firing evoked by stimuli delivered next to the capsaicin injection site. They also showed that capsaicin-induced mechanical hyperalgesia in mice was decreased by intrathecal AM251 and knockout of the CB1 receptor gene, both global and restricted to the spinal cord. Importantly, CB1 receptor deletion restricted to primary afferents did not decrease capsaicin-induced hyperalgesia, showing that the pronociceptive effect is caused by CB1 receptors in dorsal horn neurons. Our results show that this pronociceptive effect of CB1 receptors is not limited to hyperalgesia, but can also be detected during acute nociception.

In conclusion, CB1 receptors in dorsal horn interneurons produce pronociceptive effects by decreasing the release of GABA and opioids next to primary afferent terminals. The resulting decrease in the activity of the $GABA_B$ and μ-opioid receptors in these terminals facilitates substance P release by producing disinhibition.

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Abbreviations

ACEA	arachidonyl-2-chloroethylamide
aCSF	artificial cerebrospinal fluid
AM251	(*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide)
AM281	(1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide)
ANOVA	analysis of variance
CGP-55845	((2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid)
CGRP	calcitonin gene-related peptide

CI	confidence interval
CTAP	D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
Df	degrees of freedom
GPCR	G protein-coupled receptor
NA	numerical aperture
NK1R	neurokinin 1 receptor

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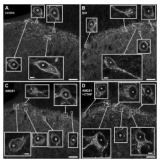

Fig. 1. Images of NK1R neurons in lamina I after dorsal root stimulation

Spinal cord slices were stimulated at the dorsal root at 100 Hz while they were superfused with aCSF alone (**A**, **B**), 100 nM AM281 (**C**), or 100 nM AM251 plus 10 μM CTAP (**D**). Images in panels **A** and **B** were taken from the same histological section and correspond to the dorsal horns contralateral (contra, **A**) and ipsilateral (ipsi, **B**) to the stimulated root. **C** and **D** are from the ipsilateral dorsal horn. Main panels: images taken with a 20x objective, with a voxel size of 488 × 488 × 1180 nm and 5 confocal planes. Insets: images of lamina I neurons taken with a 100x objective, with a voxel size of 98 × 98 × 285 nm and 3 confocal planes. Scale bars are 50 μm for the main panels and 5 μm for the insets. Neurons with NK1R internalization are indicated with "*" and neurons without internalization by "o".

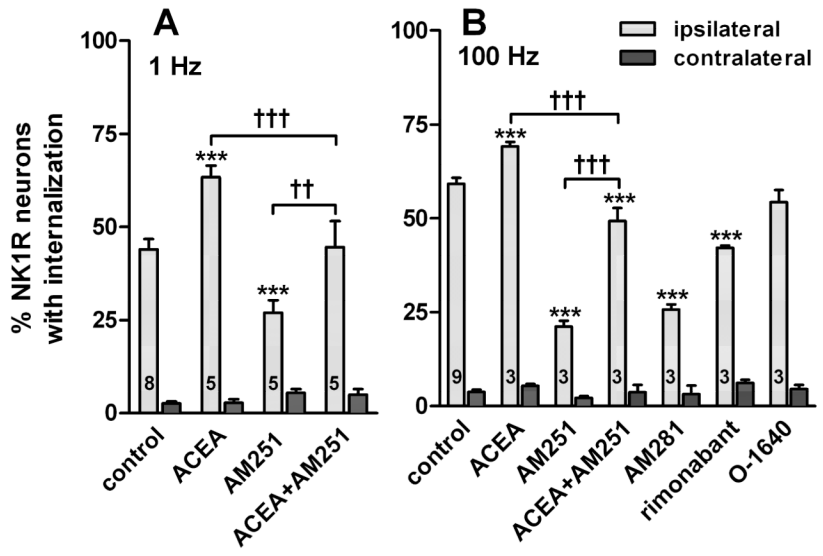

Fig. 2. Effect of CB1 receptor agonists and antagonists on NK1R internalization evoked by dorsal root stimulation

Spinal cord slices were stimulated at the dorsal root with 1000 pulses (20 V, 0.4 ms) delivered at 1 Hz (A) or 100 Hz (B) while they were superfused with the indicated compounds (all at 100 nM). Control was aCSF alone. AM251, AM281 and rimonabant are CB1 receptor antagonists, ACEA is a CB1 receptor agonist and O-2640 is a GPR55 agonist. Numbers inside the bars indicate the number of slices used for each set of data (*N*). Two-way ANOVA yielded $p<0.0001$ overall for the two variables (drugs and stimulus). Bonferroni's post-hoc tests: *** $p<0.001$; ** $p<0.01$; * $p<0.05$ compared to control; ††† $p<0.001$, †† $p<0.01$, as indicated.

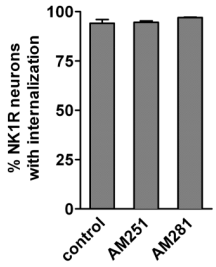

Fig. 3. NK1R internalization induced by exogenous substance P was not affected by CB1 antagonists

Slices were incubated at 35 °C for 10 min with 1 μM substance P alone (control) or with the compounds indicated (all 100 nM). One-way ANOVA: p =0.27.

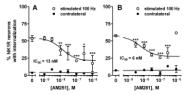

Fig. 4. Concentration-responses of the CB1 antagonists AM251 and AM281

Slices were superfused with the CB1 antagonists AM251 (A) or the CB1 inverse agonist AM281 (B) while the dorsal root was stimulated at 100 Hz. Data are the mean ± SEM of 3 slices (control, 9 slices). NK1R internalization contralateral to the root (filled symbols) was negligible and unaffected by AM251 or AM281. NK1R internalization ipsilateral to the root (empty symbols) was inhibited in a dose-dependent way by both drugs. Curves represent fitting by non-linear regression to a dose-response function: AM251, IC_{50} = 13 nM (95% CI, 2-72 nM), 'bottom' = 21 ± 5%; AM281, IC_{50} = 6 nM (95% CI, 2-16 nM), 'bottom' = 27 ± 3%. The outlier at 1 μM AM281 was excluded from the fitting. Two-way ANOVA revealed significant effects of the drugs and the stimulus (p<0.001). Bonferroni's post-hoc tests: * p<0.05, ** p<0.01, *** p<0.001.

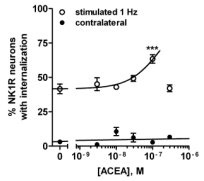

Fig. 5. Concentration-response of the CB1 agonist ACEA

Slices were superfused with the CB1 receptor agonist ACEA while the dorsal root was stimulated at 1 Hz. Data are the mean ± SEM of 3-5 slices. NK1R internalization contralateral to the root (filled symbols) was negligible and unaffected by ACEA (filled symbols). NK1R internalization ipsilateral to the root (empty symbols) was increased by ACEA. The curve represents a tentative fitting of the points (excluding the outlier at 300 nM ACEA) to a dose-response function, with the maximum effect ('top') fixed at 100%. EC_{50} = 175 nM (95% CI, 2 nM-17 μM). Two-way ANOVA revealed significant effects of ACEA (p=0.0008) and the stimulus (p<0.0001). Bonferroni's post-hoc tests: *** p<0.001.

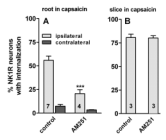

Fig. 6. Effect of AM251 on capsaicin-evoked NK1R internalization

A. The dorsal root was immersed in 1 μM capsaicin for 10 min in a compartment separated from the slice, while the slice was superfused with aCSF alone (control) or 1 μM AM251. Two-way ANOVA yielded $p<0.0001$ for the two variables (AM251 and capsaicin). Bonferroni's post-hoc test: *** $p<0.001$. **B.** Slices were incubated for 10 min at 35 °C with 0.3 μM capsaicin alone (control) or with 1 μM AM251. Numbers inside the bars indicate the number of slices used for each set of data.

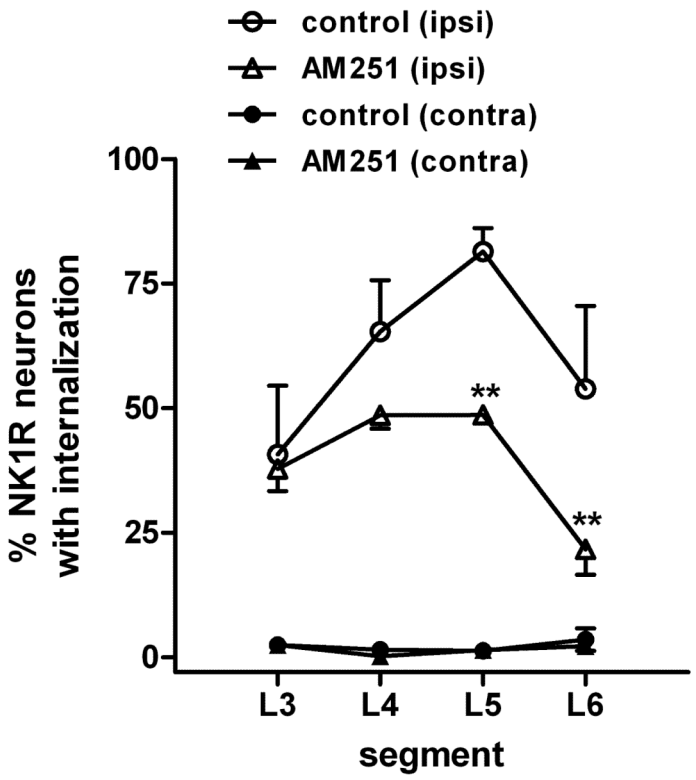

Fig. 7. AM251 inhibits NK1R internalization induced by noxious stimulation

Rats (*N* =3 per group) were injected intrathecally with 10 μl AM251 (10 nmol) or vehicle (10% DMSO, 1% Tocrisolve in saline; control). Substance P release was induced by clamping of the hind paw with a hemostat for 30 s, 10 min after the injection. After 10 min more the rats were euthanized and fixed. Two-way ANOVA yielded p=0.0014 for AM251 and p<0.0001 for spinal region. Bonferroni's post-hoc test: ** p<0.01.

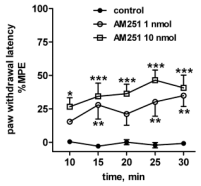

Fig. 8. Analgesia produced by AM251

Analgesia was measured as increases in latency in paw withdrawal responses to radiant heat. Baseline latencies were measured at 5 min intervals three times. Immediately after baseline determination, rats received intrathecal injections of 1 nmol AM251 (N =5) dissolved in 1% DMSO or 10 nmol AM251 (N =5) dissolved in 10% DMSO, 1% Tocrisolve. Control rats (N =7) received vehicle: 1% DMSO (4 rats) or 10% DMSO, 1% Tocrisolve (3 rats). Control values with the two vehicles were essentially the same and were pooled in the figure. Ten minutes after the injection, paw withdrawal latencies were measured at 5 min intervals. Two-way ANOVA revealed a significant effect of AM251 (p<0.0001) but not of time (p=0.19) or the interaction of the two variables (p=0.63). Bonferroni's post-hoc test: * p<0.05, ** p<0.01, *** p<0.001.

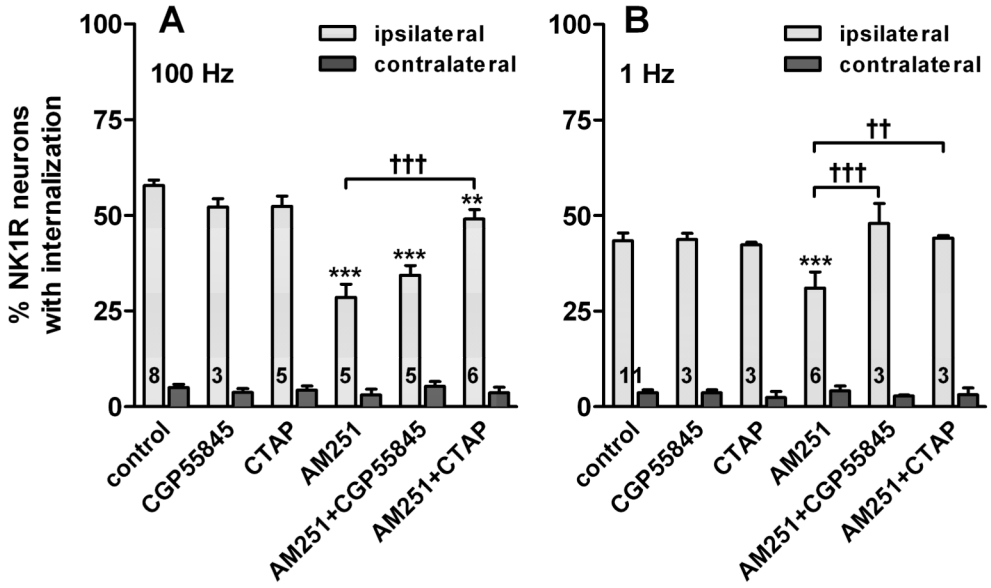

Fig. 9. Reversal by MOR or $GABA_B$ antagonists of the inhibition by AM251

Spinal cord slices were stimulated at the dorsal root with 1000 pulses delivered at 100 Hz (A) or 1 Hz (B) while they were superfused AM251 (100 nM), CGP55845 (100 nM) and CTAP (10 μM), alone or combined as indicated. Control was aCSF alone. Numbers inside the bars indicate the number of slices used (*N*). Two-way ANOVAs: A (100 Hz), $p<0.0001$ for the variables 'drugs', 'stimulus' and their interaction; B (1 Hz), $p<0.0001$ for 'stimulus', $p=0.041$ for 'drugs', $p=0.012$ for their interaction. Bonferroni's post-hoc tests: *** $p<0.001$; ** $p<0.01$, compared to control; ††† $p<0.001$, †† $p<0.01$, as indicated.

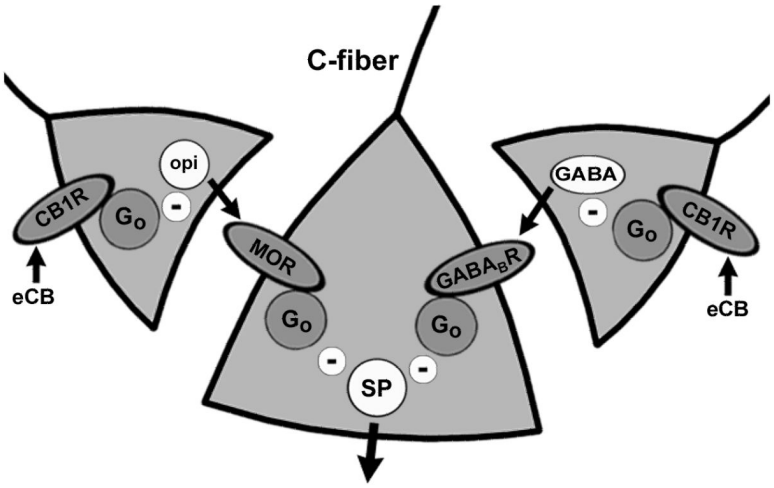

Fig. 10. Diagram showing the proposed disinhibition mechanism for the facilitation of substance P release by CB1 receptors

Dorsal horn interneurons release GABA or opioids (opi) next to substance P-containing primary afferent terminals. MORs or $GABA_B$ receptors ($GABA_BR$) coupling to α_o G proteins (G_o) inhibit substance P release. CB1 receptors (CB1R) in the GABAergic and opioidergic terminals inhibit the release of GABA and opioids, preventing the effect of the MORs and $GABA_B$ receptors.