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Spinal Cannabinoid Receptor Type 2 Activation Reduces Hypersensitivity and Spinal Cord Glial Activation after Paw Incision

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Background: Cannabinoids bind to cannabinoid receptors type 1 and 2 and produce analgesia in several pain models, but central side effects from cannabinoid 1 receptors limit their clinical use. Cannabinoid 2 receptors reduce inflammatory responses in the periphery by acting on immune cells, and they are present on glia in the central nervous system. This study tested whether spinal cannabinoid activation would induce analgesia, glial inhibition, and central side effects in a postoperative model or incisional pain.

Methods: Rats underwent paw incision surgery, with intrathecal injections of cannabinoid agonists and antagonists and assessment of withdrawal thresholds and behavioral side effects. Spinal glial activation was determined by immunohistochemistry.

Results: Intrathecal administration CP55940 reduced postoperative hypersensitivity (91 ± 9% maximum possible effect; $P < 0.05$), and this was prevented by intrathecal administration of both cannabinoid 1 receptor (AM281) and cannabinoid 2 receptor (AM630) antagonists. CP55940 also caused several behavioral side effects, and these were prevented by the cannabinoid 1 receptor but not by the cannabinoid 2 receptor antagonist. Intrathecal injection of the cannabinoid 2 receptor agonist JWH015 reversed postoperative hypersensitivity (89 ± 5% maximum possible effect; $P < 0.05$), and this was reversed by the cannabinoid 2 but not by the cannabinoid 1 receptor antagonist. JWH015, which did not induce behavioral side effects, reduced paw incision induced microglial and astrocytic activation in spinal cord ($P < 0.05$).

Conclusions: These data indicate that intrathecal administration of cannabinoid receptor agonists may provide postoperative analgesia while reducing spinal glial activation, and that selective cannabinoid 2 receptor agonists may do so without central side effects.

Δ^9-TETRAHYDROCANNABINOL, the major active ingredient of the marijuana plant (*Cannabis sativa*), is a psychotropic agent that is thought to exert most of its effects by binding to G protein–coupled cannabinoid receptors 1 and 2, both of which having been implicated in analgesia. Cannabinoid 1 receptors are expressed in neuronal structures.[1] Conversely, cannabinoid 2 receptors are expressed in immune cells[2] and keratinocytes[3] and were originally believed to be present solely in the periphery. Recent studies, however, have demonstrated the presence of cannabinoid 2 receptors in microglia and astrocytes, especially during inflammation.[4–6] In addition, nerve injury increases expression of cannabinoid 2 receptors in peripheral nerve fibers and in the spinal cord.[7]

Activation of cannabinoid 1 receptors induces antinociception.[8–10] However, cannabinoid 1 receptor agonists also produce many neurologic effects that limit their therapeutic use.[10,11] Systemic or local, peripheral administration of cannabinoid 2 receptor agonists produces antinociception without overt behavioral effects in neuropathic, inflammatory, postoperative, and acute pain models.[11–15] These studies assumed a primary action on peripheral structures, mostly immune cells, but central actions have not been previously studied in postoperative pain states. There is a clear rationale for central actions of cannabinoid 2 receptor ligands, however, because they reduce glial activation[16] and/or neuronal activity[17–19] in other pain states.

Here, we studied the importance of central cannabinoid receptor activation in a model of postoperative pain.[20] Cannabinoid 2 receptor agonists have previously been shown to reduce hypersensitivity following paw incision after systemic administration.[13,21] Whereas this could reflect peripheral immunomodulation by cannabinoid 2 receptor agonists, paw incision also activates spinal cord glia,[22] and we therefore tested whether postoperative hypersensitivity could be reduced by intrathecally administered cannabinoid receptor agonists; whether this reflected a cannabinoid 1 receptor, cannabinoid 2 receptor, or combined effect; whether selective cannabinoid activation induced central side effects; and whether this treatment reduced glial activation.

Materials and Methods

Animals and Surgeries

After approval by the Animal Care and Use Committee at Wake Forest University School of Medicine, Winston-Salem, North Carolina, and in accordance with the Guidelines for Animal Experimentation of the International Association for the Study of Pain, male Sprague-Dawley rats weighing 250–275 g (Harlan, Indianapolis, IN) underwent intrathecal catheter implantation as previously described.[23] Briefly, 5–7 days before the experiments, a sterile and nonpyrogenic 32-gauge polyurethane intrathecal catheter (ReCathCo, Allison Park, PA) was inserted 7.5 cm through the cisterna magnum until

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the tip lay near the lumbar enlargement. Animals with neurologic deficits were immediately killed by decapitation during halothane anesthesia. Lidocaine (2%, 10 μl) was injected in six animals at the end of the experiments. A transient bilateral hind limb paralysis confirmed the correct position of the catheter.

Plantar incisional surgery was performed as previous described.[20] Briefly, animals were anesthetized with 2% halothane in oxygen, and the plantar aspect of the left hind paw was prepared in a sterile manner with a 10% povidone–iodine solution. A midline incision (1 cm) from the heel to the base of the toes was performed using a No. 11 blade, using sterile technique. A small forceps was used to elevate the flexor tendon from the heel to the toes. The incision was closed using two inverted with 5.0 nylon mattress sutures.

Drugs and Treatments

The drugs used were the nonselective cannabinoid receptor agonist CP55940 (5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenol; Sigma Chemical Co., St. Louis, MO), the cannabinoid 1 receptor antagonist AM281 (1-(2,4-dichlorophenyl)-5-(4-iodo-phenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide), the cannabinoid 2 receptor antagonist AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone), and the cannabinoid 2 receptor agonist JWH015 ((2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone), all from Tocris (Ellisville, MI). Drugs were administered in the conscious animal through the intrathecal catheter 24 h after plantar incision, and were diluted in dimethylsulfoxide and saline in a ratio of 1:1 and a volume of 10 μl. CP55940 doses were 2.5, 12.5, and 50 μg/rat, respectively, as previously described.[10] AM281 doses were 2.5 and 25 μg/rat. Doses for AM630 and JWH015 were 2, 10, and 50 μg/rat and 2 and 10 μg/rat, respectively. The antagonists or vehicle were injected 30 min before the agonists or vehicle.

Two additional experiments were conducted to examine possible peripheral antinociceptive effects of intrathecal JWH015. In one, the maximum effective intrathecal dose of JWH015 or vehicle was diluted to 0.5 ml in saline and injected intraperitoneally (n = 8 each group) on the first day after incisional surgery. In the other, prevention of the antihypersensitivity effect of 10 μg intrathecal JWH015 was attempted by injection, 15 min earlier, on the dorsal surface of the incised paw of the cannabinoid 2 receptor antagonist AM630 in a dose (10 μg in 50 μl dimethylsulfoxide, 50%) shown to reverse the antinociceptive effect of cannabinoid 2 receptor agonists administered systemically.[12]

The investigator was blinded to drug treatment in all experiments. Withdrawal thresholds were converted to percentage of maximum possible effect according to the formula (withdrawal threshold after drug − withdrawal threshold 24 h after paw incision) $\times$ 100/(withdrawal threshold before paw incision − withdrawal threshold 24 h after paw incision).

Behavioral Testing

The withdrawal threshold was measured twice at 10-min intervals ipsilaterally to paw incision using calibrated von Frey filaments (Stoelting, Wood Dale, IL) and an up–down statistical method,[24] and the average of these values was used for data analysis. Withdrawal threshold was determined in each animal before the paw incision; 24 h after paw incision (immediately before antagonist or vehicle injection); and 0.5, 1, 2, and 4 h after agonist or vehicle injection. For intrathecal treatments, the groups were vehicle plus vehicle (n = 9), vehicle plus CP55940 (2.5, 12.5, and 50 μg/rat; n = 6 in all groups), AM281 plus 12.5 μg CP55940 (2.5 and 25 μg/rat of AM281; n = 5 and 7, respectively), AM630 plus 12.5 μg CP55940 (2, 10, and 50 μg/rat of AM630; n = 7, 6, and 7, respectively), vehicle plus JWH015 (2 and 10 μg/rat; n = 6 in both groups), 25 μg AM281 plus 10 μg JWH015 (n = 6), and 10 μg AM630 plus 10 μg JWH015 (n = 7). For peripheral effects of JWH015, the groups were intraperitoneal vehicle (n = 8) or 10 μg JWH015 (n = 8) and 10 μg intrapaw AM630 plus 10 μg intrathecal JWH015 or intrathecal vehicle (n = 4 each group).

Motor Function and Reflex Testing

Pinna and corneal reflexes, previously studied after intrathecal administered cannabinoid receptor agonists,[8] were ranked as present or absent and represented as percentage. The pinna reflex was evoked by touching the auditory meatus with a culture swab. The reflex was considered present if the ear was retracted and/or the head was shaken. The corneal reflex was evoked by touching the cornea with a culture swab. A blinking response was considered as a positive response.

Based on previous studies, righting and placing-stepping tests were used to evaluate motor reflexes,[8] and the bar test was used to evaluate catalepsy.[11] Cannabinoid receptor agonists have also been noted to induce vocalization and reduced exploratory activity,[10] so these were also evaluated.

The placing-stepping reflex was tested by placing the rostral aspect of the hind paws on the edge of a table and was quantified as the seconds in which the animals put the paws up and forward into a position to walk. A cutoff of 60 s was used. The bar test consists of placing the forelimbs on a bar of approximately 1 cm in diameter and 10 cm above and parallel to a table, leaving the hind paws resting on the table. A cataleptic animal will stay in that position longer than a normal animal. The time in which the animal put its forelimb on the table was recorded, using a cutoff time of 60 s.

The righting test consists of placing the animal prone and recording the ability of the animal to right itself,

studied as normal (an immediate and coordinated twisting of the body to an upright position), mild–moderate impairment (ability to right, but slowly), and severe (inability to right in 20 s). Vocalization was rated as absent, present when manipulated, or present with even light touch. Exploratory activity was rated as normal, only head movements without vertical and/or horizontal exploration, or splayed posture with no spontaneous movements. A scale of 1–3, from normal to severe impairment, was chosen to evaluate those parameters.

All behavioral measures were performed twice, and the average was used for analysis.

Tissue Preparation and Immunostaining

Naive rats (n = 4) or rats on postoperative day 1 and 4 h after intrathecal vehicle or 10 μg JWH015 (n = 5 each group) were deeply anesthetized with pentobarbital and perfused transcardially with buffer (0.01 M phosphate-buffered saline + 1% sodium nitrite, 100 ml) followed by 4% paraformaldehyde (400 ml) at room temperature. The L4–L6 portion of the spinal cord was removed and postfixed in the same fixative for 2-3 h. After cryoprotection in 30% sucrose for 48–72 h at 4°C, the tissue was cut on a cryostat at a 40-μm thickness.

Immunostaining was performed on free-floating sections. After four washes with 0.01 M phosphate-buffered saline plus 0.15% Triton X-100, sections were incubated in 50% alcohol for 45 min. Sections were washed four times with phosphate-buffered saline plus Triton X-100 and blocked with 1.5% normal goat serum. Sections were incubated overnight at 4°C with a primary antibody to ionized calcium-binding adapter molecule 1 (IBA1, rabbit polyclonal, 1:1,000; Wako Pure Chemical Industries, Richmond, VA) to mark microglia and glial fibrillary acidic protein (GFAP, rabbit polyclonal, 1:1,000; Dako Cytomation, Glostrup, Denmark) to mark astrocytes. Sections were washed two times with phosphate-buffered saline plus Triton X-100 and then incubated for 1 h with fluorescein secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). Images were captured using a digital camera attached to a light microscope.

Glial activation has been determined by immunofluorescence staining intensity comparisons.[25] Herein, we quantified the IBA1 and GFAP staining, blinded to experimental conditions, as number of pixels above a preset intensity threshold using standard commercial software. Glial activation is characterized by an increase in the number (proliferation or migration) and the complexity of the cells (rounded cell bodies and thicker processes), resulting in an increase in the immunolabeling. Therefore, an increase in the number of pixels was interpreted as a sign of glial activation. Labeling was examined in a standardized area of laminae I–II or of whole dorsal horn, with three or four slices examined per animal.

Statistical Analysis

The effects of paw incision and drug injections on withdrawal threshold, bar test, and placing-stepping test were determined using a repetitive measurements two-way analysis of variance followed by the Fisher least significant difference test using pre–paw incision thresholds as control. At 4 h after the treatment time point, the dose of CP55940 and JWH015 producing a 50% of maximum efficacy (ED_{50}) or that of AM281 and AM630 producing a 50% maximum inhibition of CP55940 (ID_{50}) and their 95% confidence intervals were calculated. Because righting test, vocalization, and exploratory activity data did not meet parametric assumptions, nonparametric analyses were conducted to examine differences between experimental conditions and changes over time. Repeated measurements were examined using the Friedman test. If significant effects were found, nonparametric Wilcoxon signed ranks tests were conducted, comparing each time point to baseline. Between-group differences were examined at each time period using the Kruskal–Wallis test. Significant effects were followed using the Mann–Whitney U test comparing only the novel treatment to control or agonist group. Comparisons among groups for microglial or astrocytic immunostaining in pixels were performed using t tests or, when normality failed, Mann–Whitney U test. Data are presented as mean ± SEM. A P value less than 0.05 was considered significant.

Results

CP55940 Antinociceptive Effects

Withdrawal threshold was reduced significantly ($P <$ 0.05) 24 h after paw incision in all groups, from 31 ± 0.9 g before surgery to 10 ± 1 g 24 h after surgery. The nonselective cannabinoid receptor agonist CP55940 produced a significant and dose-dependent reversal of paw incision–induced hypersensitivity, whereas vehicle was without effect (fig. 1A; 12.6 ± 2 g). The minimum effective dose of CP55940 was 2.5 μg (27 ± 4.7 g; $P <$ 0.05), the maximum efficacy was achieved with 50 μg (34 ± 3 g; $P <$ 0.05), and the ED_{50} (95% confidence interval) was 10 (4.3–24) μg. The cannabinoid 1 receptor antagonist AM281 prevented the antinociceptive effect of 12.5 μg CP55940 in a dose-related manner (fig. 1B). The ID_{50} of AM281 (95% confidence interval) was 0.8 (0.2–3.1) μg. The cannabinoid 2 receptor antagonist AM630 prevented the antinociceptive effects of 12.5 μg CP55940 at all doses tested (fig. 1C), and the ID_{50} (95% confidence interval) was 2.3 (1.1–4.8) μg.

JWH015 Antinociceptive Effects

The cannabinoid 2 receptor–preferring agonist JWH015 also reversed paw incision–induced hypersensitivity in a dose-related fashion when administered intrathecally and compared with the intrathecal vehicle
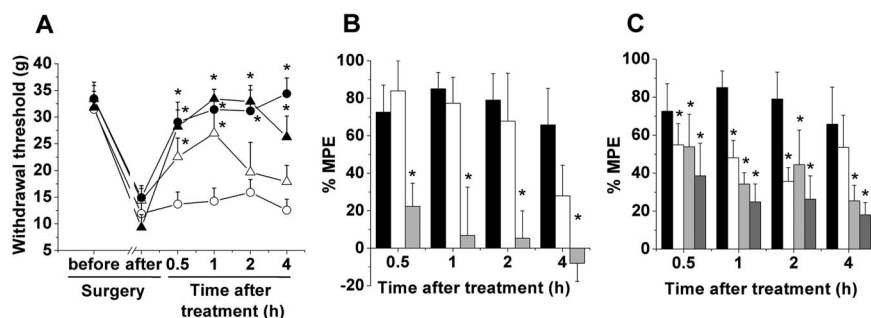
Fig. 1. Withdrawal threshold ipsilateral to paw incision before and after surgery, and after intrathecal administration of CP55940. (A) Withdrawal threshold to von Frey stimulation ipsilateral to paw incision before and 24 h after surgery and 0.5, 1, 2, and 4 h after vehicle plus vehicle (○) or CP55940 at 0.01 (△), 0.05 (▲), and 0.2 (●) mg/kg. * P < 0.05 compared with vehicle group. (B) The percent maximum possible effect (MPE) of vehicle plus CP55940 at 0.05 mg/kg (solid bars) or the cannabinoid 1 receptor antagonist AM281 at 0.01 (open bars) or 0.1 (gray bars) mg/kg plus CP55940 at 0.05 mg/kg. * P < 0.05 compared with vehicle plus CP55940 group. (C) The percent MPE of vehicle plus CP55940 at 0.05 mg/kg (solid bars) or the cannabinoid 2 receptor antagonist AM630 at 2 (open bars), 10 (light gray bars), and 50 (dark gray bars) μg plus CP55940 at 0.05 mg/kg. * P < 0.05 compared with vehicle plus CP55940 group.

group. The lowest dose, 2 μg, reversed paw incision–induced hypersensitivity 0.5, 2, and 4 h after treatment compared with after-surgery withdrawal threshold; however, no differences were found when compared with the intrathecal vehicle group. The highest dose, 10 μg, significantly reversed paw incision–induced hypersensitivity when compared with after-surgery withdrawal thresholds at all the time points tested, and also when compared with intrathecal vehicle group withdrawal thresholds 1, 2, and 4 h after treatment (fig. 2A). The ED_{50} (95% confidence interval) of JWH015 administered intrathecally was 1.6 (0.5–5.5) μg. The most effective intrathecal dose of JWH015, 10 μg, was ineffective when administered intraperitoneally compared with intraperitoneal administration of vehicle (fig. 2A). The antihypersensitivity effect of intrathecal JWH015 was prevented by intrathecal injection of the cannabinoid 2 receptor antagonist AM630 (10 μg; fig. 2B), but it was not prevented by intrathecal injection of the cannabinoid 1 receptor antagonist AM281 (0.1 mg/kg; fig. 2B) or by

intrapaw injection of the cannabinoid 2 receptor antagonist AM630 (fig. 2C). Intrapaw injection of AM630 did not change the paw incision–induced hypersensitivity observed in the intrathecal vehicle group.

Motor Function and Reflex Testing

Intrathecal CP55940 did not alter pinna or corneal reflexes, but induced catalepsy (fig. 3A), blocked the placing-stepping reflex (fig. 3B), induced vocalization (fig. 3C), and reduced exploratory activity (fig. 3D). CP55940 produced these effects in a dose-dependent manner. In contrast, the cannabinoid 2 receptor agonist JWH015 did not affect these behavioral measures (figs. 3E–H). In addition, behavioral disruption produced by 12.5 μg CP55940 was blocked in a dose-related fashion by the cannabinoid 1 receptor antagonist AM281 (figs. 4A–C), except catalepsy, which was only partially blocked by the lowest dose of AM281. Behavioral disruption produced by 12.5 μg CP55940 was not blocked by the cannabinoid 2 receptor
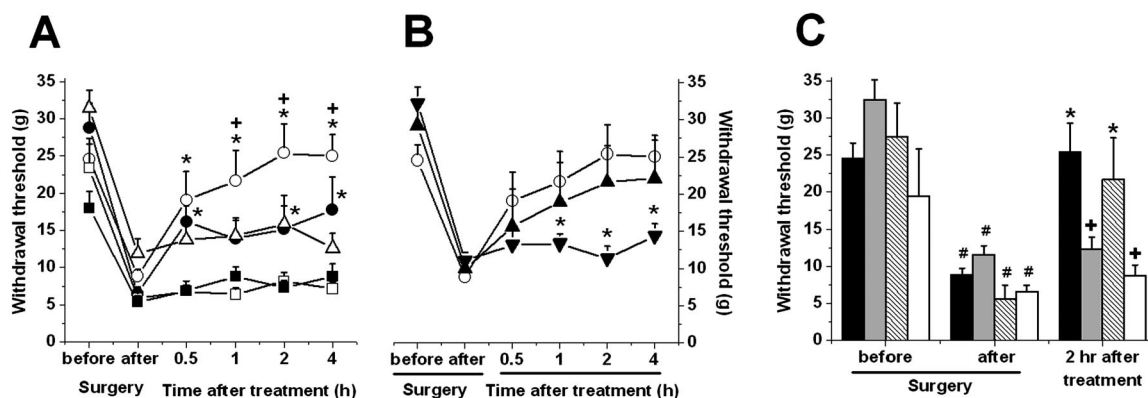

Fig. 2. Withdrawal threshold ipsilateral to paw incision before and after surgery and after the cannabinoid 2 receptor agonist JWH015. (A) Withdrawal threshold to von Frey stimulation ipsilateral to paw incision before and 24 h after surgery and 0.5, 1, 2, and 4 h after intrathecal vehicle (△) and intrathecal JWH015 at 2 (●) and 10 (○) μg, and intraperitoneal JWH015 at 10 μg (■) or intraperitoneal vehicle (□). * P < 0.05 compared with after-surgery withdrawal threshold. + P < 0.05 compared with intrathecal vehicle. (B) Withdrawal threshold to von Frey stimulation ipsilateral to paw incision before and 24 h after surgery and 0.5, 1, 2, and 4 h after JWH015 at 10 μg plus vehicle (○) or the cannabinoid 1 receptor antagonist AM281 at 0.1 mg/kg (▲) or the cannabinoid 2 receptor antagonist AM630 at 10 μg (▼). * P < 0.05 compared with vehicle plus 10 μg JWH015. (C) Withdrawal threshold to von Frey stimulation ipsilateral to paw incision before and 24 h after surgery and 2 h after intrathecal JWH015 at 10 μg plus intrathecal vehicle (solid bars), intrathecal JWH015 at 10 μg plus intrathecal AM630 at 10 μg (gray bars), intrapaw AM630 at 10 μg plus intrathecal JWH015 at 10 μg (striped bars), and intrapaw AM630 at 10 μg plus intrathecal vehicle (clear bars). # P < 0.05 compared with before surgery withdrawal threshold. * P < 0.05 compared with after-surgery withdrawal threshold. + P < 0.05 compared with intrathecal vehicle plus 10 μg intrathecal JWH015 and 10 μg intrapaw AM630 plus 10 μg intrathecal JWH015 groups.
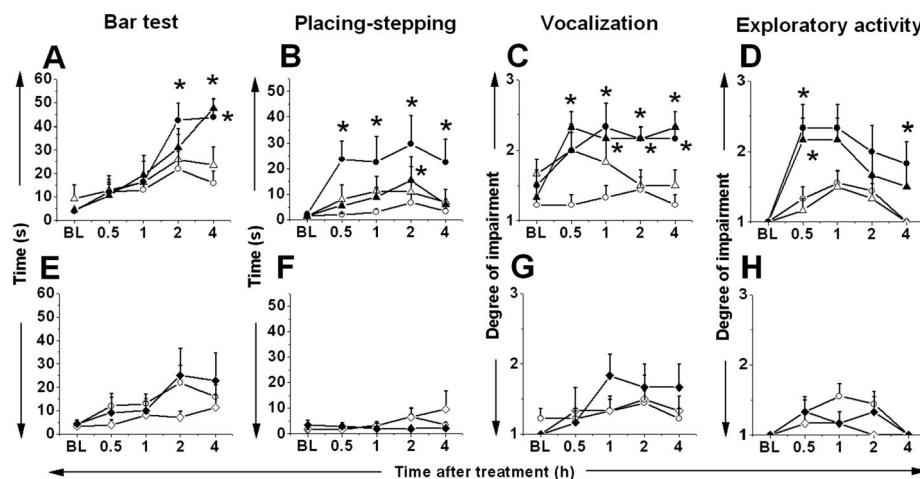
Fig. 3. Cannabinoid receptor agonist–induced neurologic impairments. (A–D) Bar test, placing-stepping reflex, vocalization, and exploratory activity before and after intrathecal injection of vehicle (○) or CP55940 at 0.01 (△), 0.05 (▲), and 0.2 (●) mg/kg, at 0, 0.5, 1, 2, and 4 h. * P < 0.05 compared with vehicle group. (E–H) Bar test, placing-stepping reflex, vocalization, and exploratory activity before and after intrathecal injection of vehicle (○) or JWH015 at 2 (◇) and 10 (◆) μg, at 0, 0.5, 1, 2, and 4 h. BL = baseline.

antagonist AM630, except catalepsy at higher doses (figs. 4D–F).

JWH015 Effects on Glial Activation

JWH015, 10 μg, administered intrathecally, reversed paw incision induced hypersensitivity compared with vehicle group (67 ± 11 *vs.* 11 ± 13% maximum possible effect; P < 0.05) in animals used for immunostaining experiments (data not shown). IBA1 and GFAP immunostaining were similar in spinal cord dorsal horn between naive animals and the dorsal horn contralateral to paw incision in intrathecal vehicle animals, whereas the number and complexity of immunostained objects were increased ipsilateral to paw incision in intrathecal vehicle-treated animals (figs. 5–7). For IBA1 immunostaining, there was a significant increase in deep, but not superficial dorsal horn (figs. 5A and 6A and B). For GFAP, both superficial and deep dorsal horn immunolabeling were increased ipsilateral to surgery (figs. 5B and 7A and B). Both IBA1 and GFAP immunostain-

ing ipsilateral to surgery were reduced in JWH015-treated animals compared with vehicle controls (figs. 5–7). Naive and paw incision plus intrathecal JWH015-treated animals showed characteristic morphology of resting microglia and astrocytes (thin and highly ramified processes), whereas paw incision with intrathecal vehicle resulted in glial cells with larger cell bodies and greatly thickened processes, a characteristic morphology of activated cells (figs. 6D–F and 7D–F).

Discussion

Central Cannabinoid 2 Receptor Activation Induces Antinociception

Spinal administration of nonselective cannabinoid receptor agonists reduces hypersensitivity in several pain models.[8,10] In this study, we have shown a dose-dependent antihypersensitivity effect after paw incision by intrathecal CP55940, a full agonist at cannabinoid recep-

Fig. 4. Cannabinoid receptor agonist–induced neurologic impairments. (A–C) Bar test, vocalization, and exploratory activity before and after vehicle plus CP55940 at 0.05 mg/kg (▲) or the cannabinoid 1 receptor antagonist AM281 at 0.01 (○) or 0.1 (●) mg/kg plus CP55940 at 0.05 mg/kg, at 0, 0.5, 1, 2, and 4 h. * P < 0.05 compared with vehicle plus CP55940 group. (D–F) Bar test, vocalization, and exploratory activity before and after vehicle plus CP55940 at 0.05 mg/kg (▲), or the cannabinoid 2 receptor antagonist AM630 at 2 (x), 10 (◇), and 50 (◆) μg, at 0, 0.5, 1, 2, and 4 h. * P < 0.05 compared with vehicle plus CP55940 group. BL = baseline.
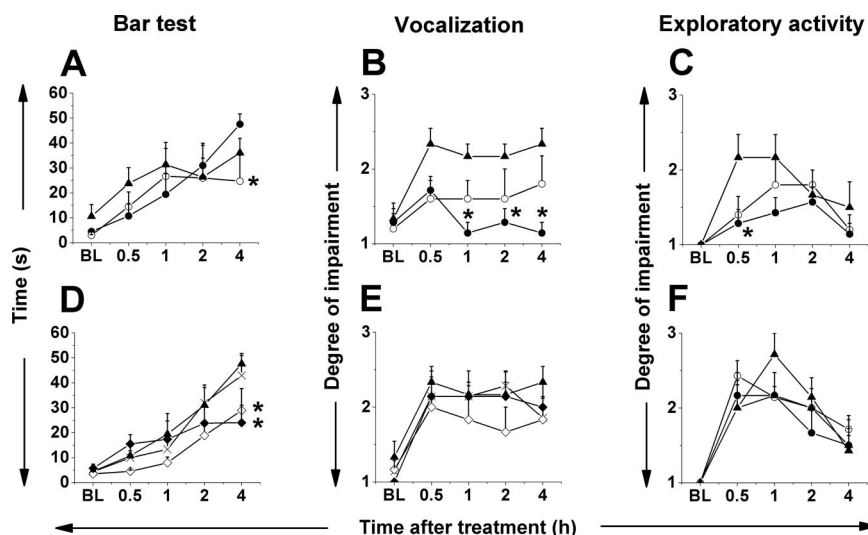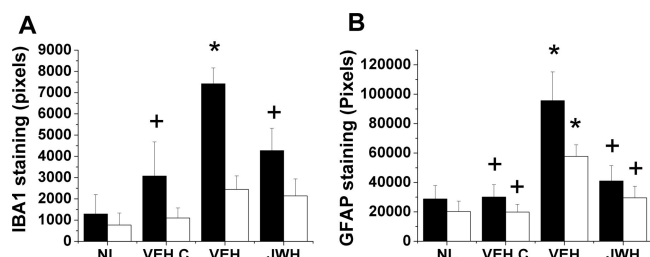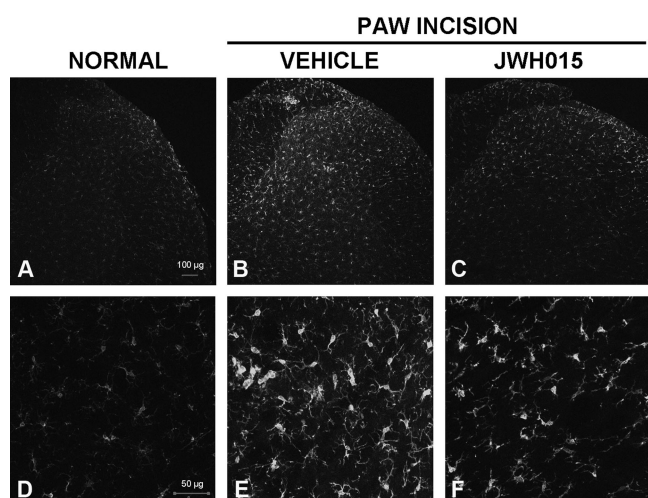
Fig. 5. Ionized calcium-binding adapter molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP) immunostaining quantification. (*A*) IBA1 (microglial marker) immunostaining in whole dorsal horn (*solid bars*) or laminae I–II (*open bars*) of normal animals without incision (NL), vehicle-treated animals contralateral (VEH C) and ipsilateral (VEH) to paw incision, and JWH015 (JWH) ipsilateral to paw incision. * $P < 0.05$ compared with normal animals. + $P < 0.05$ compared with vehicle group ipsilateral to paw incision. (*B*) GFAP (astrocytic marker) immunostaining in whole dorsal horn (*solid bars*) or laminae I–II (*open bars*) of normal animals without incision (NL), vehicle-treated animals contralateral (VEH C) and ipsilateral (VEH) to paw incision, and JWH015 (JWH) ipsilateral to paw incision. * $P < 0.05$ compared with normal animals. + $P < 0.05$ compared with vehicle group ipsilateral to paw incision.

tors. In contrast to the lack of efficacy of intrathecal administration of cannabinoid 2 receptor agonists against formalin-induced hypersensitivity,[8] the current study supports efficacy of spinal cannabinoid 2 receptor activation to diminish hypersensitivity 1 day after paw incision. Therefore, antihypersensitivity after intrathecal administration of a non–subtype-selective agonist was blocked by a cannabinoid 2 receptor–preferring antagonist, and JWH015 relieved paw incision–induced hypersensitivity in a manner that was blocked by a cannabinoid 2 receptor but not a cannabinoid 1 receptor–preferring antagonist.

Previous reports have shown antihypersensitivity with

Fig. 6. Representative confocal images of ionized calcium-binding adapter molecule 1 immunostaining. Lumbar enlargement dorsal horns of normal animals without incision (*A*), and of animals with paw incision treated with intrathecal vehicle (*B*) and intrathecal JWH015 (*C*)–treated animals. Deep dorsal horns of normal animals without incision (*D*), and of animals with paw incision treated with intrathecal vehicle (*E*) and intrathecal JWH015 (*F*)–treated animals.
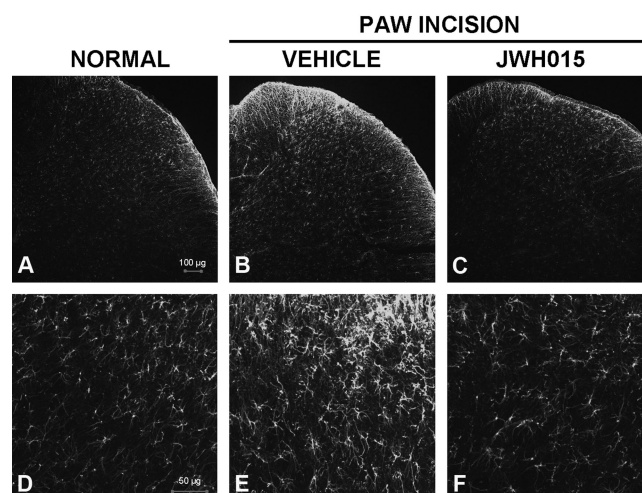
Fig. 7. Representative confocal images of glial fibrillary acidic protein immunostaining. Lumbar enlargement dorsal horns of normal animals without incision (*A*), and of animals with paw incision treated with intrathecal vehicle (*B*) and intrathecal JWH015 (*C*)–treated animals. Deep dorsal horns of normal animals without incision (*D*), and of animals with paw incision treated with intrathecal vehicle (*E*) and intrathecal JWH015 (*F*)–treated animals.

systemic administration of the cannabinoid 2 receptor agonist GW405833 after paw incision.[13,21] GW405833 substantially penetrates the central nervous system after systemic administration[13]; therefore, central cannabinoid 2 receptor could have been activated in those studies. The current study suggests that such central sites are important, because the efficacy of intrathecal injection of a cannabinoid 2 receptor agonist was unaffected by antagonist injection in the hypersensitive paw and because the maximum effective intrathecal dose of the agonist was inactive when administered systemically.

Intrathecal Administration of a Cannabinoid 2 Receptor Agonist Does Not Induce Neurologic Side Effects

The main limitation of cannabinoids for clinical uses has been their psychoactive or undesirable neurologic effects, which reflect activation of cannabinoid 1 receptors expressed in brain areas regulating motor coordination and cognition. Some authors have argued that cannabinoid 2 receptor activation after systemic administration of agonists does not induce side effects because of a lack of cannabinoid 2 receptors in the central nervous system.[25] More recent studies, however, clearly demonstrate the presence of cannabinoid 2 receptors in the brain.[26,27] Further, systemically administered cannabinoid 2 receptor ligands distribute to and act in central regions.[13,17] This suggests that although cannabinoid 2 receptors are present in the brain, they are not responsible for neurologic impairments after systemic administration of cannabinoid receptor agonists. Our observations are consistent with this hypothesis, because intrathecal

CP55940–induced side effects were blocked in a dose-related fashion by the cannabinoid 1 receptor antagonist AM281, but not by the cannabinoid 2 receptor antagonist AM630. In addition, we showed that the selective cannabinoid 2 receptor agonist JWH015 administered intrathecally did not produce neurologic side effects at antihypersensitivity doses. Because the neurologic effects examined in the current study likely reflected distribution of drug from the lumbar intrathecal space, we cannot exclude the possibility that JWH015 did not induce side effects because of limited restriction to the spinal intrathecal space. In addition, only overt effects on behavior were assessed, and we cannot exclude the possibility of cognitive side effects not tested with these simple observations.

Paw Incision Induces Glial Activation

Activation of microglia and astrocytes is involved in the initiation and maintenance of hypersensitivity in several pain models.[28–31] In agreement with a previous study,[22] we found widespread microglial and astrocytic activation in the lumbar dorsal horn ipsilateral to paw incision 24 h after surgery. Peripheral afferents are sensitized and spontaneously active after paw incision,[32] and it has been postulated that this increased input and subsequent sensitization of spinal neurons contribute to glial activation, in part due to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor stimulation in the spinal cord. Microglia can be activated to produce proinflammatory cytokines by kainate or glutamate, an effect that is blocked by an AMPA/kainate antagonist.[33] Paw incision–induced spinal neuron sensitization is known to depend on central glutamatergic signaling through AMPA/kainate rather than N-methyl-D-aspartate receptors,[34] supporting AMPA/kainate receptor stimulation in this model as a likely cause of glial activation.

JWH015 Reduces Paw Incision–induced Glial Activation

Intrathecal administration of the cannabinoid 2 receptor–preferring agonist JWH015 significantly reduced paw incision–induced microglial and astrocytic activation in parallel with its behavioral effects. Because reduction of spinal glial activation reverses or prevents hypersensitivity in other pain models,[28–31] we speculate that JWH015-induced glial inhibition is, at least in part, the cause of JWH015-induced antihypersensitivity. It is important to note that experiments in the current study were performed 1 day after surgery, a time when glial activation in the spinal cord was evident. Whether the cannabinoid receptor agonists would have been effective at earlier times, before such activation, is uncertain. Should they lack efficacy at times closer to surgery, this would lessen their clinical relevance, should they need to be administered intrathecally, because intrathecal in-

jection is most commonly performed at the time of surgery.

It is also conceivable that JWH015 could act on spinal cord neurons, or afferents because cannabinoid 2 receptors are expressed in neurons.[7] Cannabinoid 2 receptor agonists inhibit mechanically evoked responses of wide-dynamic-range dorsal horn neurons in naive rats and in rat models of inflammatory and neuropathic pain,[35] attenuate capsaicin-evoked calcium responses in primary afferents as well as mechanically evoked responses of spinal neurons in neuropathic rats,[19] suppress noxious heat–evoked activity in dorsal horn neurons after systemic or intracerebroventricular administration in normal conditions,[17] and reduce windup after intravenous and intraplantar treatments in paw-inflamed animals.[18]

On the other hand, JWH015 and other cannabinoid 2 receptor agonists inhibit microglia (regulate microglial migration, suppress interferon γ–induced CD40 expression, and attenuate tumor necrosis factor α[16,36]). Cannabinoid 2 receptor stimulation also inhibits astrocytic activation and reduces the release of nitric oxide, interleukin 1β, and tumor necrosis factor α.[5,37] Furthermore, cannabinoid 2 receptor activation induces the production of the antiinflammatory factor interleukin-1 receptor antagonist from neurons and glial cells.[38] Interestingly, tumor necrosis factor-α and interleukin-1β application in spinal cord increases windup and the electrical activity in dorsal horn neurons,[39] and glial or interleukin-1β and tumor necrosis-α inhibition in spinal cord reduces hypersensitivity.[29] This suggests that the glial inhibition by JWH015 would be enough to induce a reduction of spinal neural activity and hypersensitivity.

Conclusions

In conclusion, we show that central cannabinoid 2 receptor activation relieves paw incision–induced hypersensitivity, without overt behavioral side effects. The antihypersensitivity effect of spinal cannabinoid 2 receptor stimulation is paralleled by significant inhibition of glial activation, suggesting a primary action on glia. These data suggest that spinal cannabinoid 2 receptor activation could relieve postoperative pain.

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