

## Intrathecal cannabinoid administration suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in rat spinal cord: comparison with morphine

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**KEY WORDS** cannabinoids; pain measurement; spinal cord; nociceptors; *fos* genes; mu opioid receptors

### ABSTRACT

**AIM:** To determine whether cannabinoids suppress noxious stimulus-evoked Fos protein-like immunoreactivity (FLI) through direct actions at the spinal level.

**METHODS:** Rats were implanted with intrathecal (ith) catheters at least one week prior to evaluation in the formalin test. Effects of the cannabinoid agonist, CP55,940 (80  $\mu$ g ith) on formalin pain and FLI in rat spinal cord were compared with that of the prototypic narcotic analgesic, morphine (20  $\mu$ g ith). CP55,940 suppressed pain behavior and FLI induced by intraplantar formalin. The cannabinoid suppressed Fos in the neck region of the dorsal horn and in the ventral horn, but not in the nucleus proprius. The efficacy of the cannabinoid in suppressing FLI in these laminae and pain behavior was comparable to morphine administered via the same route. However, only morphine suppressed FLI in the superficial dorsal horn relative to vehicle treatment. **CONCLUSION:** Cannabinoids suppress nociceptive processing, in part, through actions at the spinal level. However, morphine showed greater potency and efficacy than CP55,940 in suppressing formalin-induced FLI following spinal administration.

### INTRODUCTION

Cannabinoids modulate the activity of nociceptive neurons in the spinothalamic tract<sup>[1-5]</sup> and produce antinociception<sup>[2,5-9]</sup>. Our previous work demonstrated that systemically administered cannabinoids suppress *c-fos*, a marker for neuronal activity induced by noxious stimulation, in the spinal dorsal horn<sup>[4]</sup>. However, it is unclear whether this suppression is mediated by direct or indirect actions in the spinal dorsal horn.

Cannabinoids suppress nociceptive processing, in part, through actions at the spinal level. This hypothesis is supported by the presence of cannabinoid receptors in the spinal dorsal horn<sup>[10]</sup>. Spinal cannabinoid receptors are localized, in part, presynaptically on central terminals of nociceptive primary afferents. Cannabinoid CB<sub>1</sub> receptors are synthesized in dorsal root ganglion cells that express appropriate neuropeptide phenotypes<sup>[11]</sup>. Unilateral dorsal rhizotomy depletes cannabinoid receptor binding sites in the superficial dorsal horn<sup>[12]</sup>. Moreover, depletion of sensory C-fibers following neonatal capsaicin treatment produces modest but reliable suppressions of cannabinoid binding levels in the superficial dorsal horn relative to mu opioid receptors<sup>[13]</sup>. However, absolute levels of cannabinoid receptors in spinal cord greatly exceed those of peptide receptors<sup>[12]</sup>.

Cannabinoid analgesia involves spinal and supraspinal components. Analgesic effects of intrathecally administered cannabinoids cannot be attributed to diffusion of drug to supraspinal sites<sup>[6,8]</sup>. Antinociceptive<sup>[6]</sup> and electrophysiological<sup>[6]</sup> effects of cannabinoids are attenuated following spinal transection, providing evidence for supraspinal sites of cannabinoid analgesia. Moreover, cannabinoids admi-

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nistered intraventricularly<sup>[7]</sup> or directly into brain sites<sup>[14]</sup> produce antinociception.

The present study was carried out to determine whether cannabinoids suppress noxious stimulus-evoked Fos protein expression through direct actions at the spinal level. The potent synthetic cannabinoid, CP55,940, was administered intrathecally and evaluated for antinociceptive efficacy in a model of tonic pain, the formalin test. Systemic administration of this agonist produces a potent, receptor-mediated suppression of noxious heat-evoked activity in wide dynamic range neurons in the spinal dorsal horn<sup>[3]</sup>. Effects of the cannabinoid on pain behavior and Fos expression were compared with that of the prototypic narcotic analgesic, morphine.

## MATERIALS AND METHODS

**Catheter implantation** Forty-four male Sprague Dawley (Charles River, Boston MA) rats (350–400 g) were used. Data was quantified from 40 animals receiving intraplantar formalin. Four rats were used for control experiments to examine the effects of drug or catheter placement on Fos expression in the absence of formalin. Chronic indwelling intrathecal (ith) catheters<sup>[15]</sup> were implanted under pentobarbital anesthesia at least one week prior to evaluation in the formalin test. Catheters (PE10 tubing; Clay Adams, Parsippany, NJ) were implanted through an incision in the atlanto-occipital membrane to a depth of 8.5 cm, so that the tips extended just rostral to the lumbar enlargement. Catheters were secured to the skull, and the distal ends were heat-sealed. Catheters were flushed with saline (10  $\mu$ L) post-surgery and one day prior to testing. Animals exhibiting signs of motor impairment were discarded. Locations of catheter tips were verified post-mortem.

**Drugs** CP55,940 was a gift from Pfizer Central Research (Groton, CT). Morphine sulfate was obtained from Mallinckrodt Inc (Paris, KY). Drugs were dissolved in a vehicle solution of 60 % Me<sub>2</sub>O and injected over 30 s in a volume of 10  $\mu$ L, followed by 10  $\mu$ L of normal saline to flush the catheter.

**Pain behavior** Methods were approved by the Brown University Animal Care and Use Committee. Experiments were conducted in freely moving rats so that the behavioral and neurochemical indices of

nociception could be determined concurrently in the same subjects. Formalin (4 % paraformaldehyde), administered sc into the plantar surface of the hindpaw, was used as the fos-inducing stimulus. Animals received an intraplantar injection (150  $\mu$ L) of formalin 10 min following CP55,940 (80  $\mu$ g ith;  $n = 14$ ), morphine (20  $\mu$ g ith;  $n = 12$ ) or vehicle ( $n = 12$ ). Doses of CP55,940 and morphine were determined in a pilot study to suppress pain behavior induced by intraplantar formalin. Rats were positioned above a glass mirror to facilitate observation of the formalin-injected paw. Time spent licking and lifting the injected paw was recorded in 5-min intervals for 60 min, as described<sup>[16]</sup>. This method of pain rating is superior to any single measure<sup>[17]</sup>. Rats were overdosed with pentobarbital (60 mg  $\cdot$  kg<sup>-1</sup> ip) and perfused for Fos immunocytochemistry 2 h post formalin administration.

**Immunocytochemistry** Immunocytochemical procedures were described previously<sup>[4]</sup>. Rats were perfused with ice-cold heparinized (1 MU/L) saline followed by 4 % paraformaldehyde in sodium phosphate buffer 0.1 mol  $\cdot$  L<sup>-1</sup>. The lumbar enlargement was dissected and post-fixed in the fixative for 1–2 h. The spinal cord was cryoprotected overnight by incubation in 30 % sucrose in phosphate buffered saline (PBS) 0.1 mol  $\cdot$  L<sup>-1</sup>. Alternate transverse sections (40  $\mu$ m) were cryostat-cut and collected in PBS 0.1 mol  $\cdot$  L<sup>-1</sup> (-20  $^{\circ}$ C). Sections from the three experimental conditions were processed simultaneously for Fos immunocytochemistry. Sections were washed in PBS, and incubated (40 h at 0  $^{\circ}$ C) in rabbit polyclonal Fos protein antibody (Oncogene Science, Uniondale, NY) at a concentration of 1:100. FLI was visualized by the avidin-biotin peroxidase method (ABC vectastain, Vector Laboratories, Burlingame, CA), using diaminobenzidine (0.5 %) and H<sub>2</sub>O<sub>2</sub> (0.1 %) as the chromogens. Sections were mounted, air-dried, and coverslipped. Specificity of immunostaining was verified by preabsorption of the anti-Fos antibody with peptide antigen (1:10). Control experiments examined effects of catheter placement and CP55,940 administration on FLI in the absence of formalin.

**Data analysis** Cells expressing FLI were counted on photomicrographs (100 $\times$  magnification) by an investigator blind to the experimental condition. Three sections at the L5 level, qualitatively exhibiting

the greatest number of labeled cells, were quantified for each rat. All cells were counted as labeled regardless of label intensity. The total number of labeled cells was counted in specific subdivisions of the spinal gray matter ipsilateral to the injected paw. Boundaries between laminae were marked on photomicrographs, as described previously<sup>[4]</sup>. The subdivisions employed were the superficial laminae (laminae I and II), the nucleus proprius (laminae III and IV), the neck region of the dorsal horn (laminae V and VI), and the ventral horn (laminae VII, VIII, IX and X). Data ( $\bar{x} \pm s$ ) were analyzed by ANOVA followed by Tukey post hoc tests.  $P < 0.05$  was considered significant.

## RESULTS

**Pain-related behavior** Pain behavior in control rats exhibited a characteristic biphasic pattern. The first phase peaked at 5 min followed by a sustained phase that peaked approximately 40 min after intraplantar formalin. Drug administration altered the time course of formalin-induced pain ( $P < 0.05$ ).

Both CP55,940 and morphine suppressed formalin-induced pain behavior (Fig 1). During the early phase, CP55,940 was more efficacious than morphine in attenuating pain behavior ( $P < 0.05$ ). During the late phase, the antinociceptive efficacy of the cannabinoid in general did not differ from that of morphine ( $P > 0.05$ ). However, CP55,940 was less efficacious than morphine in suppressing pain behavior during the peak of the late phase, eg 40 min post-formalin ( $P < 0.05$ ). Post-mortem evaluations of catheter placement confirmed that the tips were positioned just rostral to the lumbar enlargement.

**General features of Fos immunoreactive cells** Neurons expressing FLI were observed ipsilateral to the formalin-injected paw. The highest densities of labeled cells were observed in the superficial dorsal horn; approximately 50% of all neurons expressing FLI were concentrated in laminae I and II at the L5 level. The lowest densities of labeled cells (approximately 10%) were observed in the nucleus proprius. Intermediate densities of Fos immunoreactive cells were observed in the neck region of the dorsal horn and in the ventral horn, where approximately 25% and 15% of all labeled cells were localized, respectively.

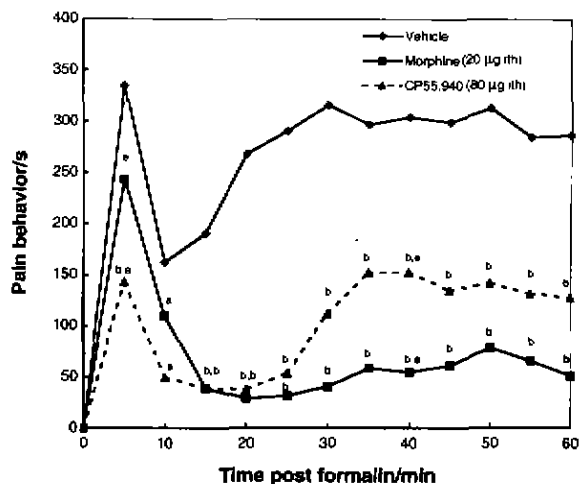


Fig 1. Time course of pain behavior following intraplantar formalin in rats administered intrathecally with vehicle, the cannabinoid agonist CP55,940, or morphine.  $n = 12 - 14$  rats per group. Mean standard deviations, omitted for clarity, were 72.6 (range: 34.1 - 141.1), 100.2 (range: 86.1 - 130.4), and 121.4 (82.3 - 142.8) s for groups receiving vehicle, morphine, and CP55,940, respectively.  $^a P > 0.05$ ,  $^b P < 0.05$  vs vehicle,  $^c P < 0.05$  vs morphine or CP55,940, by ANOVA and Tukey post hoc test.

**Effects of CP55,940 and morphine** Spinal compression was observed in rats implanted with catheters. The affected region was located rostral to the spinal segments exhibiting FLI. The presence of the catheter failed to induce Fos ( $n = 2$ ). Similarly, CP55,940 ( $n = 2$ ) failed to induce FLI in the absence of intraplantar formalin.

CP55,940 and morphine suppressed formalin-induced Fos protein expression in the neck region of the dorsal horn ( $P < 0.05$ ) and in the ventral horn ( $P < 0.05$ ), but not in the nucleus proprius (Fig 2, 3). Effects of the cannabinoid and the mu opioid agonist did not differ in these spinal cord regions. In the superficial laminae, only morphine suppressed FLI relative to vehicle treatment. The number of Fos-immunoreactive cells observed in the superficial laminae following CP55,940 did not differ from that following treatment with either morphine or vehicle ( $P > 0.05$ ).

## DISCUSSION

A potent synthetic cannabinoid, CP55,940, admi-

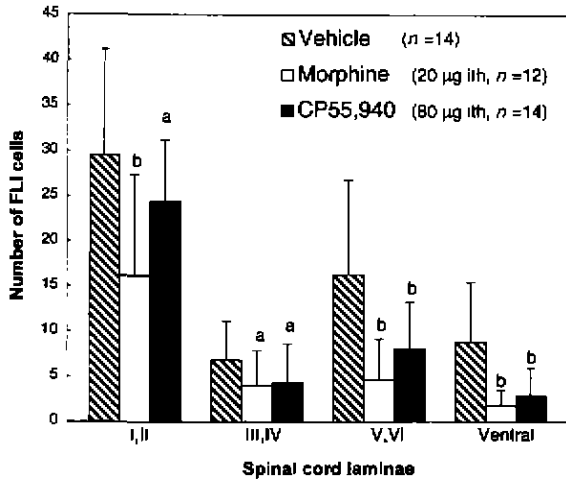


Fig 2. Formalin-induced FLI in rat spinal cord following intrathecal administration of vehicle, CP55,940, and morphine.  $n = 12 - 14$  rats per group.  $\bar{x} \pm s$ .  $^a P > 0.05$ ,  $^b P < 0.05$  vs vehicle.

nistered intrathecally, suppressed pain-related behavior and noxious stimulus evoked c-Fos protein expression in rat spinal cord. These findings are consistent with behavioral<sup>[2,6,8,9,18]</sup>, electrophysiological<sup>[2]</sup>, and anatomical<sup>[10,12,13,19]</sup> studies that suggest a spinal component in cannabinoid analgesia.

Both CP55,940 and morphine suppressed formalin-induced pain behavior. CP55,940 showed greater efficacy than morphine in suppressing pain during the early phase, but lower efficacy during the peak of the late phase. These findings suggest that sustained antinociceptive efficacy contributes to suppression of

FLI in the superficial dorsal horn following morphine.

The suppression of noxious stimulus-evoked Fos protein expression induced by CP55,940 is likely mediated by actions at cannabinoid receptors. Suppression of FLI after systemic administration of cannabinoids is enantioselective and attenuated in rats rendered tolerant to cannabinoids<sup>[4]</sup>, consistent with receptor-mediated effects. Spinal administration of cannabinoids produces receptor-mediated suppressions of noxious stimulus-evoked firing rates in spinal wide dynamic range neurons<sup>[2]</sup> and produces antinociception<sup>[2,6]</sup>. These data suggest that cannabinoid suppression of nociceptive processing involves a spinal component.

The pattern of suppression of FLI induced by morphine in the present study is similar to a previous report employing intrathecal [D-Ala<sup>2</sup>, NMePhe<sup>4</sup>, Gly<sup>5</sup>] enkephalin (DAMGO)<sup>[20]</sup>. Lower levels of suppression of Fos-like immunoreactive cells may be accounted for by higher concentration of intraplantar formalin employed in the present study. These data, together with our previous work<sup>[4]</sup>, suggest that the procedures employed in the present study have the sensitivity to detect changes in FLI induced by pharmacological manipulations.

The pattern of expression of FLI is unlikely to be confounded by technical factors such as catheter placement. The presence of the catheter and administration of CP55,940, in the absence of intraplantar formalin, failed to induce Fos.

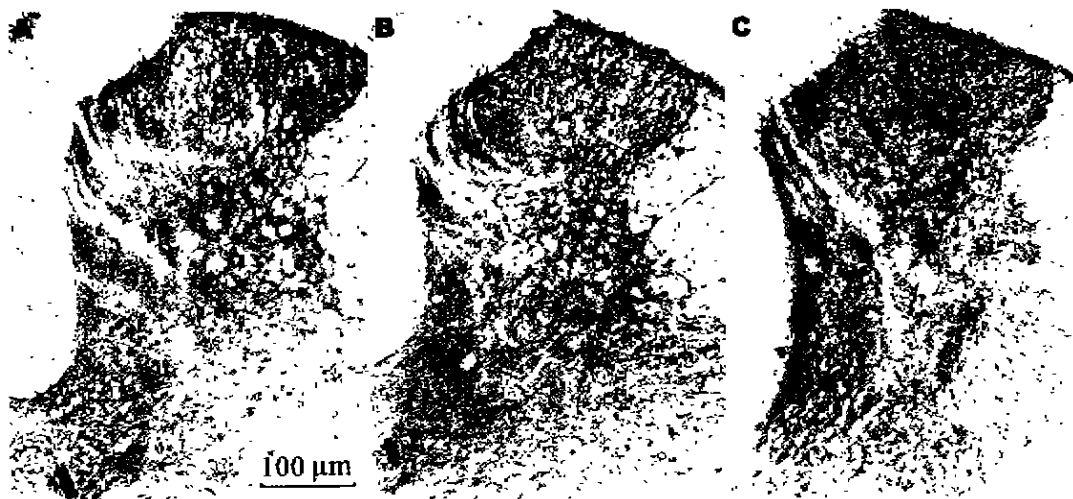


Fig 3. Representative photomicrographs show Fos protein-like immunoreactive cells in rat spinal cord hemisections following (A) vehicle (60 % Mes<sub>2</sub>SO ith), (B) CP55,940 (80 µg ith), and (C) morphine (20 µg ith).

Pain behavior in the formalin test is postulated to involve a supraspinal component. Intrathecally administered cannabinoids may exhibit greater antinociceptive efficacy in measures of pain sensitivity that are dependent upon spinally mediated reflexes (eg tail-flick test) compared with the formalin test. The observation of synergistic analgesic effects following coadministration of cannabinoids and morphine<sup>[18]</sup> also suggests that coadministration of the cannabinoid and mu opioid agonist would produce greater suppressions of FLI than either agonist alone.

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鞘内注射大麻酚类抑制大鼠脊髓内有毒刺激引起的 Fos 蛋白样免疫反应及其与吗啡的比较

R977.2

**关键词** 大麻酚类; 疼痛测定; 脊髓; 疼痛感受器; fos 基因;  $\mu$  阿片受体 (责任编辑 李颖)

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