

# Selective Activation of Cannabinoid CB<sub>2</sub> Receptors Suppresses Hyperalgesia Evoked by Intradermal Capsaicin

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## ABSTRACT

The present studies were conducted to test the hypothesis that activation of peripheral cannabinoid CB<sub>2</sub> receptors would suppress hyperalgesia evoked by intradermal administration of capsaicin, the pungent ingredient in hot chili peppers. The CB<sub>2</sub>-selective cannabinoid agonist (2-iodo-5-nitro-phenyl)-[1-(1-methyl-piperidin-2-ylmethyl)-1*H*-indol-3-yl]-methanone (AM1241) (33, 330 μg/kg i.p.) suppressed the development of capsaicin-evoked thermal and mechanical hyperalgesia and allodynia. AM1241 also produced a dose-dependent suppression of capsaicin-evoked nociceptive behavior. The AM1241-induced suppression of each parameter of capsaicin-evoked pain behavior was completely blocked by the CB<sub>2</sub> antagonist *N*-[[1*S*]-endo-1,3,3-trimethyl bicycle [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-

methylbenzyl)-pyrazole-3-carboxamide (SR144528) but not by the CB<sub>1</sub> antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A). AM1241 (33 μg/kg i.p.) suppressed capsaicin-evoked thermal and mechanical hyperalgesia and allodynia after local administration to the capsaicin-treated (ipsilateral) paw but was inactive after administration to the capsaicin-untreated (contralateral) paw. Our data indicate that AM1241 suppresses capsaicin-evoked hyperalgesia and allodynia through a local site of action. These data provide evidence that actions at cannabinoid CB<sub>2</sub> receptors are sufficient to normalize nociceptive thresholds and produce antinociception in persistent pain states.

Cannabinoid agonists suppress nociceptive transmission and inhibit pain-related behavior in animal models of acute and persistent nociception (for reviews, see Hohmann, 2002; Malan et al., 2002). CB<sub>1</sub> and CB<sub>2</sub> receptor subtypes mediate cannabinoid antinociception. Whereas CB<sub>1</sub> is expressed primarily in the central nervous system (Matsuda et al., 1990; Munro et al., 1993; Zimmer et al., 1999), CB<sub>2</sub> is expressed primarily in cells of the immune system (Lynn and Herkenham, 1994) and is absent in neurons of the central nervous system (Munro et al., 1993; Zimmer et al., 1999; Buckley et al., 2000). Thus, CB<sub>2</sub>-selective agonists fail to elicit centrally mediated cannabimimetic effects such as hypothermia, catalepsy, and hypoactivity (Hanus et al., 1999; Malan et al.,

2001) and are unlikely to be psychoactive or addictive (for review, see Malan et al., 2002).

Both CB<sub>1</sub> (Hohmann and Herkenham, 1999a,b; Khasabova et al., 2002) and CB<sub>2</sub> (Ross et al., 2001; see also Hohmann and Herkenham, 1999a; Price et al., 2003) have been identified in dorsal root ganglion cells, although it is unclear whether CB<sub>2</sub> is expressed in primary afferent neurons. Activation of CB<sub>2</sub> on nonneuronal cells in inflamed tissue is postulated to suppress the release of inflammatory mediators implicated in nociceptor sensitization (Mazzari et al., 1996). However, the mechanism by which activation of CB<sub>2</sub> suppresses nociception remains poorly understood.

Intradermal administration of capsaicin induces hyperalgesia, an increase in pain behavior evoked by suprathreshold stimuli and/or a lowered threshold for pain (Gilchrist et al., 1996). Primary hyperalgesia, observed at the site of injury, is characterized by sensitization to thermal and mechanical

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**ABBREVIATIONS:** CB, cannabinoid; DMSO, dimethyl sulfoxide; i.p., i.pl., intraplantar; ANOVA, analysis of variance; PLSD, protected least significant difference; Fr, Friedman statistic; KW, Kruskal-Wallis statistic; WIN55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; AM1241, (2-iodo-5-nitrophenyl)-[1-(1-methyl-piperidin-2-ylmethyl)-1*H*-indol-3-yl]-methanone; SR144528, *N*-[[1*S*]-endo-1,3,3-trimethyl bicycle [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide.

stimulation. Secondary hyperalgesia to mechanical stimulation is also observed in the surrounding uninjured tissue. Primary hyperalgesia, especially that elicited by noxious thermal stimulation, is mediated, in part, by sensitization of C-fiber mechanoheat (polymodal) nociceptors (Kenins, 1982; Konietzny and Hensel, 1983; Simone et al., 1987; Szolcsanyi et al., 1988; Baumann et al., 1991; LaMotte et al., 1992; Torebjork et al., 1992). By contrast, secondary (mechanical) hyperalgesia involves central nervous system sensitization rather than sensitization of peripheral nociceptors (Baumann et al., 1991; LaMotte et al., 1992). Intradermal administration of vehicle fails to induce hyperalgesia or nocifensive behavior (Gilchrist et al., 1996). Thus, intradermal capsaicin represents a useful tool for elucidating the neural mechanisms underlying CB<sub>2</sub> modulation of hyperalgesia.

The mixed CB<sub>1</sub>/CB<sub>2</sub> agonist WIN55,212-2, but not its receptor-inactive enantiomer, suppresses capsaicin-evoked thermal and mechanical hyperalgesia and nocifensive behavior (Li et al., 1999). Both spinal and peripheral mechanisms are implicated in cannabinoid modulation of capsaicin-evoked hyperalgesia. Intrathecal administration of WIN55,212-2 produces a CB<sub>1</sub>-mediated suppression of thermal and mechanical hyperalgesia but does not alter capsaicin-evoked nocifensive behavior (Johanek et al., 2001). Moreover, intraplantar pretreatment with WIN55,212-2 attenuates thermal hyperalgesia, but this effect is only partially reversed by the CB<sub>1</sub> antagonist SR141716A (Johanek et al., 2001). Furthermore, this same treatment does not attenuate capsaicin-evoked mechanical hyperalgesia or the duration of nocifensive behavior (Johanek et al., 2001). The inability of a CB<sub>1</sub> antagonist to block the suppression of nocifensive behavior and mechanical hyperalgesia by locally administered WIN55,212-2 implicates CB<sub>1</sub>-independent mechanisms in cannabinoid modulation of capsaicin-evoked hyperalgesia.

The recent development of selective agonists and antagonists for CB<sub>2</sub> has provided the pharmacological tools necessary to evaluate the role of CB<sub>2</sub> in modulating persistent nociception. CB<sub>2</sub>-selective agonists have recently been shown to induce antinociception in models of acute, inflammatory, and nerve injury-induced nociception (Hanus et al., 1999; Clayton et al., 2002; Malan et al., 2002; Ibrahim et al., 2003; Nackley et al., 2003b; Quartilho et al., 2003). AM1241 (Goutopoulos and Makriyannis, 2002), a CB<sub>2</sub>-selective agonist (Fig. 1), exhibits 340-fold selectivity for CB<sub>2</sub> over CB<sub>1</sub> ( $K_i$  [CB<sub>2</sub> versus CB<sub>1</sub>] = 2 versus 680 nM in mouse spleen versus rat brain, respectively). AM1241, administered systemically or

locally in the paw, attenuates thermal nociception and hyperalgesia (Malan et al., 2002; Quartilho et al., 2003) and suppresses carrageenan-evoked Fos protein expression and thermal and mechanical hyperalgesia (Nackley et al., 2003b). AM1241 also attenuates neuropathic pain through a CB<sub>2</sub> mechanism that is not dependent upon CB<sub>1</sub> (Ibrahim et al., 2003).

In the present work, we evaluated effects of AM1241, a selective CB<sub>2</sub> agonist, on behavioral sensitization evoked by intradermal administration of capsaicin and identified its site of action. To better understand neural mechanisms underlying antihyperalgesic actions induced by activation of CB<sub>2</sub>, we tested the hypothesis that AM1241 would suppress capsaicin-evoked primary thermal and mechanical hyperalgesia, tactile allodynia, and nocifensive behavior through a CB<sub>2</sub>-specific mechanism. Pharmacological specificity was determined using competitive antagonists for CB<sub>1</sub> and CB<sub>2</sub> (SR141716A and SR144528, respectively).

## Materials and Methods

**Subjects.** One hundred and sixty-two adult male Sprague-Dawley rats (270–350 g; Harlan, Indianapolis, IN) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983).

**Drugs and Chemicals.** Capsaicin (8-methyl-*N*-vanillyl 6-nonamide) was obtained from Sigma-Aldrich (St. Louis, MO). AM1241, a potent CB<sub>2</sub>-selective agonist, was synthesized at the University of Connecticut. SR141716A, a CB<sub>1</sub>-selective antagonist, and SR144528, a CB<sub>2</sub>-selective antagonist, were provided by the National Institute on Drug Abuse. Capsaicin was dissolved (1 mg/ml) in a vehicle of 7% Tween in 0.9% saline, sonicated, and filtered with a 0.22- $\mu$ m Millipore filter as described previously (Gilchrist et al., 1996). All other drugs were dissolved in dimethyl sulfoxide (DMSO).

**General Experimental Methods.** Responsiveness to different modalities of cutaneous (thermal and mechanical) stimulation were assessed in separate groups of rats to prevent stimulus sensitization. Rats received a unilateral intradermal injection (10  $\mu$ l) of capsaicin (10  $\mu$ g) superficially in the mid-plantar surface of the hind paw. A successful injection was confirmed by the appearance of a bleb after intraplantar capsaicin administration (Gilchrist et al., 1996). Drug or vehicle was administered either systemically (1 ml/kg i.p.) 20 min before capsaicin administration or locally (50  $\mu$ l i.p.) in the plantar surface of the hindpaw immediately before capsaicin administration.

**Experiment 1: Assessment of Thermal Hyperalgesia after Systemic Administration of AM1241.** Thermal hyperalgesia was evaluated using the radiant heat method (Hargreaves et al., 1988). Rats were placed in Plexiglas cages on an elevated glass platform. Subjects were acclimated to their environment for 15 to 25 min before testing. Radiant heat was presented to the midplantar region of the hind paw through the floor of the glass platform. Stimulation was terminated upon paw withdrawal or after 25 s if the rat failed to withdraw from the stimulus. After establishing stable baseline responsiveness to thermal stimuli, rats received intraperitoneal injections of AM1241 (33 or 330  $\mu$ g/kg;  $n$  = 6 and  $n$  = 5/group, respectively), SR144528 (1 mg/kg;  $n$  = 5), SR141716A (1 mg/kg;  $n$  = 5), SR144528 (1 mg/kg) coadministered with AM1241 (330  $\mu$ g/kg;  $n$  = 4), SR141716A (1 mg/kg) coadministered with AM1241 (330  $\mu$ g/kg;  $n$  = 5) or vehicle ( $n$  = 5) 20 min before capsaicin administration. Paw withdrawal latencies were subsequently measured in duplicate, alternating between paws, and recorded at 5, 20, 35, and 50 min after capsaicin administration.

**Experiment 2: Assessment of Tactile Allodynia after Systemic Administration of AM1241.** Rats were placed in Plexiglas cages positioned over an elevated wire mesh platform and habitu-

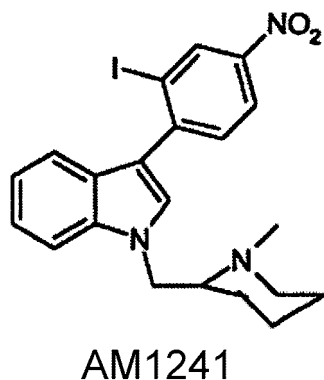


Fig. 1. Chemical structure of AM1241.

ated to the testing environment for 15 to 25 min before testing. Tactile allodynia was assessed using the up-down method (Chaplan et al., 1994). Tactile allodynia refers to a nocifensive behavior elicited by a light touch or innocuous (here, mechanical) stimulus and was operationally defined as a lowering of the threshold for paw withdrawal from punctate mechanical stimulation. A series of nine calibrated filaments (with bending forces of 0.35, 0.47, 0.67, 2.9, 4.2, 5.9, 9.0, 12.5, and 23.4 g; Stoelting, Wood Dale, IL) with approximately equal logarithmic spacing between stimuli (mean  $\pm$  S.E.M.;  $0.201 \pm 0.06$  and  $0.199 \pm 0.06$  units in experiment 2 and 3, and 5, respectively) were presented to each hind paw in successive order, whether ascending or descending. Filaments were positioned in contact with the hindpaw for a duration of 5 s or until a withdrawal response occurred. Testing was initiated with the middle filament of the series (4.2 g). In the absence of a paw withdrawal response, an incrementally stronger filament was presented and in the event of a paw withdrawal, an incrementally weaker filament was presented. After the initial response threshold was crossed, this procedure was repeated four times to obtain a total of six responses in the immediate vicinity of the threshold. The pattern of withdrawals (X) and absence of withdrawal (O) was noted together with the terminal filament used in the series of six responses. The 50% g threshold was interpolated using the formula  $50\% \text{ g threshold} = (10^{(X_f + k\delta)})/10,000$ , where  $X_f$  is the value (in log units) of the final von Frey hair used;  $k$  is the tabular value of pattern of positive (X) and negative (O) responses, as described by Chaplan et al. (1994); and  $\delta$  is the mean difference (in log units) between stimuli.

**Experiment 3: Assessment of Mechanical Hyperalgesia after Systemic Administration of AM1241.** Immediately after determination of the response threshold, a von Frey monofilament (with a calibrated bending force of 12.45 and 11.28 g in experiment 3 and 5, respectively) was presented to the hind paw 10 times for a duration of 1 s with an interstimulus interval of approximately 1 s. The frequency of paw withdrawal (percentage) to punctate mechanical stimulation was assessed in the capsaicin-injected (ipsilateral) and noninjected (contralateral) paws. Only immediate, robust withdrawal responses from the stimulus were recorded as positive responses. Mechanical hyperalgesia was defined as an increase in the percentage frequency (i.e., [no. of paw withdrawals/10]  $\times$  100) of paw withdrawal evoked by stimulation with the von Frey monofilament.

After establishing stable baseline responsiveness to punctate mechanical stimulation, rats received intraperitoneal injections of AM1241 (33 or 330  $\mu\text{g}/\text{kg}$ ;  $n = 6/\text{group}$ , respectively), SR144528 (1 mg/kg;  $n = 6$ ), SR141716A (1 mg/kg;  $n = 6$ ), SR144528 (1 mg/kg) coadministered with AM1241 (330  $\mu\text{g}/\text{kg}$ ;  $n = 6$ ), SR141716A (1 mg/kg) coadministered with AM1241 (330  $\mu\text{g}/\text{kg}$ ;  $n = 6$ ), or vehicle ( $n = 6$ ) 20 min before capsaicin administration. Responsiveness to von Frey monofilaments was reassessed at 5, 30, and 120 min post-capsaicin.

**Experiment 4: Assessment of Nocifensive Behavior after Systemic Administration of AM1241.** Capsaicin-evoked nocifensive behavior was defined as guarding behavior that consisted of

licking and failure to bear weight on the injected paw (Gilchrist et al., 1996). Rats received intraperitoneal injections of AM1241 (33 or 330  $\mu\text{g}/\text{kg}$ ;  $n = 5$  and 13/group), SR144528 (1 mg/kg;  $n = 6$ ), SR141716A (1 mg/kg;  $n = 6$ ), SR144528 (1 mg/kg) coadministered with AM1241 (330  $\mu\text{g}/\text{kg}$ ;  $n = 6$ ), SR141716A (1 mg/kg) coadministered with AM1241 (330  $\mu\text{g}/\text{kg}$ ;  $n = 6$ ) or vehicle ( $n = 7$ ) 20 min before capsaicin administration. The total time rats exhibited nocifensive behavior was measured over 5 min immediately after intradermal administration of capsaicin.

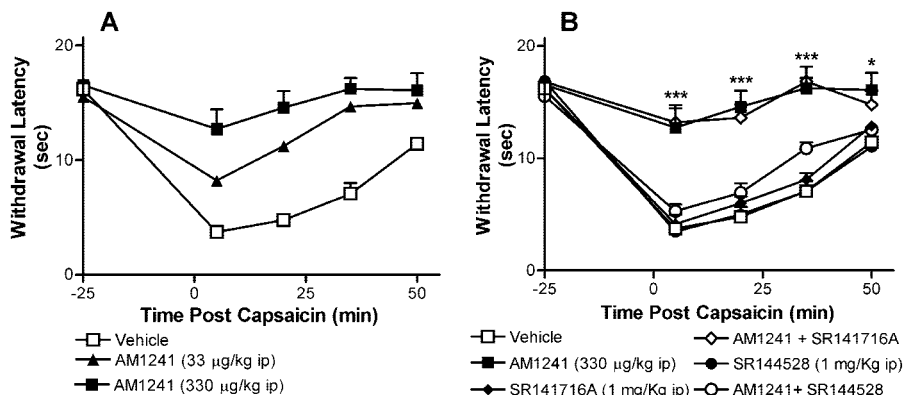
**Experiment 5: Site of Action of AM1241.** To address the site of action, AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.;  $n = 6/\text{group}$  per stimulus modality) or vehicle ( $n = 6/\text{group}/\text{stimulus modality}$ ) was administered locally in the ipsilateral paw just prior to intradermal administration of capsaicin. A separate group of rats received the same dose of AM1241 in the paw contralateral to the site of capsaicin injection ( $n = 6/\text{group}/\text{stimulus modality}$ ). Separate groups of rats were subsequently evaluated for thermal (at 10, 25, 40, and 55 min postcapsaicin) and mechanical hyperalgesia and allodynia (at 10, 35, and 125 min postcapsaicin) as described above.

**Statistical Analysis.** Behavioral data were analyzed parametrically using analysis of variance (ANOVA) for repeated measures (to assess thermal paw withdrawal latency and mechanical paw withdrawal frequency) and ANOVA (to assess nocifensive behavior). The Greenhouse-Geisser correction (Greenhouse and Geisser, 1959) was applied to all repeated factors to avoid spurious significance due to lack of homogeneity of variance and covariance in repeated factors. Mechanical thresholds within each group were analyzed by one-way nonparametric repeated measures ANOVA (the Friedman test). The nonparametric Kruskal-Wallis ANOVA by ranks was subsequently used to assess group differences in capsaicin-evoked paw withdrawal thresholds at time points characterized by maximal capsaicin-evoked allodynia. Post hoc comparisons after parametric and nonparametric ANOVA were performed using Fisher's protected least significant difference (PLSD) and Dunn's multiple comparison post hoc tests, respectively.  $P < 0.05$  was considered to be statistically significant.

## Results

**Experiment 1: Assessment of Thermal Hyperalgesia after Systemic Administration of AM1241.** Paw withdrawal latencies in response to radiant heat did not differ between groups in either paw before administration of capsaicin (mean  $\pm$  S.E.M.;  $16.1 \pm 0.18$  versus  $15.6 \pm 0.19$  s in left and right paws, respectively). In all studies, intraplantar capsaicin reduced paw withdrawal latencies to thermal stimulation ( $P < 0.0002$ ).

AM1241 (33 or 330  $\mu\text{g}/\text{kg}$  i.p.) induced a dose-dependent suppression of thermal hyperalgesia relative to vehicle ( $F_{2,13} = 107.85$ ,  $P < 0.0002$ ;  $P < 0.002$  for each comparison; Fig. 2A). At the time point of maximal capsaicin-evoked hyperal-



**Fig. 2.** A,  $\text{CB}_2$  agonist AM1241 suppresses the development of capsaicin-evoked thermal hyperalgesia. B, AM1241-induced suppression of thermal hyperalgesia was blocked by the  $\text{CB}_2$ -selective antagonist SR144528 (1 mg/kg i.p.), but not by the  $\text{CB}_1$ -selective antagonist SR141716A (1 mg/kg i.p.). Data (mean  $\pm$  S.E.M.) are shown for the capsaicin-injected paw. Capsaicin was administered at time 0. \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$  AM1241, AM1241 + SR141716A different from all other comparisons by ANOVA and Fisher's PLSD post hoc test.  $n = 4$  to 6 rats/group.



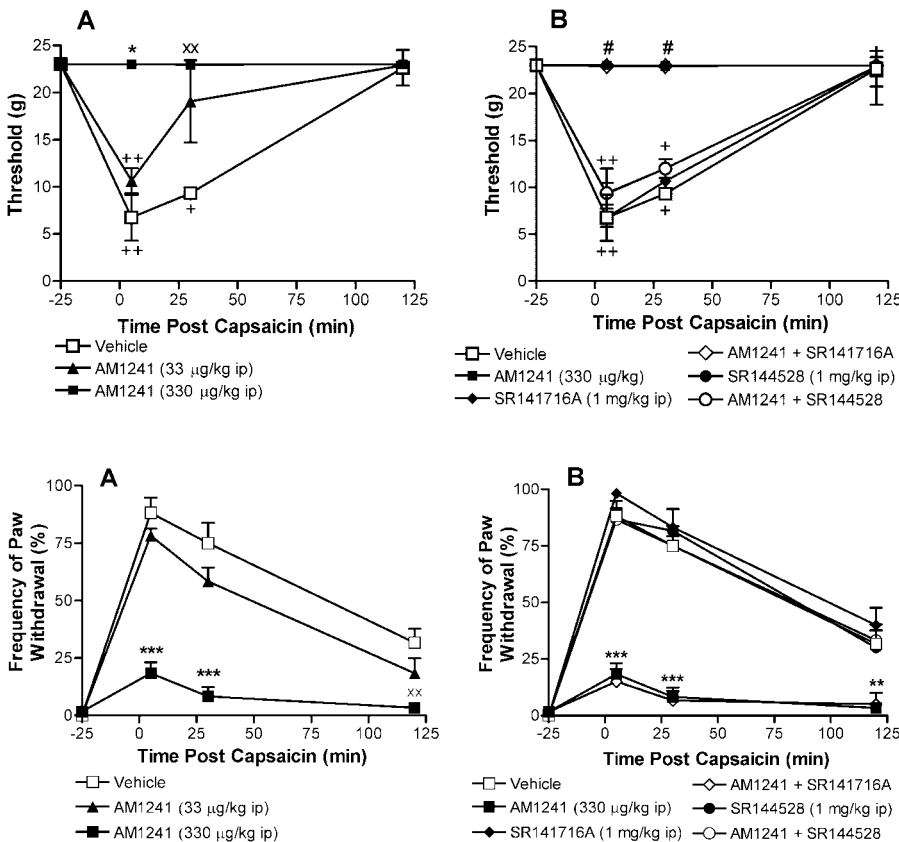
gesia (5 min postcapsaicin), paw withdrawal latencies, relative to preinjection (baseline) levels, were reduced 77% in vehicle-treated rats but only 47 and 23% in rats receiving the low and the high dose of AM1241. Thermal withdrawal latencies in rats receiving the high dose of AM1241 were similar to precapsaicin levels throughout the observation interval. The antihyperalgesic effect of AM1241 (330 μg/kg i.p.) was blocked by the CB<sub>2</sub> antagonist SR144528, but not by the CB<sub>1</sub> antagonist SR141716A ( $F_{5,23} = 57.32, P < 0.002$ ; Fig. 2B). Administration of SR144528 or SR141716A alone failed to alter thermal hyperalgesia. By contrast, after capsaicin administration, withdrawal latencies in the noninjected paw did not differ between groups (average mean ± S.E.M., 17.8 ± 0.22 s).

**Experiment 2: Assessment of Tactile Allodynia after Systemic Administration of AM1241.** The threshold and frequency of paw withdrawal elicited by von Frey monofilaments did not differ between groups prior to intradermal administration of capsaicin (Figs. 3 and 4, respectively). Capsaicin lowered the threshold for paw withdrawal relative to baseline levels in rats receiving vehicle (Friedman statistic,  $Fr = 16.12, P < 0.002$ ) or the low dose (Fr = 16.53,  $P < 0.001$ ) of AM1241 (Fig. 3, A and B). In vehicle-treated rats, the threshold for paw withdrawal was 71 and 59% lower, relative to baseline, at 5 ( $P < 0.05$ ) and 30 ( $P < 0.05$ ) min postcapsaicin, respectively. In rats receiving the low dose of AM1241, paw withdrawal thresholds were 54% lower, relative to baseline, at 5 min ( $P < 0.05$ ) postcapsaicin. By contrast, paw withdrawal thresholds were normalized to baseline levels and did not change over time after systemic administration of the high dose of AM1241 (Fig. 3, A and B). At the time point of maximal alteration in paw withdrawal thresholds (5 min),

the high dose of AM1241 suppressed capsaicin-evoked tactile allodynia (Kruskal-Wallis statistic,  $KW = 12.66, P < 0.002$ ) relative to vehicle ( $P < 0.01$ ) or the low ( $P < 0.05$ ) dose of AM1241 (Fig. 3A). Only the high dose of AM1241 increased paw withdrawal thresholds relative to vehicle at 30 ( $P < 0.01$ ) min postcapsaicin.

Capsaicin also lowered the threshold for paw withdrawal relative to baseline in groups receiving AM1241 (330 μg/kg i.p.) coadministered with SR144528 (Fr = 14.37,  $P < 0.003$ ) or either SR144528 (Fr = 16.88,  $P < 0.0008$ ) or SR141716A (Fr = 16.93,  $P < 0.0008$ ) administered alone (Fig. 3B). Capsaicin-evoked allodynia was observed in each group at 5 ( $P < 0.01$ ) and 30 min ( $P < 0.05$ ) postcapsaicin. By contrast, paw withdrawal thresholds were similar to baseline levels in groups receiving AM1241 either alone or together with SR141716A (Fig. 3B). At 5 min postcapsaicin, the antiallodynic effect of AM1241 ( $KW = 23.09, P < 0.0004$ ) was blocked by the CB<sub>2</sub> antagonist SR144528 ( $P < 0.05$ ), but not by the CB<sub>1</sub> antagonist SR141716A (Fig. 3B). At 30 min postcapsaicin, the AM1241-induced suppression of tactile allodynia ( $KW = 24.60, P < 0.0003$ ) was similarly blocked by SR144528 ( $P < 0.05$ ; Fig. 3B).

**Experiment 3: Assessment of Mechanical Hyperalgesia after Systemic Administration of AM1241.** Capsaicin increased the frequency of paw withdrawal elicited in response to repetitive testing with the 12.45 g von Frey monofilament ( $F_{2,30} = 59.16, P < 0.0002$ ). The frequency of paw withdrawal increased by approximately 80%, relative to baseline, in vehicle-treated rats at 5 min postcapsaicin. AM1241 (33 and 330 μg/kg i.p.) induced a dose-dependent suppression of mechanical hyperalgesia relative to vehicle ( $F_{2,15} = 54.21, P < 0.0002$ ;  $P < 0.0002$  for each comparison;



**Fig. 3.** A, CB<sub>2</sub> agonist AM1241 suppresses the development of capsaicin-evoked tactile allodynia. B, SR144528 (1 mg/kg i.p.), but not SR141716A (1 mg/kg i.p.), blocked capsaicin-evoked tactile allodynia when coadministered with AM1241 (330 μg/kg i.p.). A and B, data [median ± semi-interquartile range (SIQR)] are shown for the capsaicin-injected paw only. ++,  $P < 0.01$ ; +,  $P < 0.05$  threshold different from baseline (within group) by nonparametric one-way repeated measures ANOVA (Friedman test) and Dunn's multiple comparison post hoc test. \*,  $P < 0.05$  different from all other groups; XX,  $P < 0.01$  different from vehicle; #,  $P < 0.05$  AM1241 different from AM1241 + SR144528 by Kruskal-Wallis nonparametric ANOVA and Dunn's multiple comparison post hoc test.  $n = 6$  rats/group.

**Fig. 4.** A, CB<sub>2</sub> agonist AM1241 suppresses the frequency of paw withdrawal elicited in the capsaicin-injected paw in response to successive presentations of a von Frey monofilament (bending force of 12.45 g). B, suppression of mechanical hyperalgesia was blocked by the CB<sub>2</sub> antagonist SR144528 (1 mg/kg i.p.) but not by the CB<sub>1</sub> antagonist SR141716A (1 mg/kg i.p.). Data (mean ± S.E.M.) are shown for the capsaicin-injected paw only. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$  AM1241, AM1241 + SR141716A different from all other comparisons, XX,  $P < 0.01$  different from vehicle by ANOVA and Fisher's PLSD post hoc test.  $n = 6$  rats/group.

Fig. 4A). The high dose of AM1241 reduced the frequency of paw withdrawal from 80 to 17% at the time point of maximal capsaicin-evoked mechanical hyperalgesia, whereas the low dose reduced paw withdrawal frequency to only 71%. The suppression of mechanical hyperalgesia induced by the high dose of AM1241 outlasted that of the low dose ( $F_{4,30} = 7.26$ ,  $P < 0.0006$ ) at 120 min postcapsaicin.

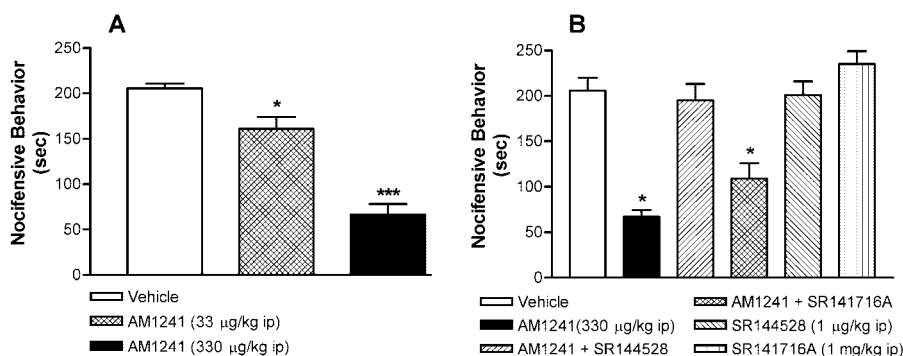
The attenuation of mechanical hyperalgesia induced by AM1241 (330  $\mu\text{g}/\text{kg}$  i.p.) was blocked by the  $\text{CB}_2$  antagonist SR144528 ( $F_{5,30} = 38.39$ ,  $P < 0.0002$ ) but not by the  $\text{CB}_1$  antagonist SR141716A (Fig. 4B). Mechanical hyperalgesia was attenuated in groups receiving AM1241 alone or coadministered with SR141716A compared with groups receiving vehicle, either antagonist administered alone or AM1241 coadministered with SR144528 ( $P < 0.0002$  for each comparison; Fig. 4B). The frequency of capsaicin-evoked paw withdrawal was also similar in groups receiving AM1241 together with SR141716A compared with groups receiving AM1241 alone. SR144528 and SR141716A did not alter mechanical hyperalgesia at any time point relative to vehicle. Withdrawal threshold and frequency in the untreated paw did not differ between groups at any time point.

**Experiment 4: Assessment of Nocifensive Behavior after Systemic Administration of AM1241.** A single phase of nocifensive behavior was observed immediately after intradermal administration of capsaicin and terminated within 4.3 min. AM1241 (33 and 330  $\mu\text{g}/\text{kg}$  i.p.) induced a dose-dependent suppression of capsaicin-evoked nocifensive behavior relative to vehicle ( $F_{2,22} = 53.14$ ,  $P < 0.0002$ ;  $P < 0.02$  for each comparison; Fig. 5A). The low and high doses of AM1241 suppressed the duration of nocifensive behavior by approximately 22 and 68%, relative to groups receiving vehicle. The duration of nocifensive behavior was reduced in groups receiving the high dose of AM1241 relative to groups receiving SR144528 coadministered with AM1241 or either antagonist administered alone ( $P < 0.0002$  for each comparison; Fig. 5B). The duration of nocifensive behavior was attenuated in groups receiving the high dose of AM1241 relative to groups receiving AM1241 coadministered with SR141716A ( $P < 0.03$ ). Nocifensive behavior was greater in groups receiving AM1241 coadministered with SR144528 compared with groups receiving AM1241 together with SR141716A ( $P < 0.0003$ ; Fig. 5B).

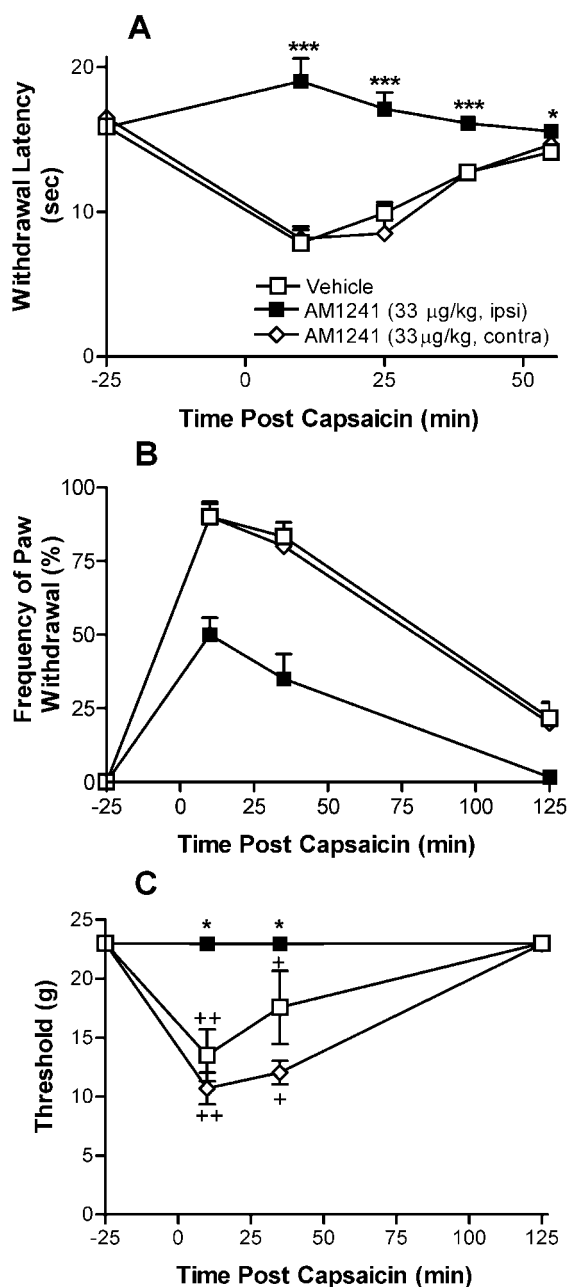
**Experiment 5: Site of Action of AM1241.** Baseline responses to thermal and mechanical stimuli did not differ between groups in either paw before intradermal administration of capsaicin (mean thermal withdrawal latency  $\pm$  S.E.M.;  $16.1 \pm 0.22$  versus  $15.6 \pm 0.22$  s in right and left paws, respectively).

Administration of AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.) locally in the capsaicin-injected paw blocked the development of thermal ( $F_{2,15} = 37.21$ ,  $P < 0.0002$ ; Fig. 6A) and mechanical ( $F_{2,15} = 35.49$ ,  $P < 0.0002$ ; Fig. 6B) hyperalgesia. This attenuation was observed relative to groups receiving vehicle locally in the capsaicin-treated paw or AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.) locally in the capsaicin-untreated (contralateral) paw ( $P < 0.0002$  for each comparison). Thermal withdrawal latencies were similar to preinjection levels at all time points in groups receiving AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.) in the ipsilateral hind paw. Local administration of AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.) reduced the frequency of capsaicin-evoked paw withdrawal to punctate mechanical stimulation from 90 to 50% at 10 min postcapsaicin (Fig. 6B); this same dose induced less than a 10% reduction in mechanical hyperalgesia after systemic administration at 5 min postcapsaicin (Fig. 4A).

Capsaicin lowered the threshold for paw withdrawal in groups receiving vehicle in the capsaicin-treated paw ( $\text{Fr} = 15.32$ ,  $P < 0.002$ ) or AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.) in the capsaicin-untreated paw ( $\text{Fr} = 16.20$ ,  $P < 0.002$ ) relative to baseline levels (Fig. 6C). Allodynia was detected at 10 ( $P < 0.01$  for each comparison) and 35 min ( $P < 0.05$ ) postcapsaicin in each condition. In vehicle-treated rats, capsaicin reduced paw withdrawal thresholds by 41% and 24%, relative to baseline, at 10 and 35 min postcapsaicin, respectively. By contrast, mechanical thresholds did not differ from preinjection levels at any time point in groups receiving AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.) locally in the capsaicin-injected paw. Local injections of AM1241 to the ipsilateral paw suppressed capsaicin-evoked allodynia at 10 ( $\text{KW} = 13.62$ ,  $P < 0.002$ ) and 35 min ( $\text{KW} = 11.21$ ,  $P < 0.004$ ) postcapsaicin (Fig. 6C). The median paw withdrawal threshold was higher in groups receiving AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.) locally in the capsaicin-treated paw relative to groups receiving equivalent injections of vehicle ( $P < 0.05$ ) at each time point. Paw withdrawal thresholds were also higher in groups receiving AM1241 locally in the capsaicin-treated paw relative to the capsaicin-untreated contralateral paw at 10 ( $P < 0.001$ ) and 35 min ( $P < 0.01$ ) postcapsaicin (Fig. 6C). Suppressions of thermal and mechanical hyperalgesia and allodynia were absent when this same dose was applied locally in the paw contralateral to capsaicin administration (Fig. 6, A–C). Capsaicin-evoked pain behavior did not differ between groups receiving vehicle locally in the capsaicin-treated paw or AM1241 locally in the capsaicin-untreated paw in any study. Moreover, no group differences were detected in the capsaicin-untreated (contralateral) paw for any dependent measure.



**Fig. 5.** A, AM1241 suppresses the development of capsaicin-evoked nocifensive behavior. B,  $\text{CB}_2$  antagonist SR144528 (1  $\text{mg}/\text{kg}$  i.p.) produced a greater suppression of nocifensive behavior than the  $\text{CB}_1$  antagonist SR141716A (1  $\text{mg}/\text{kg}$  i.p.). Data are mean  $\pm$  S.E.M. \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$  different from all comparisons by ANOVA and Fisher's PLSD post hoc test.  $n = 5$  to 13 rats/group.



**Fig. 6.** Local administration of AM1241 (33 µg/kg i.pl.) ipsilateral but not contralateral to the capsaicin-injected paw attenuates the development of hyperalgesia and allodynia. A, AM1241 suppresses capsaicin-evoked thermal hyperalgesia. B, AM1241 reduces the frequency of paw withdrawal elicited in the capsaicin-injected paw in response to stimulation with a von Frey monofilament (bending force of 11.28 g). A and B, data (mean ± S.E.M.) are shown for the capsaicin-injected paw only. \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$  different from all comparisons by ANOVA and Fisher's PLSD post hoc test;  $n = 6$ /group. C, AM1241 raised the threshold for paw withdrawal to punctate mechanical stimulation after intraplantar administration in the capsaicin-injected paw. Data [median ± semi-interquartile range (SIQR)] are shown for the capsaicin-injected paw only. ++,  $P < 0.01$ ; +,  $P < 0.05$  threshold different from baseline (within group) by nonparametric one-way repeated measures ANOVA (Friedman test) and Dunn's multiple comparison post hoc test. \*,  $P < 0.05$  different from all other groups by Kruskal-Wallis nonparametric ANOVA and Dunn's multiple comparison post hoc test.

## Discussion

In the present study, selective activation of cannabinoid CB<sub>2</sub> receptors attenuated the development of behavioral sen-

sitization to thermal and mechanical stimulation in the capsaicin model of neurogenic inflammation. The CB<sub>2</sub>-selective agonist AM1241 induced a dose-dependent suppression of capsaicin-evoked thermal and mechanical hyperalgesia and tactile allodynia. These actions were mediated by a peripheral mechanism because administration of AM1241 directly to the capsaicin-injected paw suppressed the development of thermal and mechanical hyperalgesia and allodynia, whereas the same dose administered to the contralateral (capsaicin-untreated) paw was inactive. These data are in agreement with other studies showing that CB<sub>2</sub>-selective agonists are antinociceptive in models of acute and inflammatory nociception (Hanus et al., 1999; Clayton et al., 2002; Malan et al., 2002; Nackley et al., 2003b; Quartilho et al., 2003). Our findings demonstrate that AM1241 suppresses capsaicin-evoked mechanical and thermal hyperalgesia, tactile allodynia, and nocifensive behavior through a CB<sub>2</sub>-specific mechanism and extend recent observations made by Quartilho et al. (2003). This latter work showed that systemic administration of AM1241 induces a CB<sub>2</sub>-mediated suppression of thermal hyperalgesia (at 10 min postcapsaicin) and flinching evoked by capsaicin administration to the dorsal hind paw surface (Quartilho et al., 2003). Our data additionally demonstrate 1) that local administration of AM1241 to the site of injury suppresses behavioral sensitization to capsaicin, consistent with a peripheral site of action; 2) that the suppressive effects of systemically and locally administered AM1241 generalize to multiple modalities of stimulation (mechanical as well as thermal); 3) that behavioral sensitization to each stimulus modality is completely blocked by the CB<sub>2</sub> antagonist SR144528 but not by the CB<sub>1</sub> antagonist SR141716A; and 4) reveal the time course of AM1241-induced suppressions of capsaicin-evoked thermal and mechanical hyperalgesia and tactile allodynia. The duration of capsaicin-evoked mechanical hyperalgesia outlasts that of capsaicin-evoked thermal hyperalgesia (Gilchrist et al., 1996). Our data show that the antihyperalgesic and antiallodynic effects of AM1241 (330 µg/kg i.p.) outlast the duration of capsaicin-evoked behavioral sensitization. More work is necessary to determine whether AM1241 suppresses capsaicin-evoked plasma extravasation through activation of peripheral CB<sub>2</sub> receptors.

Intradermal administration of capsaicin to the plantar hind paw surface induced a single phase of nocifensive behavior consistent with other published reports (Gilchrist et al., 1996). Because nocifensive behavior terminated by 5 min postcapsaicin, this behavior could not confound assessments of hyperalgesia or allodynia in the present work. AM1241 induced a dose-dependent suppression of capsaicin-evoked nocifensive behavior. The AM1241-induced attenuation of nocifensive behavior was completely blocked by the CB<sub>2</sub> antagonist SR144528. In our study, a modest but significant blockade of the AM1241-induced suppression capsaicin-evoked nocifensive behavior was also observed after coadministration with the CB<sub>1</sub> antagonist SR141716A. Thus, our data are also consistent with observations by Simone and colleagues that SR141716A only partially blocks the attenuation of capsaicin-evoked nocifensive behavior induced by the mixed CB<sub>1</sub>/CB<sub>2</sub> agonist WIN55,212-2 (Johanek et al., 2001).

The antihyperalgesic and antiallodynic actions of AM1241 were blocked by the CB<sub>2</sub> antagonist SR144528 but not by the CB<sub>1</sub> antagonist SR141716A. These findings are consistent



with recent observations by our group demonstrating that pretreatment with AM1241 suppresses C-fiber-mediated responses and wind-up during the development of inflammation without reliably altering A- $\beta$  or A- $\delta$  fiber-evoked responses; these electrophysiological effects were more pronounced in carrageenan-inflamed rats relative to noninflamed rats and were blocked selectively by the CB<sub>2</sub> antagonist SR144528 (Nackley et al., 2003a). Intraplantar anandamide also attenuates noxious and nonnoxious mechanically evoked responses in spinal wide dynamic range neurons in the presence of carrageenan inflammation, but not in its absence; these actions were blocked by the CB<sub>2</sub> antagonist SR144528 (Sokal et al., 2003). The lack of efficacy of SR141716A in blocking the attenuation of capsaicin-evoked hyperalgesia induced by AM1241 is consistent with the absence of a neuronally expressed CB<sub>2</sub> receptor in the central nervous system (Munro et al., 1993; Zimmer et al., 1999; Buckley et al., 2000). The functional significance of CB<sub>2</sub> mRNA expression in nonneuronal cells that coincides with the appearance of activated microglia after injury (Zhang et al., 2003) awaits further investigation.

A CB<sub>2</sub> mechanism does not tonically modulate nociceptive thresholds in the capsaicin model of persistent nociception. Neither SR144528 nor SR141716A, administered before intradermal capsaicin, altered nociceptive thresholds relative to vehicle. This observation is consistent with our failure to observe facilitation of carrageenan-evoked spinal Fos protein expression or pain behavior after preemptive systemic or intraplantar administration of SR144528 (Nackley et al., 2003b,c). Moreover, systemic pretreatment with CB<sub>1</sub> and CB<sub>2</sub> antagonists, also failed to enhance A $\beta$ -, A $\delta$ -, and C-fiber-evoked responses and wind-up in spinal wide dynamic range neurons during the development of carrageenan inflammation (Nackley et al., 2003a).

Intraplantar administration of the CB<sub>2</sub> agonist suppressed capsaicin-evoked hyperalgesia and allodynia relative to rats receiving an equivalent intraplantar injection of vehicle. Support for a local site of action for AM1241 in the present work is derived from the observation that AM1241 suppressed hyperalgesia and allodynia after local administration in the capsaicin-treated paw but was inactive after local administration in the contralateral (capsaicin-untreated) paw. Pharmacological specificity of AM1241-induced actions was established using antagonists that were administered systemically, rather than locally in the paw. In the carrageenan model of inflammation, a local dose of AM1241 that is 121-fold higher than the local dose used in the present study (4000 versus 33  $\mu\text{g}/\text{kg}$  i.p.) was blocked selectively by a CB<sub>2</sub> but not by a CB<sub>1</sub> antagonist (Quartilho et al., 2003). These findings suggest that the lower dose of AM1241 employed here is unlikely to be acting nonselectively at CB<sub>1</sub>.

Both the high dose of AM1241 (330  $\mu\text{g}/\text{kg}$  i.p.) administered systemically and the low dose (33  $\mu\text{g}/\text{kg}$  i.p.) administered locally in the paw effectively normalized thermal and mechanical withdrawal latencies thresholds to precapsaicin levels. For each route of drug administration, thermal paw withdrawal latencies and mechanical paw withdrawal thresholds were similar in rats receiving AM1241 (330  $\mu\text{g}/\text{kg}$  i.p. or 33  $\mu\text{g}/\text{kg}$  i.p.) to baseline (precapsaicin) levels. Moreover, AM1241 (33  $\mu\text{g}/\text{kg}$  i.p.), administered locally in the paw, was more potent than the same dose administered systemically. These data are similarly consistent with a local

site of action. However, the local dose of AM1241 was not sufficient to eliminate capsaicin-evoked mechanical hyperalgesia elicited by repetitive stimulation with von Frey monofilaments.

In the present study, capsaicin-evoked reductions in thermal paw withdrawal latencies were similar in rats receiving a local injection of the DMSO vehicle to those observed in naive rats receiving the same concentration of capsaicin (51% in Fig. 6A versus 50% decrease in latency reported by Gilchrist et al., 1996). Moreover, in the present study, thermal and mechanical hyperalgesia and allodynia in the capsaicin-injected paw did not differ in groups receiving vehicle locally in the ipsilateral paw or AM1241 locally in the contralateral (capsaicin-untreated) paw. In addition, no group differences were detected in the contralateral hind paw in any dependent measure. Importantly, intraplantar injections of the DMSO vehicle did not prevent detection of capsaicin-evoked hyperalgesia and allodynia or the antihyperalgesic and antiallodynic effects of locally administered AM1241 in the present study. Thus, possible alterations in sensory thresholds after injections of vehicle cannot account for the local antihyperalgesic and antiallodynic effects of AM1241 observed here.

Our data indicate that selective activation of CB<sub>2</sub> with AM1241 is sufficient to suppress the activation of C-polymodal heat nociceptors that results in primary (thermal) hyperalgesia in the capsaicin model of persistent nociception. More work is necessary to determine whether activation of CB<sub>2</sub> is sufficient to suppress the central nervous system sensitization that underlies secondary hyperalgesia to mechanical stimulation outside the zone of injection. Our data could parsimoniously be attributed, in part, to a direct effect of the CB<sub>2</sub>-selective agonist on primary afferent C-fibers. However, levels of CB<sub>2</sub> mRNA are similar to background in native dorsal root (Hohmann and Herkenham, 1999a) and trigeminal (Price et al., 2003) ganglia under conditions in which CB<sub>1</sub> mRNA was clearly demonstrated and CB<sub>2</sub> mRNA was highly expressed in rat spleen. Of course, different expression levels could be observed in acute or persistent pain states, or levels of CB<sub>2</sub> mRNA could be near the threshold for detection. CB<sub>2</sub> does not couple to calcium-Q or inward rectifying K<sup>+</sup> channels in CB<sub>2</sub>-transfected cell lines (Felder et al., 1995), suggesting that CB<sub>2</sub> mechanisms regulate neuronal excitability through other signal transduction systems (e.g., mitogen activated protein kinase; Bouaboula et al., 1996) and/or modulation of nonneuronal cells (e.g., satellite glial cells).

The present work provides evidence that activation of a cannabinoid CB<sub>2</sub> mechanism in the periphery is sufficient to suppress thermal and mechanical hyperalgesia, tactile allodynia, and nocifensive behavior evoked by intradermal capsaicin. Our data suggest that the failure of CB<sub>1</sub> activation to block mechanical hyperalgesia and nocifensive behavior after intraplantar administration of WIN55,212-2 (Johanek et al., 2001) can be attributed to mediation by CB<sub>2</sub>. Our data support a newly emerging literature that suggests that CB<sub>2</sub>-selective agonists may be exploited as a novel pharmacotherapy for pain in the absence of unwanted central side effects.

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