

Effects of a Cannabinoid Agonist on Spinal Nociceptive Neurons in a Rodent Model of Neuropathic Pain

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Liu, Cheng and J. Michael Walker. Effects of a cannabinoid agonist on spinal nociceptive neurons in a rodent model of neuropathic pain. *J Neurophysiol* 96: 2984–2994, 2006. First published August 30, 2006; doi:10.1152/jn.00498.2006. The effects of the synthetic cannabinoid WIN 55,212–2 on heat-evoked firing of spinal wide dynamic range (WDR) neurons were examined in a rodent model of neuropathic pain. Fifty-eight WDR neurons (1 cell/animal) were recorded from the ipsilateral spinal dorsal horns of rats with chronic constriction injury (CCI) and sham-operated controls. Relative to sham-operated controls, neurons recorded in CCI rats showed elevations in spontaneous firing, noxious heat-evoked responses, and afterdischarge firing as well as increases in receptive field size. WIN 55,212–2 (0.0625, 0.125, and 0.25 mg/kg, intravenous) dose-dependently suppressed heat-evoked activity and decreased the receptive field areas of dorsal horn WDR neurons in both nerve injured and control rats with a greater inhibition in CCI rats. At the dose of 0.125 mg/kg iv, WIN 55,212–2 reversed the hyperalgesia produced by nerve injury. The effect of intravenous administration of WIN 55,212–2 appears to be centrally mediated because administration of the drug directly to the ligated nerve did not suppress the heat-evoked neuronal activity in CCI rats. Pretreatment with the cannabinoid CB₁ receptor antagonists SR141716A or AM251, but not the CB₂ antagonist SR144528, blocked the effects. These results provide a neural basis for reports of potent suppression by cannabinoids of the abnormal sensory responses that result from nerve injury.

INTRODUCTION

Chronic constriction of the sciatic nerve (CCI) in rats, which mimics persistent nerve entrapment (Bennett and Xie 1988), is a widely used model of neuropathic pain. CCI rats show symptoms that are common in human neuropathic pain patients including allodynia, mechanical and thermal hyperalgesia, extraterritorial pain, and guarding behavior suggestive of spontaneous pain (Attal et al. 1990; Bennett and Xie 1988). Electrophysiological studies revealed that spinal dorsal horn neurons ipsilateral to the constricted nerve become sensitized, this manifested by increased spontaneous activity, exaggerated responses to afferent input, and prolonged afterdischarge to noxious stimuli (Laird and Bennett 1993; Palecek et al. 1992a,b; Sotgiu et al. 1992). One advantage of this experimental model lies in the possibility of correlating behavioral signs of pain with physiological changes that occur in the nerve itself or its central connections.

Cannabinoids suppress pain reactions in animal models of acute pain, an early example being that provided by Dixon (1899), who observed that dogs failed to respond to pinpricks

after exposure to cannabis smoke. The subsequent preclinical literature is uniform in showing that naturally occurring cannabinoids (e.g., Δ^9 -tetrahydrocannabinol) and synthetic cannabinoids (e.g., WIN 55,212–2, CP-55,940) inhibit responses to all types of acute noxious stimuli (Lichtman and Martin 1991; Martin et al. 1996; Smith et al. 1998; Sofia et al. 1973; Welch et al. 1998). Although the profound suppression of pain behavior by cannabinoids is impressive, cannabinoids also produce motor dysfunction that could be misinterpreted as a loss of sensory responsiveness (reviewed in Sañudo-Peña et al. 1998). However, neurophysiological studies showed that cannabinoids suppress the responsiveness of nociceptive neurons in the spinal cord and thalamus indicating a reduction in nociceptive processing independent of any motor effects (Hohmann et al. 1995, 1998, 1999; Martin et al. 1996; Strangman and Walker 1999; Walker et al. 1999).

Cannabinoids are also effective against persistent nociceptive processes, reducing the spontaneous pain behavior as well as the hyperalgesia and allodynia that result from noxious chemical stimuli and peripheral inflammation (Bicher and Mechoulam 1968; Kosersky et al. 1973; Li et al. 1999; Martin et al. 1999; Moss and Johnson 1980; Richardson et al. 1998a,b; Tsou et al. 1996). Herzberg et al. (1997) reported that relatively low doses of the cannabinoid agonist WIN 55,212–2 eliminated the hyperalgesia and allodynia produced by CCI, a finding confirmed by others (Bridges et al. 2001; Fox et al. 2001). Both CB₁ (Bridges et al. 2001; Fox et al. 2001; Herzberg et al. 1997; Mao et al. 2000;) and CB₂ (Elmes et al. 2004; Ibrahim et al. 2005; Malan et al. 2003; Sagar et al. 2005) receptors at peripheral and central sites were proposed to mediate cannabinoid-induced antinociception sites in neuropathic pain.

To gain insight into the mechanisms by which cannabinoids suppress neuropathic pain, we used electrophysiological methods to examine the effects of WIN 55,212–2 (0.0625, 0.125, and 0.25 mg/ml iv) on spontaneous and heat-evoked activity of lumbar dorsal horn neurons and their receptive field sizes in the CCI model of neuropathic pain. Subtype-specific cannabinoid receptor antagonists were used to determine whether the observed effects were mediated by CB₁ or CB₂ receptors. The relationships between the neurophysiological and behavioral effects of the drug were studied to provide insight into the neurophysiological parameters that predict pain behavior.

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METHODS

Drug preparation

The mixed CB₁/CB₂ cannabinoid agonist WIN 55,212-2 (Research Biochemicals International, Natick, MA), the CB₁ antagonists SR141716A (Research Triangle Inst., RTP, NC, 1 mg/ml) and AM 251 (Cayman Chemical, Ann Arbor, MI, 1 mg/ml), and the CB₂ antagonist SR 144528 (Sanofi, Recherche, France, 1 mg/ml) were prepared in an emulphor:ethanol:saline (1:1:18) vehicle and administered through the lateral vein in a volume of 1 ml/kg.

Subjects/CCI surgery

Sprague-Dawley rats weighing 250–350 g initially were anesthetized with pentothal (50 mg/kg ip) and supplemented if necessary. Forty-eight rats comprised the CCI groups, and 28 rats served as sham-operated controls. CCI was produced as follows: the right sciatic nerve was exposed at mid-thigh level immediately proximal to the point of trifurcation; it was freed from the surrounding connective tissue for a 25-mm length, and four chromic gut (4–0) ligatures were tied loosely around the nerve trunk. Sham-operated control animals underwent similar procedures except that no ligatures were placed around the nerve.

Behavioral testing

Heat hyperalgesia was assessed in sham-operated control and CCI rats each day after the surgery. Prior to CCI surgery, the intensity of the radiant heat stimulus was adjusted to obtain a paw withdrawal latency of ~10 s. Paw withdrawal latencies were recorded at 5-min intervals. Data are reported as difference scores calculated as (latency of withdrawal of the paw ipsilateral to the CCI) – (latency of withdrawal of the paw contralateral to the CCI). Thus negative scores indicate hyperalgesia, whereas positive scores indicate hypoalgesia. Behavioral changes were usually observed 3 days after CCI surgery and were most pronounced 7–14 days after the nerve injury as reported by Herzberg et al. (1997). Each electrophysiology experiment was conducted immediately after behavioral testing but only if the mean difference score was in the range of –1.5 to –3.0 s. For studies of the effect of WIN 55,212-2 on neuropathic pain behavior, baseline paw withdrawal latencies were established before drug or vehicle was administered, and paw withdrawal latencies were obtained at 5-min intervals during the subsequent 25 min.

Physiological recording of single neuron responses

Each rat was anesthetized with urethan (1.5 g/kg ip, supplemented as needed) and mounted in a stereotaxic frame. A 26-gauge hypodermic needle was inserted into the lateral tail vein for subsequent drug treatments. Throughout the experiment, body temperature was held at 37°C. A laminectomy was performed at L₅–L₆, the spine was immobilized with clamps, and the spinal cord was bathed in warm mineral oil. Electrophysiological responses of single dorsal horn neurons with receptive fields (RFs) located on the hindpaw were recorded extracellularly using tungsten parylene-coated microelectrodes (Microprobe, Potomac, MD, 0.8–1.5 MΩ). A microelectrode was lowered into the spinal cord using a hydraulic microdrive. Action potentials were amplified, audio monitored and discriminated according to their amplitudes. The hindpaw was brushed with a camel hair artist's brush to activate neurons that exhibited low levels of spontaneous firing. Neurons were classified as wide dynamic range (WDR) if they responded in a graded fashion to increasing intensities of mechanical stimulation (Price 1999).

Noxious thermal stimulation

After isolation and characterization of a neuron, the receptive field was mapped by gentle touching of the ipsilateral hindpaw with an

insect pin before and after drug. A computer-controlled Peltier-device (7 × 7 mm) was used to rapidly heat and cool various spots of the mapped receptive fields to confirm its location and size. The thermode was positioned in contact with the hindpaw and maintained in its position throughout the experiment so that thermal-evoked responses would not be confounded with mechanical stimulation. Stimulation trials consisted of a 2-s prestimulus interval, 20 s of thermal stimulation consisting of a linear temperature ramp from 32 to 53°C with rapid, active cooling to 32°C, which was maintained during 10 s of data collection after stimulus offset. During each 32 s trial, the time of occurrence of each action potential (1-ms resolution) was recorded on a computer (Fig. 1). For each neuron, the spontaneous firing rate was assessed as the mean firing rate during the 2 s prior to stimulus onset. Evoked responses were quantified after subtracting the frequency of spontaneous discharge. Afterdischarge was quantified as the mean firing rate during the final 7 s of the trial, during which the paw temperature was maintained at 32°C. Consecutive stimulation trials were separated by 5 min to avoid peripheral and central sensitization.

Experimental procedure

Baseline responses were determined by applying the thermal stimulus five times at 5-min intervals. A single i.v. injection of either drug or vehicle was performed, and the stimulus was applied at 5-min intervals for 25 min. In experiments that examined the receptor subtype mediating the effect of WIN 55,212-2, the subtype selective antagonist SR141716A (1 mg/kg iv, CB₁), AM251 (1 mg/kg iv, CB₁), or SR144528 (1 mg/kg iv, CB₂) was administered 5 min before administration of WIN 55,212-2. In one set of experiments, subsequent to recordings of baseline responses, the injured section of the nerve was bathed in 1 ml of a 0.3-mg/ml solution of WIN 55,212-2.

Histology

At the end of each experiment, a current of 20 μA was injected through the electrode (tip negative) for 15 s to mark the location of the recording site. Animals were perfused transcardially with 0.9% NaCl,

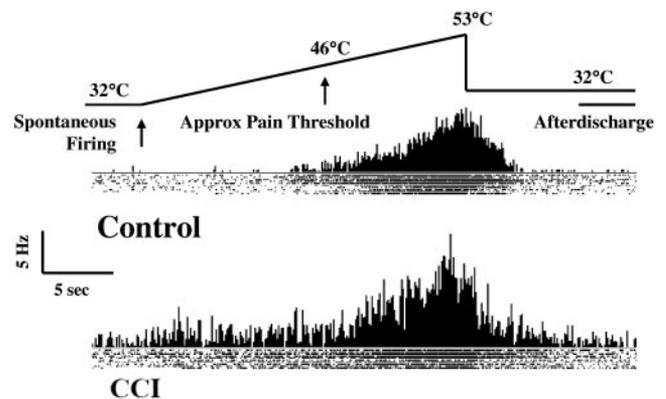


FIG. 1. Sample recording of a single spinal wide dynamic range (WDR) neuron before and after thermal stimulation of its receptive field. Stimulation trials consisted of a 2-s prestimulus interval, 20 s of thermal stimulation consisting of a linear temperature ramp (initiated at leftmost arrow) from 32 to 53°C with rapid active cooling to 32°C, which was maintained for 10 s (top solid line). Nociceptive temperatures were assessed as those ranging from 46°C (center arrow) to 53°C. *Bottom*: histograms and raster plots of firing in register with paw temperature show increased spontaneous and evoked firing in a chronic constriction injury (CCI) rat compared with a control rat. During each 32-s trial, the time of occurrence of each action potential (1-ms resolution) was recorded on a computer. Spontaneous firing rates were assessed as the mean firing rate during the 2 s prior to stimulus onset. Evoked responses were quantified after subtracting the frequency of spontaneous discharge. Afterdischarge was quantified as the mean firing rate during the final 7 s of the trial, during which paw temperature was held constant at 32°C.

followed by 10% formalin. Spinal cords were removed and stored overnight in a 30% sucrose-formalin solution. Frozen sections (40 μm) were mounted, stained with cresyl violet, and recording sites were localized microscopically.

Data analysis

The mean applied temperatures during successive time intervals in each trial were combined with the calculated firing rates to construct stimulus-response functions. Nonnoxious and noxious stimuli were defined as 32–45.9 and 46–53°C, respectively. The effects of drug treatment on stimulus-response functions of single neurons were determined by calculating the mean response for each subject. A C-language computer program was constructed to analyze scanned images of the paw and calculate the areas of RFs, which had been identified with an indelible marker. These data were transferred to a Hewlett-Packard (Palo Alto, CA) UNIX workstation for visualization and statistical analysis using custom C-language programs and BMDP Statistical Software (Los Angeles, CA). Multifactor repeated measures analyses of variance (ANOVA) were used to analyze treatment effects. The Greenhouse-Geiser (Greenhouse and Geiser 1959) correction was applied to interaction terms containing repeated factors to avoid inflated significance estimates produced by violations of the assumption of homogeneity of variance and covariance.

RESULTS

Effect of WIN 55,212–2 on behavioral responses of CCI rats to a thermal stimulus

Sham-operated control animals ($n = 23$) showed no significant change in paw withdrawal latency compared with pre-surgical baseline. By contrast, decreased paw withdrawal latencies were observed after stimulation of the paw ipsilateral to the ligated nerve in CCI animals ($P < 0.05$; $n = 24$, Fig. 2). Prior to electrophysiology experiments (postsurgical day 8), the thermal hyperalgesia score for CCI group was -2.7 ± 0.54 s.

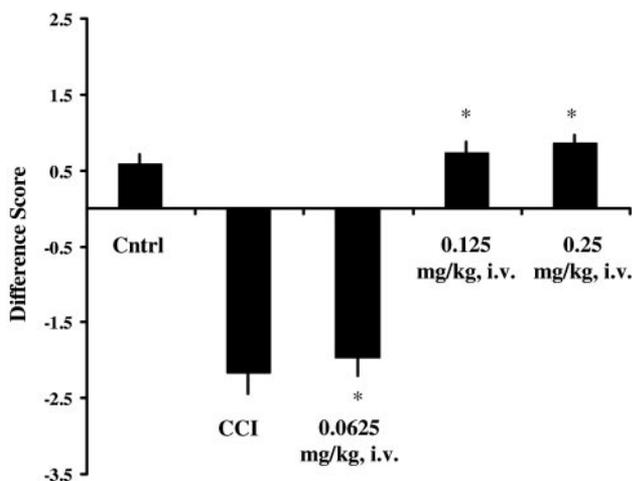


FIG. 2. Effects of WIN 55,212–2 on neuropathic hyperalgesia 7–8 days after surgery. WIN 55,212–2 dose-dependently reversed the hyperalgesia produced by peripheral nerve injury in rats. Difference scores were calculated as: (latency of withdrawal of the paw ipsilateral to the CCI) – (latency of withdrawal of the paw contralateral to the CCI). Thus negative scores indicate hyperalgesia, whereas positive scores indicate hypoalgesia. CCI, nerve injured animals; Cntrl, sham-operated animal; doses shown are for WIN 55,212–2 in CCI animals. Tests were performed at 5-min intervals for 25 min after administration of the drug or vehicle. Each bar represents means \pm SE ($n = 5$ per group); *, $P < 0.05$ compared with CCI group by ANOVA.

WIN 55,212–2 (0.0625, 0.125, and 0.25 mg/kg iv) was administered in nerve-injured rats the paw withdrawal latency difference scores of which were in the range of -1.5 to -3.0 s. The effects of WIN 55,212–2 were apparent 5 min after administration and lasted at least for 25 min. ANOVA revealed that WIN 55,212–2 dose-dependently reversed hyperalgesia in nerve-injured rats [$F(8,48) = 25.8$, $P < 0.01$, Fig. 2].

Electrophysiological characterization of spinal neurons in nerve-injured and sham-operated control rats

Fifty-six WDR neurons (33 CCI, 23 control) in L₅–L₆ of the spinal dorsal horn were recorded at a depth of 450–560 μm (control group: 545 ± 30 μm , CCI group: 512 ± 50). Histological reconstruction revealed that these cells were mainly located in Rexed's laminae V–VI; their receptive fields were located in the plantar surface of the paw and responded with increasing vigor to graded thermal stimulation of the RF. Hence all neurons recorded in this study fit the classification of WDR neurons. The mean frequencies of spontaneous and afterdischarge firing of dorsal horn neurons in CCI rats were significantly higher than those of control rats [$F(1,32) = 95.3$; $P < 0.05$, $F(1,32) = 107.7$, $P < 0.05$, respectively, Table 1]. The magnitude of both nonnoxious and noxious heat-evoked neuronal responses (the overall firing during the stimulation between 46°C–53°C) of CCI rats was significantly greater than that of control rats [$F(1,32) = 427.7$, $P < 0.005$, $F(1,32) = 159.4$, $P < 0.05$, respectively]. As expected from previous reports (Devor and Wall 1978), the areas of receptive fields of WDR neurons in CCI rats were significantly larger than those of control rats [$F(1,32) = 49.3$, $P < 0.005$; Table 1]. Spontaneous and evoked firing rates did not vary across baseline trails, suggesting that the stimulus parameters employed were appropriate for the determination of drug effects.

Lack of effect of vehicle on lumbar dorsal horn WDR neurons

The vehicle was administered intravenously for each experimental condition using a separate group of animals. The electrophysiological responses (spontaneous, heat-evoked firing, and afterdischarge) and receptive field areas of neurons after vehicle administration were compared with those before drug treatments by ANOVA. These analyses failed to reveal any effect of the vehicle. Because the vehicle failed to alter neuronal firing, analyses were conducted comparing pre- and postdrug treatment conditions using repeated-measures ANOVA, which provides greater statistical power than between group analyses.

Effect of WIN 55,212–2 on lumbar dorsal horn WDR neurons

SPONTANEOUS FIRING. ANOVA revealed that WIN 55,212–2 (0.0625, 0.125, and 0.25 mg/kg iv) dose-dependently inhibited spontaneous firing in CCI rats [$F(3,32) = 3.75$; $P < 0.05$, Fig. 3A]. At the dose of 0.25 mg/kg, WIN 55,212–2 decreased the rate of spontaneous firing in CCI rats to approximately that of the control group. The significant interaction between drug treatment and lesion condition [$F(3,32) = 3.31$; $P < 0.05$]

TABLE 1. Pre-treatment neurophysiological parameters in sham-operated control and nerve-injured CCI rats

	Control	CCI
Spontaneous firing rate, Hz	3.9 ± 0.12	18.5 ± 0.9*
Heat evoked response, Hz		
Non-noxious stimuli	4.8 ± 1.3	18.1 ± 2.4*
Noxious stimuli	15.1 ± 1.7	42.4 ± 3.0*
Afterdischarge activity, Hz	4.4 ± 0.3	20.8 ± 1.3*
Receptive field area, mm ²	115.0 ± 3.1	142.0 ± 3.1*

Values are means ± SE, CCI, chronic constriction injury. * $P < 0.05$.

revealed that the drug produced a larger effect in the CCI group compared with the control group.

Experiments with the selective CB₁ antagonists SR141716A and AM251 indicated that the suppression of spontaneous firing by WIN 55,212–2 was mediated by cannabinoid CB₁ receptors. The antagonists alone did not reliably alter spontaneous firing in either control (3.9 ± 1.5 and 4.1 ± 2.0 Hz pre- and postdrug, respectively) or CCI (18.8 ± 3.6 and 21.4 ± 2.5 Hz pre- and postdrug, respectively) group. Pretreatment with either SR141716A or AM251 blocked the suppression induced by WIN 55,212–2 in CCI groups [$F(1,4) = 47.39$; $P < 0.05$, Fig. 3B]. There was no significant difference between the two CB₁ antagonists ($P > 0.05$). By contrast, the CB₂ antagonist SR144528 did not alter the suppression of spontaneous firing by WIN 55,212–2 in nerve-injured rats ($P > 0.05$, Fig. 3B). These data indicate that CB₁ receptors mediated the suppressive effects of WIN 55,212–2 on spontaneous firing.

HEAT-EVOKED FIRING. The stimulus-response functions of neurons recorded in CCI rats were left-shifted compared with those of control rats, and the magnitude of the noxious heat evoked response of neurons recorded in CCI rats was significantly greater than that of control rats [$F(1,32) = 83.6$, $P < 0.005$; Fig. 4]. These observations are consistent with the observations of allodynia and hyperalgesia in nerve-injured rats.

WIN 55,212–2 decreased both nonnoxious and noxious heat-evoked activity of spinal neurons in CCI rats [$F(3,32) = 74.37$, $P < 0.05$; $F(3,32) = 45.90$, $P < 0.05$, respectively; Figs. 4–6]. At the dose of 0.125 mg/kg, WIN 55,212–2 normalized the responses of the neurons to noxious heat stimuli in CCI rats by right shifting the curve to overlap with that obtained from untreated control rats (Fig. 4B). In contrast, the effects of WIN 55,212–2 on stimulus-response functions of control rats occurred only at higher doses. At doses that suppressed neuronal responses of both CCI rats and control rats, the degree of suppression was greater in CCI rats compared with control rats. The heightened potency and efficacy of WIN 55,212–2 in CCI rats suggests that nerve injury leads to sensitization of the endocannabinoid system.

When administered alone, neither of the CB₁ antagonists (SR141716A, AM251) altered noxious thermal evoked responses in either control (15.9 ± 3.9 and 16.6 ± 6.0 Hz for pre- and postdrug, respectively) or CCI (43.76 ± 8.59 and 37.36 ± 7.49 Hz pre- and postdrug, respectively) rats. However, the suppression by WIN 55,212–2 of heat-evoked responses in CCI rats was blocked by pretreatment with either SR141716A or AM251 [$F(1,4) = 479.03$; $P < 0.005$], indicating a cannabinoid CB₁ receptor-mediated effect (Fig. 6B). There was no significant difference in efficacy between

the two CB₁ antagonists ($P > 0.05$). By contrast, the CB₂ antagonist SR144528 failed to alter the ability of WIN 55,212–2 to suppress the responses of neurons in CCI rats ($P > 0.05$, Fig. 6B).

AFTERDISCHARGE. ANOVA revealed that WIN 55,212–2 dose-dependently decreased afterdischarge in spinal neurons of CCI rats [$F(3,32) = 6.4$; $P < 0.05$, Fig. 7A]. Similar effects in control rats were found only at higher doses. WIN 55,212–2 produced a larger suppression of afterdischarge in CCI rats as compared with that of control rats [$F(3,32) = 5.4$; $P < 0.05$].

As with the other dependent measures, the suppressive effects of WIN 55,212–2 on afterdischarge were blocked by pretreatment with either SR141716A or AM251 in CCI rats [$F(1,4) = 49.4$; $P < 0.05$], indicating that the effect was mediated by CB₁ receptors (Fig. 7B). No significant difference was found between the two CB₁ antagonists. The antagonists alone did not alter the afterdischarge of neurons recorded from either control (4.7 ± 3.8 and 4.5 ± 2.8 Hz pre- and postdrug,

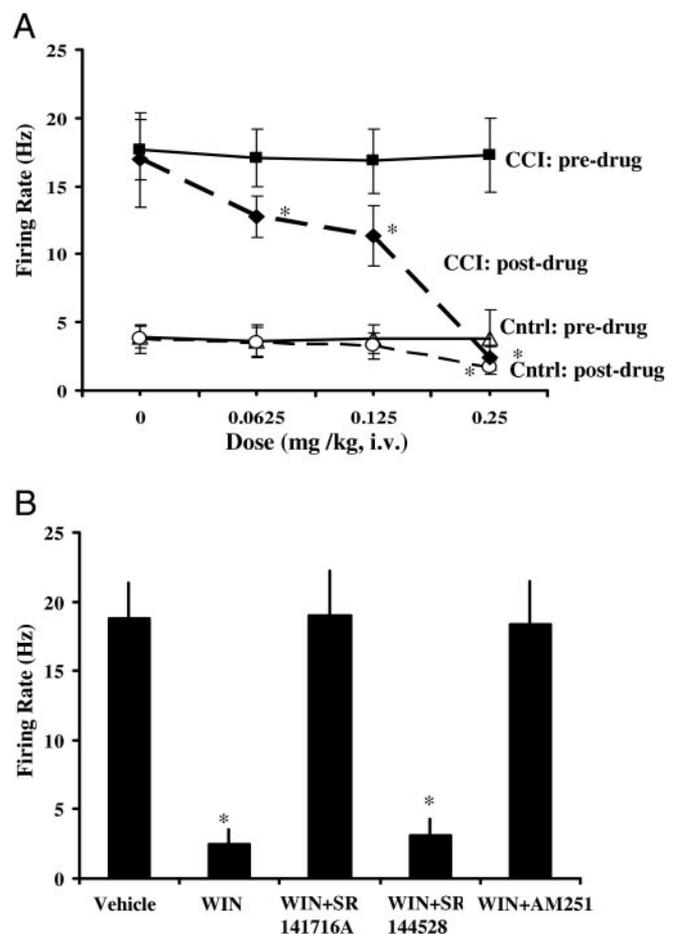


FIG. 3. A: effects of WIN 55,212–2 on spontaneous firing rates in CCI and control rats. Drug or vehicle was administered 5 min after a baseline was established. Each point reflects the mean spontaneous firing ($n = 5$ per group) for each condition; *, $P < 0.05$ compared with predrug condition by ANOVA. B: pretreatment with the selective CB₁ antagonists SR141716A (1 mg/kg) or AM251 (1 mg/kg) inhibited the suppression of spontaneous firing by WIN 55,212–2 (0.25 mg/kg) in CCI rats. By contrast, the CB₂ antagonist SR141428 (1 mg/kg) did not affect the ability of WIN 55,212–2 to suppress spontaneous firing in CCI rats. Antagonists or vehicle were administered intravenously 5 min prior to WIN 55,212–2 administration. SR141716A alone did not affect spontaneous firing (not shown, $P > 0.05$). Each point or bar represents means ± SE; $n = 5$ per group; *, $P < 0.05$.

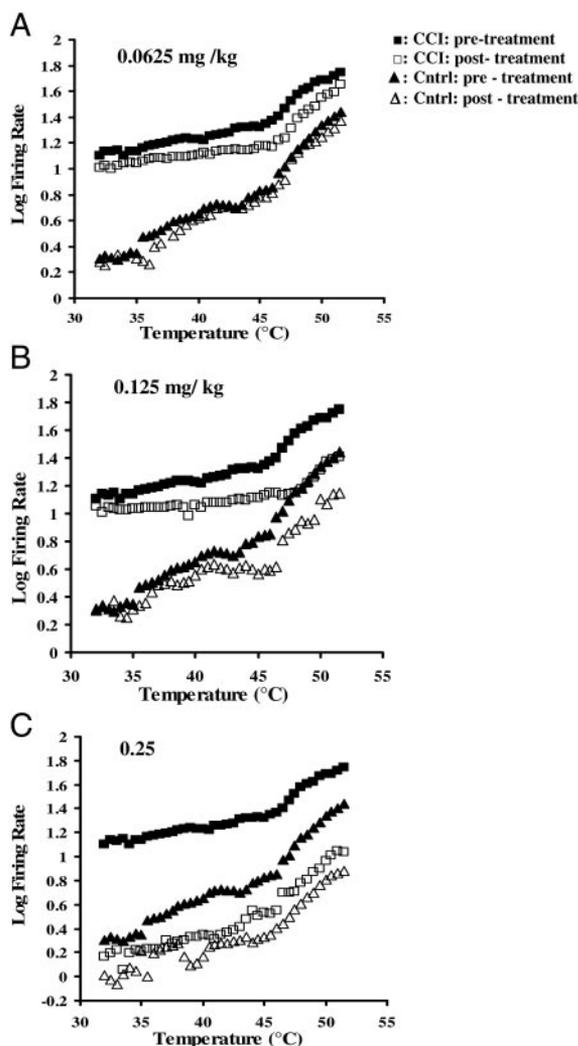


FIG. 4. Effect of various doses of WIN 55,212-2 on heat-evoked activity of spinal neurons in control and CCI rats. WIN 55,212-2 dose-dependently suppressed heat-evoked firing in control and CCI animals. The responses of neurons to the temperature ramp (32–53°C, 20 s; <46°C = nonnoxious stimuli; >46°C = noxious stimuli) were recorded before and after drug administration. *A*: at the dose of 0.0625 mg/kg iv, WIN 55,212-2 decreased heat-evoked firing of neurons in CCI rats without altering evoked firing in control rats. *B*: at the dose of 0.125 mg/kg iv, WIN 55,212-2 decreased heat-evoked activity of neurons in CCI rats with minimal effect on firing rates in control rats; it normalized responses of neurons to noxious heat in CCI rats shifting the stimulus-response curve to resemble that observed in untreated control rats; and it decreased the responses of neurons to noxious heat in control animals. *C*: at the dose of 0.25 mg/kg, WIN 55,212-2 suppressed both nonnoxious and noxious heat-evoked firing of neurons recorded in both CCI and control rats with a greater effect in the CCI rats. Each point represents the mean response of neurons recorded during successive 500-ms intervals ($n = 5$ –7 per group, 1 neuron per subject).

respectively) or CCI (22.8 ± 4.7 and 16.4 ± 5.9 Hz pre- and postdrug, respectively) rats. The CB₂ antagonist SR144528 failed to produce a significant change in the actions of WIN 55,212-2 ($P > 0.05$, Fig. 7B).

RECEPTIVE FIELD SIZE. The areas of the receptive fields of neurons recorded in CCI rats were significantly larger than those of control rats [$F(1,32) = 49.3$, $P < 0.005$]. WIN 55,212-2 dose-dependently decreased the sizes of receptive fields of WDR neurons in both control and CCI rats [$F(3,32) = 3.9$; $P < 0.05$, Fig. 8A]. The decrease in receptive field size

was clear at the dose of 0.125 mg/kg WIN 55,212-2, and ANOVA revealed a larger effect in CCI rats as compared with control rats [$F(3,32) = 4.7$; $P < 0.05$]. The suppressive effect of WIN 55,212-2 on receptive field size was blocked by pretreatment with either SR141716A or AM251 in both control and CCI groups [$F(1,4) = 279.6$; $P < 0.005$, $F(1,4) = 328.4$; $P < 0.005$], indicating that the effects were mediated by CB₁ receptors (Fig. 8B). There was no significant difference between the two CB₁ antagonists ($P > 0.05$). The antagonists alone did not alter receptive field size in either control (118.6 ± 13.8 and 125.5 ± 16.8 mm² pre- and postdrug, respectively) or CCI (142.8 ± 17.4 and 158.4 ± 15.5 Hz pre- and postdrug, respectively) rats. The CB₂ antagonist SR144528 failed to block the reduction in receptive field size by WIN 55,212-2 in CCI rats (Fig. 8B).

Time course of the effect of WIN 55,212-2 in control and CCI rats

WIN 55,212-2, at the dose of 0.125 mg/kg, significantly suppressed noxious heat-evoked activity of neurons in both control and CCI rats with greater effects in CCI rats [$F(3,32) = 5.89$, $P < 0.01$]. Throughout the 25-min interval of recording, the suppression of evoked firing produced by WIN 55,212-2 in CCI rats was maintained (Fig. 9). In contrast, control rats recovered from the suppression 20 min after drug injection (Fig. 9).

Site of action of WIN 55,212-2 in CCI rats

When directly administered around the injured sciatic nerve, WIN 55,212-2 (1 ml, 0.3 mg/ml = 574 μ M bathing solution) failed to alter noxious heat-evoked firing rates ($P > 0.05$, compared with 0.25 mg/kg iv, Fig. 10). In Chinese hamster ovary (CHO) cells overexpressing human CB₁ or CB₂ receptors, WIN 55,212-2 was reported to have Ki values of 1.9 and 0.3 nM, respectively. The lack of effect of the high dose used, which was employed to ensure full activation of CB₁ and CB₂ receptors in the vicinity of the injury site, strongly suggests a site of action other than the injury site.

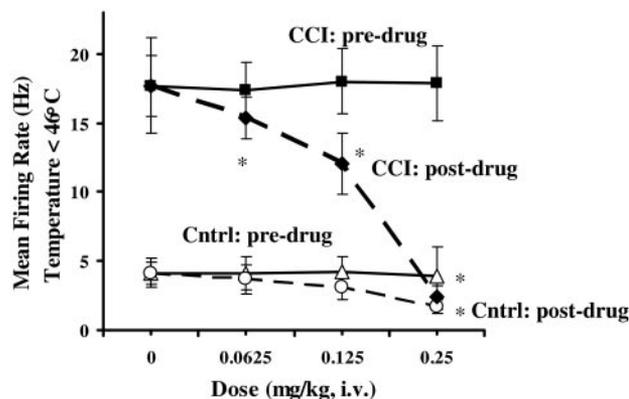


FIG. 5. Effects of WIN 55,212-2 on spinal neuronal responses to nonnoxious heat-evoked firing (<46°C) in control and CCI rats. WIN 55,212-2 dose-dependently decreased nonnoxious heat-evoked activity of neurons in CCI animals. At the highest dose tested (0.25 mg/kg), WIN 55,212-2 decreased the responses of neurons to nonnoxious heat stimuli in control rats. Each point reflects the mean responses of neurons to stimuli ($n = 5$ per group); *, $P < 0.05$ compared with predrug condition by ANOVA.

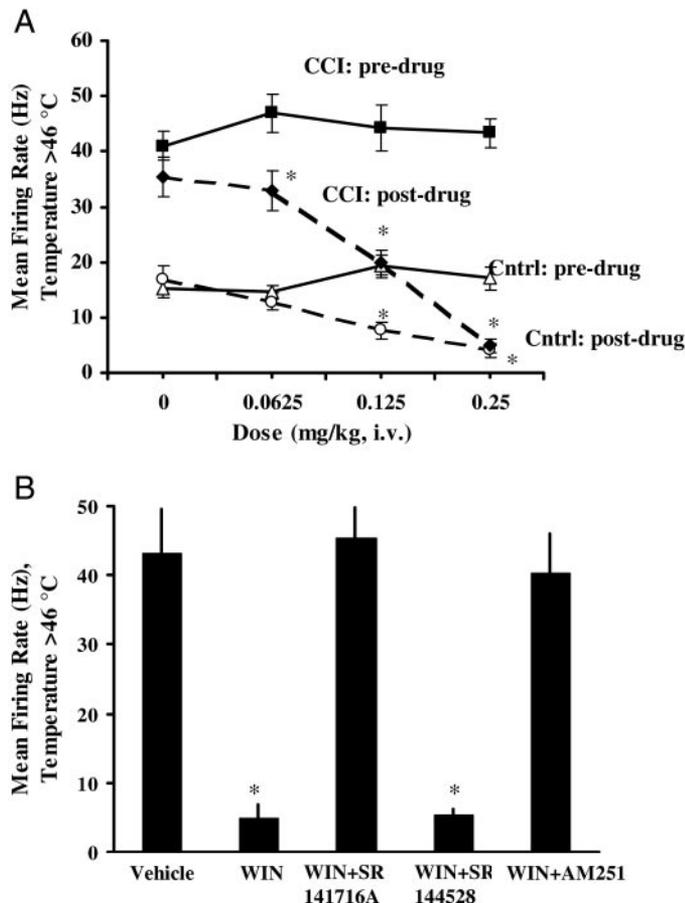


FIG. 6. Effects of WIN 55,212-2 on spinal neuronal responses to noxious heat-evoked stimuli ($>46^{\circ}\text{C}$) in control and CCI rats. Drug was administered 5 min after the baseline was established. Each point reflects the mean response of neurons ($n = 5$ per group); *, $P < 0.05$ compared with predrug by ANOVA. **A:** WIN 55,212-2 dose dependently suppressed the noxious heat-evoked activity of WDR neurons in both CCI and control rats with a greater effect in CCI rats. At the dose of 0.125 mg/kg iv, WIN 55,212-2 normalized the responses of neurons recorded from CCI rats. **B:** pretreatment with selective CB₁ antagonists, SR141716A (1 mg/kg) or AM251 (1 mg/kg), but not the CB₂ antagonist, SR144528 (1 mg/kg), blocked the ability of WIN 55,212-2 to inhibit noxious heat-evoked responses of the neurons in CCI animals, suggesting a CB₁ receptor-mediated effect.

Relationships among neurophysiological indices and behavioral responses

As noted in the preceding text, CCI rats withdrew their paws from a radiant thermal stimulus with a shorter latency than that of sham-operated control rats. Comparison of the neurophysiological and behavioral dose curves of WIN 55,212-2 in CCI rats may provide insight into the critical determinants of the abnormal sensitivity to thermal stimuli in nerve injury. A cursory examination shows that the behavioral response showed a major change toward normalcy at the dose of 0.125 mg/kg, specifically a 37% change from the next lower dose, 0.0625 mg/kg. By contrast, the changes in spontaneous firing and nonnoxious heat-evoked firing were only 11 and 5%, respectively. Correlations between the dose curves of the behavioral and the neurophysiological indices revealed a 0.95 correlation between paw withdrawal latency and receptive field size and a 0.89 correlation for behavior and neurophysiological response at noxious temperatures. Although the latter correlation is difficult to explain, because the animals never experi-

enced these temperatures in the behavioral testing paradigm, the strong correlation between receptive field size and behavior suggests that the exaggerated population response of spinal neurons consequent to neuropathic pain is a major factor in the exaggerated behavioral responses to both noxious and nonnoxious stimuli.

DISCUSSION

Confirming previous reports (Chapman 2001; Devor and Wall 1978; Palecek et al. 1992a,b; Pertovaara et al. 1997; Sotgiu et al. 1995), this study showed that WDR neurons in the spinal dorsal horn ipsilateral to nerve injury exhibited significant pathophysiological responses manifested as increased spontaneous activity, decreased thresholds for stimulus-evoked firing, expansion of RFs, increased responsiveness to noxious thermal stimuli, and prolonged afterdischarge as compared with control rats. Systemic administration of the synthetic cannabinoid WIN 55,212-2 induced dose-dependent, CB₁ re-

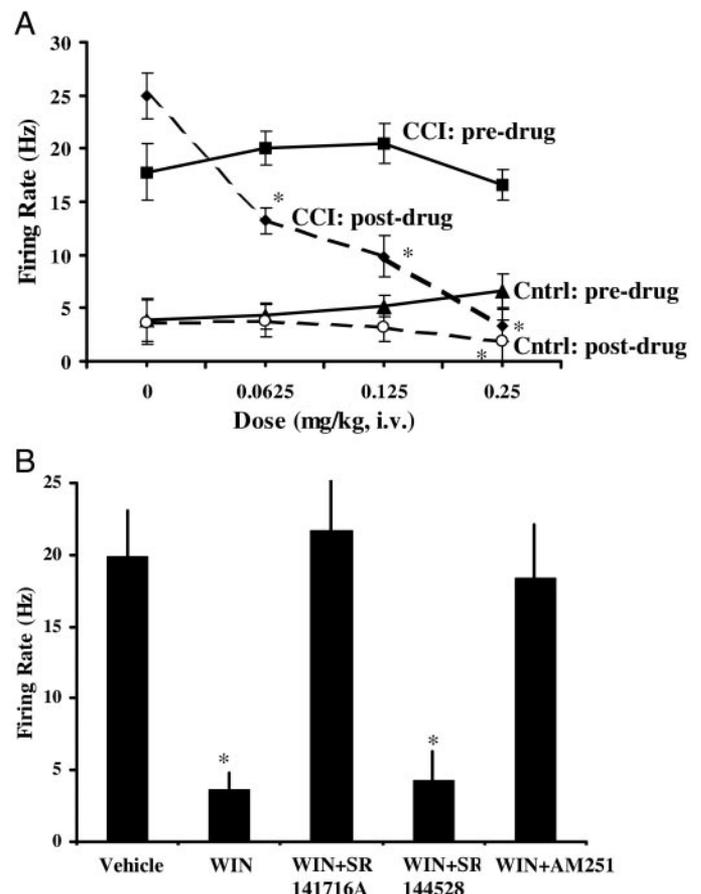


FIG. 7. Effects of WIN 55,212-2 on afterdischarge of spinal WDR neurons in CCI and control rats. **A:** WIN 55,212-2 dose-dependently suppressed afterdischarge recorded in CCI rats, but in control rats, this was observed only at the highest dose (0.25 mg/kg). WIN 55,212-2 was administered 5 min after the baseline was established. Each point reflects the mean afterdischarge rate ($n = 5$ per group); *, $P < 0.05$ compared with predrug condition for by ANOVA. **B:** pretreatment with the selective CB₁ antagonists SR141716A (1 mg/kg) or AM251 (1 mg/kg), but not the CB₂ antagonist SR144528 (1 mg/kg), blocked the ability of WIN 55,212-2 (0.25 mg/kg iv) to inhibit afterdischarge in CCI animals, indicating a CB₁ receptor-mediated effect. Antagonists or vehicle were administered intravenous 5 min prior to injection of WIN 55,212-2. Each bar represents means \pm SE ($n = 5$ per group); *, $P < 0.05$ compared with vehicle group by ANOVA.

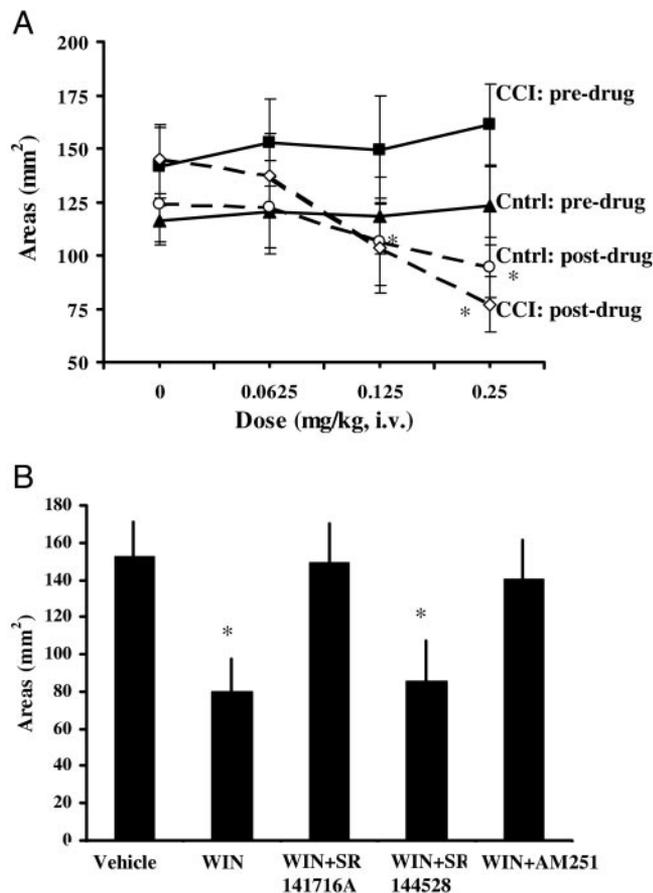


FIG. 8. Effects of WIN 55,212-2 on receptive field sizes of spinal neurons in CCI and control rats. In CCI rats, WIN 55,212-2 dose-dependently decreased the sizes of receptive fields, which were abnormally large. At the dose of 0.25 mg/kg, WIN 55,212-2 also decreased receptive field size in control rats. Drug or vehicle was administered 5 min after the baseline was established. Each point represents mean receptive field size (mm^2 , $n = 5$ per group); *, $P < 0.05$ compared with predrug conditions by ANOVA. *B*: pretreatment with the selective CB₁ antagonist SR141716A (1 mg/kg) or AM251 (1 mg/kg), but not the CB₂ antagonist SR144528 (1 mg/kg), blocked the ability of WIN 55,212-2 (0.25 mg/kg iv) to decrease receptive field sizes in CCI animals, indicating a CB₁ receptor-mediated effect. Antagonists or vehicle were administered intravenous 5 min prior to injection of WIN 55,212-2. Each bar represents means \pm SE ($n = 5$ per group); *, $P < 0.05$ compared with vehicle group by ANOVA.

ceptor-mediated inhibition of each of these abnormalities. These findings are consistent with the antihyperalgesic and antiallodynic effects of cannabinoids in behavioral models of neuropathic pain (Bridges et al. 2001; Costa et al. 2004; Fox et al. 2001; Herzberg et al. 1997; Wallace et al. 2003).

Our study suggested that the suppressive effects of WIN 55,212-2 on neuropathic pain were not due to actions at the site of injury because direct administration of the drug on the injured nerve failed to produce an effect. In a previous study, Fox et al. (2001) found that intraplantar administration of WIN 55,212-2 suppressed mechanical hyperalgesia in a rodent model of neuropathic pain (Fox et al. 2001), suggesting that a cannabinoid agonist may produce effects at the peripheral terminals. By contrast, using acute noxious stimuli, Hohmann et al. (1999) demonstrated that transection of the spinal cord drastically reduced the effects of systemically administered WIN 55,212-2, supporting the conclusion that cannabinoids suppress spinal responses to acute noxious stimuli by modula-

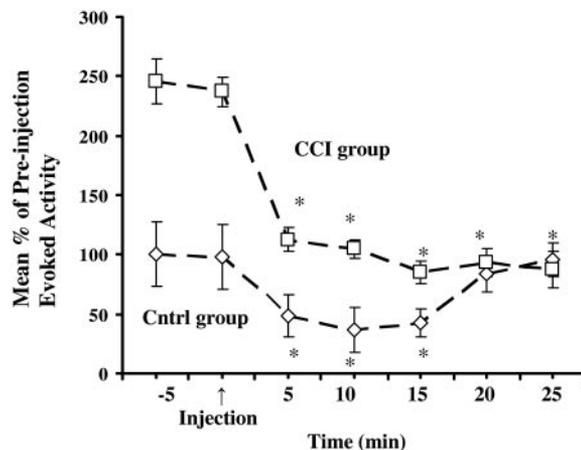


FIG. 9. Mean time course of the effects of WIN 55,212-2 on noxious heat-evoked activity of neurons in control and CCI rats. WIN 55,212-2 (0.125 mg/kg iv) was injected intravenous 5 min after baseline was established in control and CCI rats. Then heat-evoked activity was recorded at 5-min intervals for 25 min. In CCI rats, WIN 55,212-2 suppressed heat-evoked firing throughout the 25-min duration of the experiment. However, control rats recovered from the suppression \sim 20 min after injection. Each point represents the means \pm SE, $n = 5$ per group; *, $P < 0.05$ compared with predrug condition for each group by ANOVA.

tion of descending inhibitory pathways. Hence, although our data do not rule out peripheral or spinal sites of action, the observed effects may be the result of descending inhibition produced by actions of the drug in the brain.

Alternatively, cannabinoid agonists may decrease descending facilitation. The notion that midbrain and bulbar regions facilitate nociception in persistent pain states has been demonstrated repeatedly during the past decade (Guan et al. 2002; Porreca et al. 2002; Ren and Dubner 2002; Urban and Gebhart 1999; Vanegas and Schaible 2004; Wei et al. 1999). This raises the possibility that the cannabinoid effects observed in our experiments were due to suppression of descending facilitation. In light of the multiple sites of action reported for cannabinoid suppression of pain, it would be unsurprising for there to be synergistic interactions among them. Future research should be able to distinguish among these possibilities.

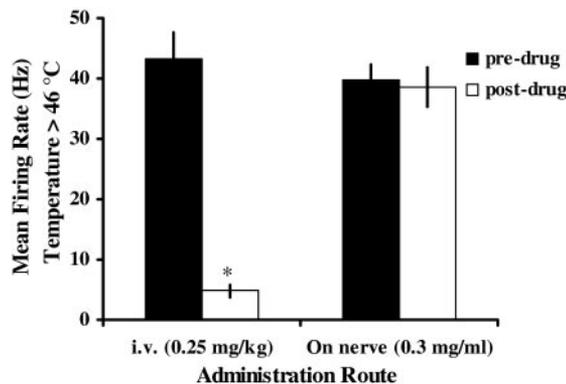


FIG. 10. Application of a bathing solution of WIN 55,212-2 (0.3 mg/ml) around the injured sciatic nerve did not suppress noxious heat-evoked responses of spinal neurons in CCI rats. One milliliter of the solution was applied to the injured nerve 5 min after baseline responses were established. The responses of neurons were monitored for 25 min after drug administration. Each bar represents the means \pm SE, $n = 5$ per group; *, $P < 0.05$ compared with predrug condition for each group by ANOVA.

Several recent studies reported the involvement of CB₂ receptors in the suppression of neuropathic pain by cannabinoids (Elmes et al. 2004; Ibrahim et al. 2003, 2005; Malan et al. 2003; Sagar et al. 2005). CB₂ receptor activation reduced tactile allodynia and thermal hyperalgesia in neuropathic rats (Ibrahim et al. 2003), and intraplantar administration (Elmes et al. 2004) or direct application onto spinal cord (Sagar et al. 2005) of the CB₂ receptor agonist JWH 133 reduced mechanically evoked responses of spinal WDR neurons in neuropathic rats. The authors of the latter study proposed that CB₂ receptor-mediated antinociception in neuropathic pain resulted from neural mechanisms at primary afferent terminals and/or spinal dorsal horn neurons. Several studies showed CB₂ expression in the dorsal root ganglion (Beltramo et al. 2006; Ross et al. 2001) and the superficial laminae of the spinal cord after peripheral nerve injuries probably associated with activated microglia (Wotherspoon et al. 2005; Zhang et al. 2003). In the current study, systemic administration of the CB₂ receptor antagonist SR144528 failed to reduce any of the effects of WIN 55,212-2, suggesting a CB₁ receptor-mediated effect. The lack of CB₂-mediated effects cannot be explained in terms of the selectivity of WIN 55,212-2 because it is a ~20-fold more potent at CB₂ compared with CB₁ (Showalter et al. 1996). Furthermore, Herzberg et al. (1997) reported that SR141716A reversed the effect of WIN 55,212-2 in a behavioral study of neuropathic pain, and the increased potency of WIN 55,212-2 in neuropathic pain may be explained by the upregulation of CB₁ receptors in contralateral thalamus and ipsilateral spinal dorsal horn after nerve injury (Lim 2003; Siegling et al. 2001).

The upregulation of CB₁ receptors by nerve injury may also explain why WIN 55,212-2 produced greater inhibition of spontaneous firing, noxious stimulus-evoked responses, after-discharge, and receptive field size in CCI rats compared with control rats. The enhanced sensitivity to cannabinoids in nerve-injured rats reported here is consistent with reports that anti-hyperalgesic and -allodynic effects of WIN 55,212-2 occurred at doses that did not affect pain-related behavior under non-pathological conditions (Bridges et al. 2001; Fox et al. 2001; Herzberg et al. 1997; Richardson et al. 1998b). This enhanced sensitivity may be selective for nociception because we did not observe behavior indicative of psychotropic effects (e.g., sedation) at the any of doses of WIN 55,212-2 used, an observation consistent with previous studies (Herzberg et al. 1997).

Examination of the dose-curves of WIN 55,212-2 in measures of pain behavior in comparison to those of the various electrophysiological parameters suggests the relative importance of particular neurophysiological anomalies in the maintenance of neuropathic pain. In considering this issue, it should be noted that skin temperature rises steadily as a logarithmic function of the duration of application of a fixed source of radiant heat (Hargreaves et al. 1988). Hence, treatments that reduce the paw withdrawal latency indicate the occurrence of a behavioral response at a temperature lower than normal. Hence, any neurophysiological effects of WIN 55,212-2 on temperatures above the normal thermal pain threshold are irrelevant because in the behavioral experiments, these temperatures were not reached due to paw withdrawal. In attempting to understand how the neurophysiological parameters correspond to pain perception after treatment with the cannabi-

noid, of particular note was the contrast between the doses of 0.0625 and 0.125 mg/kg WIN 55,212-2. The lower dose produced virtually no effect on pain behavior, whereas the higher dose resulted in virtually normal pain behavior. Only one of the relevant neurophysiological variables, receptive field size, showed a highly similar dose-response relationship. A marked decrease (normalization) in receptive size occurred between the two doses, and the size of the receptive field exhibited a 0.95 correlation with the behavioral response. By contrast, there was little difference between the effects of the two doses on stimulus-evoked firing rate at temperatures <46°C. Similarly, the effects of the cannabinoid on spontaneous firing at the doses 0.0625 and 0.125 mg/kg were small and similar in magnitude. The observation that the doses that reduced injury-induced exaggeration of receptive field size were the same as those that caused a normalization of pain behavior suggest that it is the larger population response of spinal neurons resulting from nerve injury that accounts for the decreased withdrawal latencies to a thermal stimulus in CCI rats. Conversely, these findings suggest that in subjects with painful neuropathy, a treatment that reduces the sizes of the receptive fields of spinal WDR neurons may markedly reduce the perception of thermal pain, even if the treatment fails to affect spontaneous firing rate or magnitude of evoked firing in response to nonnoxious temperatures.

The implications of the data discussed in the preceding text deserve a rigorous test in future experiments, especially because of factors that limit interpretation of the present data. First, the electrophysiological experiments were conducted with a contact thermode that produced a controlled temperature ramp, whereas the behavioral experiments were conducted with a fixed source of radiant heat. Previously reported differences in psychophysical stimulus-response functions for contact heat versus radiant heat (reviewed by Price 1988) and the presence of mild mechanical stimulation due to placement of the contact thermode limit the ability to generalize between the two experimental conditions. Second, the electrophysiological experiments were conducted in anesthetized animals, whereas the behavioral experiments were conducted in waking animals. The generality of results obtained from anesthetized subjects to those of awake subjects is uncertain. Finally, although this study focused on thermal stimulation, future studies would benefit from comparing thermal and mechanical stimuli, as the mechanisms for thermal hyperalgesia and mechanical allodynia likely differ. Nevertheless, a working hypothesis for future investigations may be gained from the present experiments, and cannabinoid agonists may be a useful tool for such investigations because of the demonstration of different dose requirements for altering the various physiological aberrations in neuropathic pain.

In this study, pretreatment with either SR141716A or AM251 did not change the responses of WDR neurons in either CCI or control rats. This contrasts with the previously reported observation that administration of SR141716A alone increased allodynia and hyperalgesia in rats with peripheral nerve injury (Herzberg et al. 1997; but see Bridges et al. 2001). The increased pain-related behavior after administration of these receptor antagonists suggested endocannabinoid tone in neuropathic pain. Alternatively, these observations may be the result of inverse agonism by the compounds. Although several reports suggested the presence of a physiological analgesic

tone exerted by the endocannabinoid system (Martin et al. 1999; Richardson et al. 1998b; Strangman et al. 1999), others investigators failed to observe enhancement of pain subsequent to administration of SR141716A (Beaulieu et al. 2000; Bridges et al. 2001; Rinaldi-Carmona et al. 1995), suggesting that endocannabinoid modulation of pain has multiple controls.

In contrast to the findings in this experiment, Chapman (2001) reported that systemically administered cannabinoids did not significantly reduce electrically evoked responses of spinal neurons in nerve-injured rats. The different results of the two experiments may be due to the use of natural stimuli in the present study, whereas electrical stimuli were used in the previous study.

Recent evidence suggested the existence of a novel non-CB₁, non-CB₂ cannabinoid receptor (Breivogel et al. 2001; Di Marzo et al. 2000). This putative G-protein-coupled receptor can be activated by anandamide and WIN 55,212-2 but not by other CB₁/CB₂ agonists, and the cannabinoid antagonist SR141716A does not distinguish between the canonical CB₁ and the putative new CB receptor (Hajos et al. 2001; Hoffman and Lupica 2000). However, the selective CB₁ antagonist AM251 (2 μM) blocked WIN 55,212-2-induced inhibition of evoked IPSCs, but not of EPSCs, in the hippocampus providing evidence for its ability to separate CB₁ from the new CB receptor (Hajos and Freund 2002). In our experiment, the effect of AM251 was not significantly different from that of SR141716A, further suggesting CB₁-mediated effects.

In summary, the electrophysiological studies described here demonstrate normalization of neurophysiological responses of spinal nociceptive neurons in animals with neuropathic pain. This, together with the behavioral data of Herzberg et al. (1997), provided evidence that cannabinoids exhibit increased potency in neuropathic pain compared with nociceptive pain. Further study is needed to determine the site(s) of action of cannabinoids and the means by which cannabinoids produce such profound effects on each of the pathological responses observed in neuropathic pain.

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