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November 22, 1997

Todd McCormack
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Dear Mr. McCormack:

Enclosed, please find our published papers on the effects of cannabinoid agonists on pain processing in the nervous system and on the reactions to painful stimuli by rats. The literature on humans is contradictory and confusing, partly because the work was done at a time when the methods for pain measurement in humans was quite crude. I would however refer you to papers by Professor Noyes (e.g. Noyes R Jr, et al. Analgesic effect of delta−9−tetrahydrocannabinol. J Clin Pharmacol. 1975 Feb 1; 15(2−3): 139−143). Most of the work done on healthy people with cannabinoids has not resulted in positive findings, but this may relate in part to changes that occur during chronic pain. In particular, it appears that the number of cannabinoid receptors in spinal cord increases following nerve injury, which would likely lead to greater analgesia with fewer side effects in such cases.

Thank you for your interest in our work, and good luck with your pain therapy!

Sincerely Yours,

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Endogenous cannabinoids as an aversive or counter-rewarding system in the rat

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Abstract

Human use of marijuana (*Cannabis sativa*) is widely assumed to have rewarding properties, a notion supported by its widespread recreational use. However, no study has clearly demonstrated such effects in animal models. The purpose of this study was to test for the presumed rewarding effect of cannabinoids using a conditioned place preference paradigm. The results showed that animals failed to develop place conditioning at a low dose (1.5 mg/kg) and developed a place aversion at a high dose (15 mg/kg) of the active principle in marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), a finding consistent with most previous studies. Moreover, the administration of the cannabinoid antagonist SR141716A induced a conditioned place preference at both a low (0.5 mg/kg) and a high (5 mg/kg) dose. In summary, cannabinoid antagonism produced place preference while cannabinoid agonism induced place aversion. These results suggest that endogenous cannabinoids serve normally to suppress reward or to induce aversion. © 1997 Elsevier Science Ireland Ltd.

Keywords: Marijuana; Cannabinoid; Δ^9-Tetrahydrocannabinol; SR141716A; Place preference; Reward; Aversion

Habit-forming drugs are thought to produce their effects by acting on brain reward pathways. This notion is supported by their ability to induce self-administration in experimental animals, enhance brain stimulation reward (or lower brain reward thresholds), and induce conditioned place preference. One of the oldest botanicals used by humans for recreational purposes is cannabis (marijuana) [9,11]. Its psychotropic actions derive primarily from Δ^9-tetrahydrocannabinol (Δ^9-THC) [18].

Several lines of evidence from animal studies support the conclusion that as drugs of abuse, cannabinoids (substances with a pharmacology similar to that of Δ^9-THC) are atypical. Cannabinoid agonists produce conditioned place aversion [17,22], taste aversion [17,22], and anxiogenic effects in the elevated plus maze in rodents [20]. They also fail to lower the threshold for electrical self-stimulation [26], and they fail to support self administration ([3,6,14] but see [5,15]). Thus, cannabinoid agonists do not appear to produce rewarding effects in animals. Also, dysphoria, fear and anxiety are sometimes observed in humans during therapeutic use of cannabinoids although at lesser doses a 'high' feeling is also reported [13,19,23].

Cannabinoids also fail to induce strong drug seeking behavior or a withdrawal syndrome in humans, as is observed with other drugs of abuse [11]. However, it was recently demonstrated by ourselves [27] and independently by a second group [1] that profound precipitated withdrawal occurs in animals treated chronically with Δ^9-THC when they receive the competitive cannabinoid receptor antagonist SR141716A [24]. During these experiments we observed that behavioral activation and docile behavior occurred when rats were treated with SR141716A, in contrast to the hyperreactivity, rigidity and aggressiveness of animals treated with Δ^9-THC. These observations raised the possibility that the cannabinoid antagonist produced rewarding effects, presumably by blocking either a dysphoric action or an inhibition of reward circuits produced by endogenous cannabinoids. To test this possibility, the effects of Δ^9-THC and SR141716A were examined in a conditioned place preference para-

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digm [2].

Male Sprague Dawley rats ($n = 60$; Charles River Labs.), 240–280 g served as subjects. The animals were individually housed in metal cages in a temperature-regulated (22–23°C) room. Food and water were freely available. Artificial lighting was provided from 0700 to 1900 h.

The cannabinoid agonist Δ^9-THC and the cannabinoid antagonist SR141716A were generously provided by the National Institute of Drug Abuse, Rockville, MD, USA. Both drugs were suspended in an ethanol/alkamuls-emulphor/saline solution (1:1:18 and 1:1:3, respectively for Δ^9-THC and SR141716A).

The conditioned place preference (CPP) apparatus consisted of two 30 × 30 × 40 cm. Plexiglas compartments separated by a central guillotine door. One of the compartments had 0.5 cm thick horizontal black and white lines on the four walls including the side of the guillotine door facing that compartment. The other compartment had 2.5 cm thick vertical black and white lines. The floor was smooth or rough black Plexiglas.

Prior to the experiment, the animals were habituated to handling. The experimental design consisted of three phases: pretesting, conditioning, and testing. In the pretesting phase, animals were placed in the middle of the chamber with the guillotine door raised approximately 12 cm and allowed 10 min to explore both chambers of the CPP apparatus. The conditioning phase consisted of two pairings of one of the distinctive compartments with either Δ^9-THC or SR141716A, alternated with two pairings of the other compartment with the vehicle. Animals in each drug group were randomly assigned to compartments and injection orders, both of which were counterbalanced. Each animal was placed in the appropriate compartment immediately after receiving an i.p. injection of the drug or vehicle. The guillotine door was closed to confine the animal to the compartment for 30 min. Test sessions were separated by 48 h to allow clearance of the drugs. In the testing phase, no drug or vehicle was injected and the guillotine door was raised 12 cm to allow access to either chamber. The animals were placed in the middle and allowed to move about freely for 20 min. The amount of time spent in each compartment was recorded.

A paired differences t-test was used to compare the time spent in each of the compartments for each drug treatment. An overall analysis of variance (ANOVA) was performed to compare differences between groups treated with SR141716A and Δ^9-THC followed by post hoc pairwise comparisons using the Newman–Keuls test. The difference between the time spent in the paired compartment and the time spent in the unpaired compartment was used in the later statistical analysis.

A paired differences t-test revealed no preference in the control-vehicle group for either compartment (time spent in the compartment with wide vertical black and white lines: 617 ± 127 (SEM) versus 583 ± 127 s spent in the compartment with thin horizontal black and white lines, ns).

Both doses of SR141716A induced a preference for the drug-paired compartment ($P \leq 0.005$). By contrast, the group treated with the high dose of Δ^9-THC preferred the drug-unpaired compartment ($P \leq 0.03$). No differences in the time spent in the two compartments were observed in the group treated with the low dose of Δ^9 THC (ns) (Fig. 1).

An overall two-way ANOVA (2 drug × 2 dose) revealed significant differences between the drug treatments ($F_{1,47} = 41.9$, $P \leq 0.00001$) and the doses of drug employed ($F_{1,47} = 6.0$, $P \leq 0.01$). Post hoc pairwise comparisons using the Newman–Keuls test showed that animals treated with either dose of the cannabinoid antagonist spent more time in the drug-paired compartment than those treated with either dose of the cannabinoid agonist ($P \leq 0.01$). The two doses of the cannabinoid antagonist did not produce significantly different effects. However, the group treated with the higher dose of the cannabinoid agonist spent less time in the drug-paired compartment than the group treated with the low dose ($P < 0.05$).

In this study, Δ^9 THC produced place aversion at a high dose, while a low dose had no effect in an unbiased conditioned place preference paradigm. In contrast, the cannabinoid antagonist SR141716A induced a place pre-

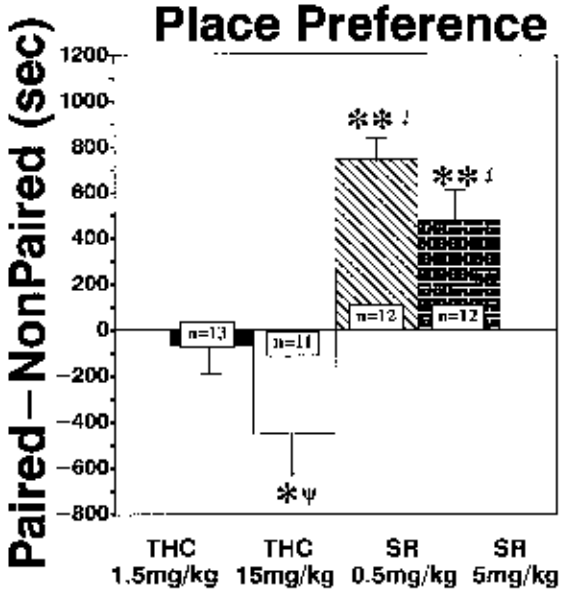

Fig. 1. Place conditioning as expressed by time spent in the drug paired compartment minus time spent in the unpaired compartment produced by a high or a low dose of the cannabinoid agonist Δ^9-THC or the cannabinoid antagonist SR141716A. The administration of the cannabinoid agonist induced placed aversion at the higher dose, while both doses of the cannabinoid antagonist induced place preference (* $P < 0.05$; significantly different from all other groups; ** $P < 0.01$; significantly different from the groups treated with Δ^9-THC; $^{\psi}P < 0.05$, significant preference for the unpaired compartment (or aversion); $^{\uparrow}P \leq 0.005$, significant preference for the drug-paired compartment.

ference at both doses tested. Presumably, lower doses of the antagonist were required because of its higher affinity for cannabinoid receptors in the brain (CB1 and CB1A) compared to the agonist Δ^9-THC [24,25]. The relatively high doses of Δ^9-THC that were necessary for this effect may indicate that the place preference test is not very sensitive.

This work confirms previous observations that cannabinoid agonists produce conditioned place aversion [17,22] and is consistent with other reports suggesting that cannabinoid agonists produce aversive motivational states. The findings include the production of taste aversion [17,22] and reduced exploration of an elevated plus maze [20]. The place aversion produced by Δ^9-THC are consistent with its failure to support self-administration [3,6,14] or lower the threshold for electrical self-stimulation [26].

The finding that a cannabinoid antagonist induces a place preference suggests that endogenous cannabinoids act as a counter-reward mechanism or produce aversive motivational states. These possibilities, which are not mutually exclusive, reflect the multiple substrates for motivated behavior. Work during the last 50 years revealed the existence of brain circuits that mediate reward [21] and many details on how drug reinforcers produce pleasure by activating these same circuits [29]. Other investigators [12] demonstrated the existence of neural substrates for fear and stress and the role of these circuits in the actions of fear- and stress-producing drugs such as benzodiazepine inverse agonists [4] and corticotropin releasing factor [7]. The role of cannabinoid receptors in either of these circuits is poorly understood, but their presence in both is clearly established. Cannabinoid receptors are present in localized zones of the nucleus accumbens and the lateral septum [8,16,28], two areas that support electrical self-stimulation and are targets of abused drugs. Cannabinoid receptors are also localized in the central and basolateral amygdaloid nuclei and in the periaqueductal gray, two areas that support electrically-induced fear responses and are presumed sites of action of certain anxiogenic drugs. The observation by Herkenham and Brady [10] that Δ^9-THC induces c-Fos expression in stress-responsive nuclei of the rat brain further supports an anxiogenic role of cannabinoids. The present data raise the speculation that endogenous cannabinoids may serve normally to inhibit reward or induce aversion by actions in within these circuits. Further work is needed to confirm or reject this hypothesis. For example, it would be of interest to determine whether the cannabinoid antagonist supports self-administration, lowers the threshold for intracranial self stimulation, or elevates extracellular dopamine levels in brain reward circuits.

The pleasurable effects of Δ^9-THC in humans might appear to be in disagreement with the present work. An obvious possibility is that there may be significant species differences in functions of the cannabinergic system in rats and humans. Perhaps the recreational use of cannabis by humans results from complex cognitive effects that cannot be reproduced in the simpler brains of other species. Alternatively, low doses of cannabinoid agonists may have an antagonist-like effect due to an autoreceptor action, as observed in other neurotransmitter systems. This would be consistent with the finding in clinical studies that low doses of Δ^9-THC produce a 'high' feeling, whereas higher doses produce aversive effects [13,19,23].

The finding that cannabinoid antagonism rather than agonism is rewarding may change our views of cannabinoids in relation to drug abuse. If confirmed, a counter-reward action of cannabinoid agonists would help explain controversies found in literature concerning actions of cannabinoids within reward systems. Furthermore, it could explain why cannabinoids do not induce the typical pattern of obsessive drug seeking and compulsive drug taking behavior that is associated in humans with other drugs of abuse.

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INHIBITION OF NOXIOUS STIMULUS-EVOKED ACTIVITY OF SPINAL CORD DORSAL HORN NEURONS BY THE CANNABINOID WIN 55,212–2

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Summary

The effects of a potent synthetic cannabinoid WIN 55,212–2 on nociceptive responses of wide dynamic range (WDR) neurons in the lumbar spinal cord were investigated in anesthetized rats. WDR neurons were identified by their responses to innocuous brushing and to a range of pressure stimuli from innocuous to noxious. Noxious pressure was applied to regions of the ipsilateral hind paw corresponding to the receptive field of the neuron. WIN 55,212–2 (125 μg/kg and 250 μg/kg, i.v.) produced a profound inhibition of firing evoked by the noxious pressure stimulus. By contrast, the cannabinoid did not alter the evoked activity of non–nociceptive neurons in response to non–noxious levels of stimulation. Treatment with either vehicle or the inactive enantiomer WIN 55,212–3 (250 μg/kg) failed to alter noxious stimulus–evoked activity of WDR neurons. These data provide direct evidence for cannabinoid–mediated inhibition of pain neurotransmission in the spinal dorsal horn. The site of action for these effects remains to be determined.

The cloning of a G–protein coupled cannabinoid receptor (1) and the isolation of the putative endogenous ligand anandamide (2,3) provide a strong argument for the existence of a cannabinoidergic neurotransmitter or neuromodulatory system in the central nervous system (see 4 for review). Although the functions of endogenous cannabinoids are largely unknown, several lines of evidence suggest a role of cannabinoid receptors in pain modulation. Cannabinoids produced powerful inhibition of pain responses in behavioral tests that examined noxious thermal (5–11), mechanical (12) and chemical stimuli (12,13). In fact, the potency and efficacy of cannabinoids in suppressing behavioral responses to painful stimuli is comparable to that of the opiates (6,7). The role of cannabinoid receptors in this effect is suggested by the high correlation between the potencies of cannabinoids in producing analgesia and the binding affinities of these compounds for the cannabinoid receptor (r=0.92, see 14) as well as by the failure of enantiomers of active compounds to produce antinociception (see 4, 9,10,15). A role of cannabinoid receptors in pain modulation receives further support from the presence of cannabinoid receptors in brain areas that are know to exert control of nociceptive tracts (16), such as the spinal dorsal horn (see 17 for review) and periaqueductal gray (18,19). Furthermore, administration of cannabinoids intrathecally (8,10,20) or locally in the periaqueductal gray (21,22) produces antinociception. This body of evidence raises the possibility that endogenous cannabinoids may function in part as an endogenous nonopiate system that modulates pain sensitivity.

In spite of the above, the lack of data demonstrating effects of cannabinoids on neural systems that process noxious stimuli remains a significant gap in the literature. The demonstration of such an effect is important because cannabinoids produce certain behavioral effects such as catalepsy and hypothermia (see 14) that can complicate the interpretation of behavioral measures of analgesia. Consequently, the present experiments were carried out to determine whether intravenous administration of the cannabinoid WIN 55,212–2 alters the responses of nociceptive neurons in the lumbar dorsal horn. These studies examined the effects of the synthetic cannabinoid WIN 55,212–2 and the inactive enantiomer WIN 55,212–3 on the responses of wide dynamic range (WDR) neurons (23) to a noxious pressure stimulus. A considerable body of literature has demonstrated that these second order neurons transmit information about the intensity and location of noxious stimuli to rostral centers of nociceptive proces-

sing (see 24 for review, 25,26). Therefore, an effect of a cannabinoid on these neurons would demonstrate an interruption of pain neurotransmission at an early stage of nociceptive processing.

Methods

Subjects

All the experiments were carried out using male (250–350 g) Sprague–Dawley rats (Charles River, Boston MA). The experimental protocols were reviewed and approved by the Brown University Institutional Animal Care and Use Committee.

Drug Preparation

WIN 55,212–2-mesylate was obtained from Research Biochemicals Inc. (Natick, MA). WIN 55,212–3-mesylate was a gift from Sterling–Winthrop (Rensselaer, NY). Drugs were dissolved in emulphor:ethanol:saline (1:1:18). Drug or vehicle was administered in a volume of 1 ml/kg bodyweight.

Administration of Noxious Pressure Stimuli

Pressure stimuli were administered using a computer controlled miniature air–cylinder (see Fig. 1A). Computer equipment provided a pulse trigger that opened an electronically controlled solenoid gas valve for a 3 sec interval and allowed nitrogen to flow into the air cylinder. A voltage proportional to the pressure in the cylinder was produced by a solid state pressure transducer (SenSym, Sunnyvale, CA; model LX 1820GBN), monitored on computer by an analog to digital converter, and used to quantify the pressure stimulus over time. The rise and fall times of the pressure pulse were limited by adjustable check valves to obtain the pressure waveforms illustrated in Fig. 1B and 3.

Behavioral Experiments: Determination of Noxious Pressure Threshold

In order to determine the threshold for pain, 12 rats were anesthetized with a dose of urethane (1 g/kg) that reduced motor tone but did not affect the animal's reflexes. At this dose, the animals permitted insertion of the hind paw into the pressure device but were capable of making a vigorous response at noxious levels of stimulation. Pressure stimuli were presented five times at 3 min intervals. The stimulus increased gradually over 3 seconds to a peak of 4.6 kg/cm^2 (waveform shown in Fig. 1B). The pressure at which a withdrawal reaction occurred was recorded by a computer, and the stimulus was immediately removed. The mean pressure at which a nociceptive response occurred was calculated for each rat.

Electrophysiological Methods

Glass or stainless steel microelectrodes were used in these experiments. Single barrel glass electrodes were prepared from 2.0 mm omega dotstock capillary tubing (Glass Co. of America, Millville, NJ) using a Narashige PE2 puller. The electrode tip was broken back under a microscope to 1 μm. Stainless steel microelectrodes were purchased from Frederick Haer & Co. (Brunswick, ME). Amplified action potentials were passed through low and high pass filters into a window comparator and a computer. Electrical signals were monitored on an audio amplifier and displayed on an oscilloscope.

Recordings of the extracellular activity of 21 single neurons in the lumbar spinal cord were obtained in separate rats. Following the induction of deep surgical anesthesia with urethane (1.5 g/kg, i.p.), rats were mounted in a stereotaxic head holder. A laminectomy was performed at the level T12 to L1 to expose the spinal cord, which was then bathed in 37°C saline or mineral oil. The spinal cord was immobilized for extracellular recordings by clamping the spinous processes rostral and caudal to the recording site. Throughout the experiment, body temperature was maintained by means of a heating pad controlled by a device that monitored the output of a thermistor probe.

WDR neurons were identified by their increasing responses to various stimuli ranging from very mild to noxious (23). Because these neurons typically exhibit little or no spontaneous firing, we used a search stimulus, which consisted of light stroking of the hind paw with a camel hair brush. After a responsive neuron was isolated, its responses to a graded series of mechanical pressures ranging from innocuous to noxious (0.5 kg/cm^2 to 3.75 kg/cm^2) were characterized (see Results). Pressure was applied to the center of the excitatory receptive field of the hind paw at 1 min intervals by the air

cylinder which produced a 3 sec pressure stimulus (Fig. 3). Neurons that responded with increasing firing rates to stimuli ranging from mild to noxious were classified as WDR neurons and tests with drug or vehicle commenced.

A single nociceptive pressure (3.75 kg/cm^2, Fig. 3) was used to examine the effects of WIN 55,212–2 on WDR neurons based upon the results of the behavioral experiments. After establishing stable evoked responses to the nociceptive stimulus, the stimulus was presented 5 times at 2 min intervals. A single intravenous injection of WIN 55,212–2 (125 μg/kg, n=5, or 250 μg/kg, n=4), WIN 55,212–3 (250 μg/kg, n=4) or vehicle (n=5) was then administered. Subsequently, the response of the neuron to the noxious stimulus was reassessed at 2 min intervals. Stable recordings were generally maintained until recovery was observed. However, in 3 cases the recording was lost before full recovery was attained.

The electrophysiological effects of WIN 55,212–2 (250 μg/kg) on the responses evoked by a mild pressure stimulus (1 kg/cm^2) were examined in three non–nociceptive mechanosensitive neurons. These neurons were classified as non–nociceptive based on their lack of a differential response to non–noxious and noxious stimulation. The pressure employed in these experiments was defined as non–noxious, because stimulation at this intensity failed to elicit limb withdrawal in lightly anesthetized rats (described above) and was not perceived as painful by the investigators. Stimuli were presented at the same intervals used to assess the effects of drug or vehicle on noxious stimulus–evoked activity in WDR neurons.

Data were quantified by collecting the time of occurrence of each action potential and generating peristimulus time histograms and raster plots using software developed in our laboratory. Each stimulus trial consisted of a 1 sec pre–stimulus interval, a 3 sec interval corresponding to the period of pressure application (noxious stimulus–evoked) and a 6 sec interval immediately following offset of the stimulus. At the end of each experiment, recording sites were marked by ejecting fast green dye by iontophoresis from glass microelectrodes. A 50–150 μm green spot identified the approximate location of the recording site. Recording sites obtained using stainless steel microelectrodes were marked via iron deposition (2 μA for 20–30 sec) and perfusion with Prussian Blue marking solution. Frozen sections (40 μm) were obtained using a cryostat, mounted onto glass slides and counterstained with Neutral red. Appropriate sections were stained for iron via the Perls method prior to counterstaining with Neutral red. Recording sites were verified under a light microscope to be in the lumbar dorsal horn with the exception of one non–nociceptive neuron, which was localized in the surrounding white matter.

Statistical Methods

Data were analyzed by analysis of variance (ANOVA) and t–tests using BMDP statistical software (Los Angeles, CA). Post hoc comparisons were performed using the Tukey test. Statistical results were considered significant if $p < 0.05$.

Results

Behavioral Experiments: Determination of Noxious Pressure Threshold

Limb withdrawal reflexes in lightly anesthetized rats were elicited at 3.2 ± 0.2 kg/cm^2. Baseline responses to noxious stimulation were elicited at a pressure above the limb withdrawal threshold (3.75 kg/cm^2) in the electrophysiological experiments. This intensity of noxious pressure did not result in overt tissue damage in the subjects tested but was perceived as painful by the investigators.

Electrophysiological Experiments

WDR neurons are characterized by increasingly strong responses to increasingly intense stimuli ranging from non–noxious to noxious levels (23). The neurons we recorded in the lumbar dorsal horn clearly fit this description since evoked activity was significantly greater at noxious (3–3.75kg/cm^2) compared to non–noxious (0.5–1 kg/cm^2) levels (46.98 ± 4.6 vs. 22.78 ± 4.46 Hz: mean firing rate $\pm$ SEM; $t_{15} = 8.55$, $p < 0.0001$).

The populations of neurons sampled in the active enantiomer (125 μg/kg and 250 μg/kg doses), inactive enantiomer and vehicle groups were very similar. There were no significant differences be-

tween vehicle and drug groups for either pre–injection stimulus–evoked responses or pre–injection spontaneous firing rate. The failure to observe a shift in the baseline firing across repeated trials demonstrates that firing had returned to normal prior to commencement of a new stimulation, and thus the interval between stimulation was appropriate for the determination of drug effects.

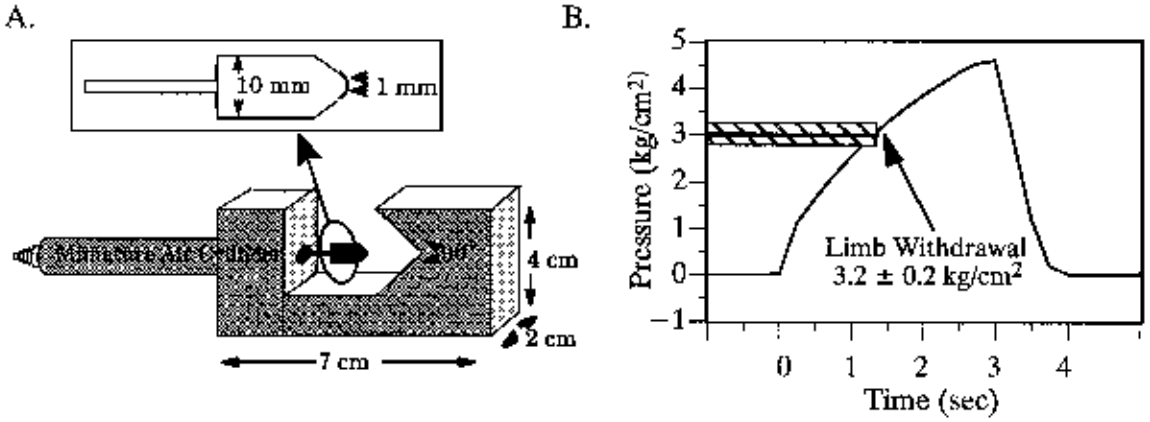

Fig. 1

A. Diagram of the pressure device used in the behavioral and electrophysiological experiments. B. Pressure waveform representing the mean pressure eliciting limb withdrawal in lightly anesthetized rats (n=12). The hatched area represents the mean ± SEM.

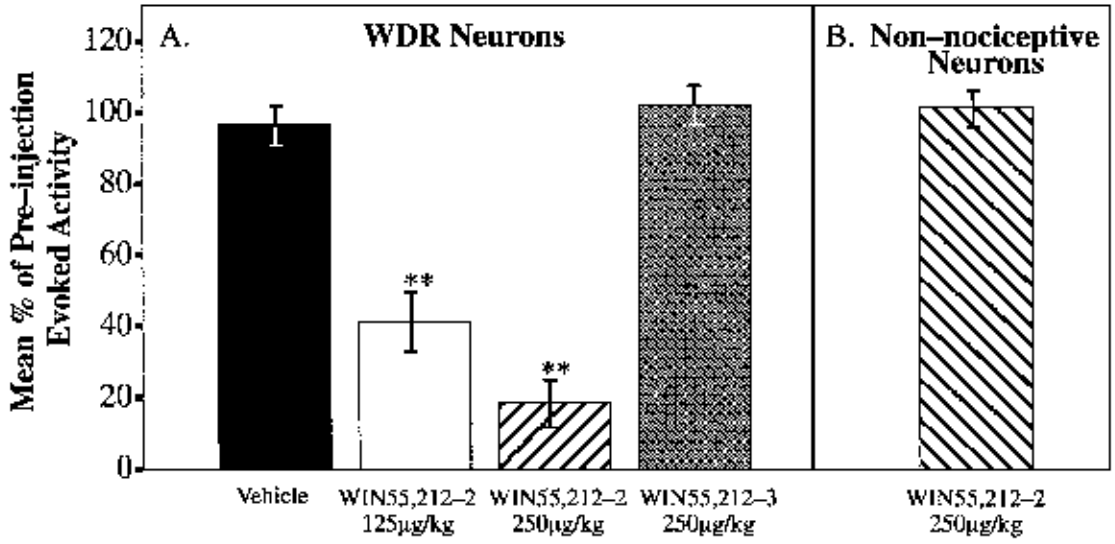

Fig. 2

A. Effects of WIN 55,212–2 and WIN 55,212–3 on noxious stimulus–evoked activity of WDR neurons in lumbar dorsal horn. B. The effects of WIN 55,212–2 on non–noxious stimulus–evoked activity of non–nociceptive neurons in the lumbar spinal cord. (Mean ± SEM for 5 consecutive post–injection trials). Significant difference from Vehicle and WIN 55,212–3, **$p < 0.01$.

The most important finding of these experiments was that administration of WIN 55,212–2 produced a potent and enantioselective decrease in noxious stimulus–evoked firing compared to vehicle treatment (ANOVA on 5 post–injection trials: $F_{3,14}= 40.58$, $p < 0.0001$, see Fig. 2A). The low dose of WIN 55,212–2 (125 µg/kg) produced a 58.8% inhibition of noxious stimulus–evoked activity whereas the high dose (250 µg/kg) produced an 82.4% inhibition of noxious stimulus–evoked activity relative to pre–injection levels of evoked activity (see Fig. 2A). By contrast, administration of either vehicle or inactive enantiomer did not significantly alter evoked activity. Post hoc comparisons performed on the percentage of pre–injection levels of evoked activity revealed a significant suppression of noxious stimulus–evoked activity at each dose of WIN 55,212–2 relative to vehicle treatment ($p < 0.01$). Treatment with the inactive enantiomer WIN 55,212–3 (250 µg/kg) did not differ significantly from treatment with vehicle but differed significantly from treatment with either dose of WIN 55,212–2 ($p < 0.01$, see Fig. 2A). The effects of the low and high dose of WIN 55,212–2 on nox-

ious stimulus–evoked activity did not differ significantly from each other. A representative experiment (selected on the basis of proximity to the mean for that group) is shown in Fig. 3 to illustrate the effects of i.v. administration of 250 μg/kg WIN 55,212–2 on noxious stimulus–evoked activity.

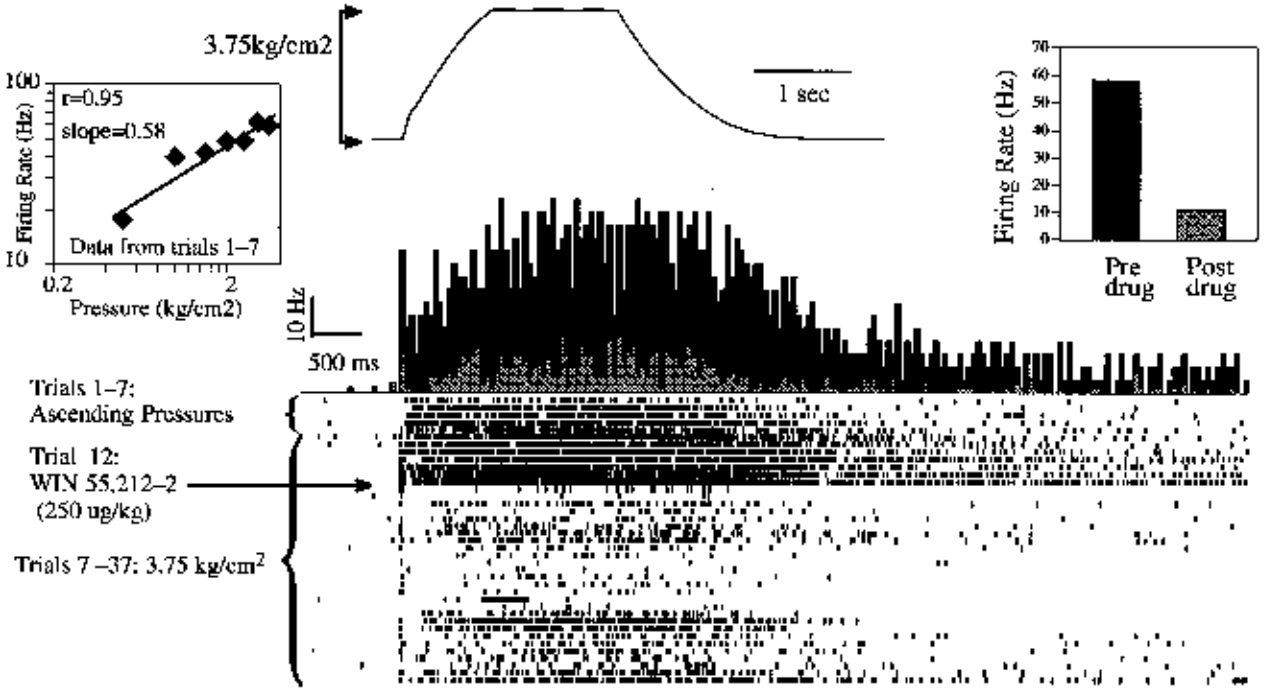

Fig. 3

Example of inhibition of evoked activity in a WDR neuron by the cannabinoid WIN 55,212–2. The responses of the neuron to the pressure device shown in Fig. 1A were examined during 37 trials corresponding to each row of dots in the raster plot (top row=trial 1); each dot represents the time of occurrence of a single action potential relative to the stimulus onset. Trials 1 to 7 consisted of applications of increasingly strong mechanical stimulation ranging from non–noxious to noxious levels (0.5, 1, 1.5, 2, 2.5, 3, 3.75 kg/cm^2). The concomitant increases in density of dots under the stimulus in the first 7 rows are indicative of the increasingly strong response of the neuron. LEFT (INSET): The mean firing rates of the neuron during a graded series of stimulations are plotted (log–log coordinates) against the applied pressure. The neuron's systematic change in responsiveness was the basis for classifying this cell as a WDR neuron. CENTER: The noxious stimulus illustrated by the pressure waveform (top center) was administered every 2 min for trials 7 to 37. Trials 8–12 constituted baseline trials; after trial 12 (arrow), WIN 55,212–2 (250 μg/kg, i.v.) was administered. A marked decrease in the responsiveness of the neuron is indicated by the sharply decreased density of dots in subsequent rows of the raster plot. RIGHT (INSET): Comparison of the mean firing rate during the stimulus for the 5 baseline trials to the firing rate during the stimulus for the first 10 post–injection trials illustrating, approximately, an 82% decrease in responsiveness. The black peristimulus time histogram between the raster plot and the pressure waveform represents the baseline firing rate prior to injection, whereas the grey peristimulus time histogram represents the firing rate for the first 10 postinjection trials.

Treatment with WIN 55,212–2 (250 μg/kg) did not significantly alter the firing rate evoked by non–noxious stimulation in three non–nociceptive neurons (see Fig 2B).

The mean time course of the effects of WIN 55,212–2, WIN 55,212–3 and vehicle throughout the recording period is shown in Fig. 4. These data indicate that the cannabinoid–induced suppression of noxious stimulus–evoked activity was long–lasting, enantioselective and reversible, although in 3 cases the cell was lost before recovery occurred in animals treated with 125 μg/kg of WIN 55,212–2. Cells recorded in animals receiving 250 μg/kg doses of WIN 55,212–2 recovered to greater than 60% of baseline responding by 45 min. Mean noxious stimulus–evoked activity changed less than 5% throughout a 45 minute interval following administration of either vehicle or inactive enantiomer.

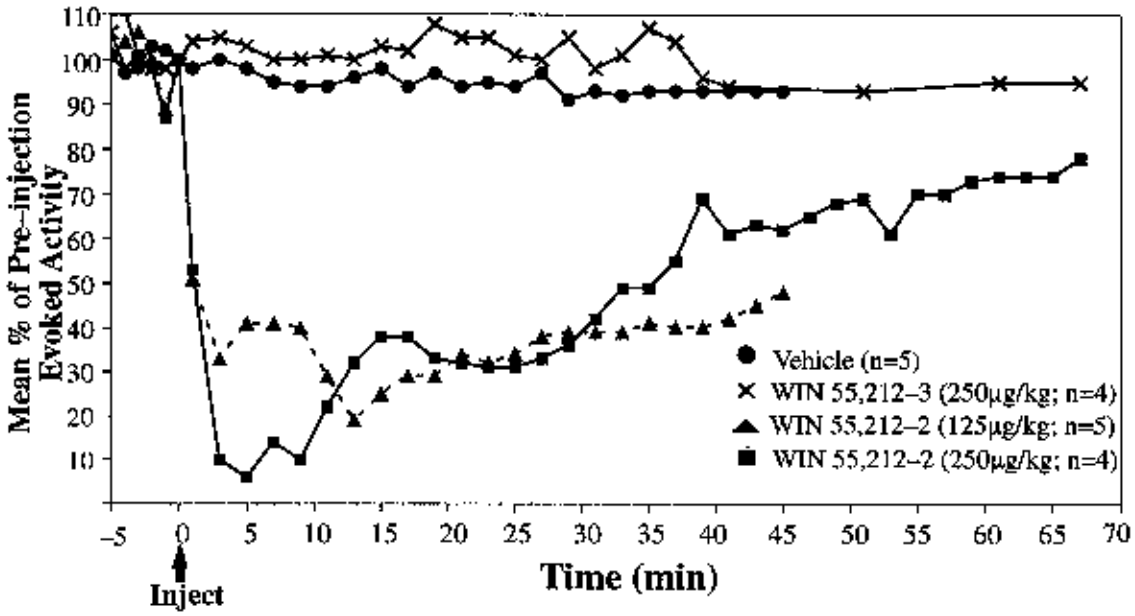

Fig. 4

Time course of the effects of WIN 55,212–2, WIN 55,212–3 and vehicle on noxious stimulus–evoked activity of WDR neurons in lumbar dorsal horn.

Discussion

The synthetic cannabinoid WIN 55,212–2 produced a potent, reversible and enantioselective inhibition of noxious stimulus–evoked activity in lumbar dorsal horn neurons. By contrast, the activity of non–nociceptive neurons in the lumbar spinal cord evoked by non–noxious pressure was unchanged by a dose of WIN 55,212–2 (250µg/kg, i.v.) that produced the maximal suppression of noxious stimulus–evoked activity in WDR neurons. These data provide direct evidence that cannabinoids modulate the activity of nociceptive neurons. These findings are consistent with the hypothesis that endogenous cannabinoids act as nonopiate modulators of pain neurotransmission by a direct or indirect action on spinal nociceptive neurons.

The firing rate of the WDR neurons recorded in the present study increased when stimulus strength was increased from very mild through noxious levels. This finding, together with the excitatory responses of these cells to a light brush stimulus, and the afterdischarges seen at noxious levels of stimulation suggest that the neurons we recorded in the lumbar dorsal horn were indeed WDR neurons, rather than mechanosensitive cells or some other type of non–nociceptive neuron. By contrast, the cannabinoid agonist failed to alter the stimulus–evoked activity in the non–nociceptive neurons we recorded. These data support the hypothesis that cannabinoids selectively suppress pain neurotransmission.

The suppression of noxious stimulus–evoked activity induced by WIN 55,212–2 in this experiment is likely to be mediated by actions of this compound at cannabinoid receptors. The inactive enantiomer WIN 55,212–3 failed to alter noxious stimulus–evoked activity in WDR neurons. Moreover, the high potency of the effects, the rapid onset, and the timely recovery of normal responding are consistent with receptor–mediated effects. Likewise, the data from the present study are consistent with the receptor–mediated suppression by WIN 55,212–2 of Fos protein (27) in the lumbar dorsal horn that is induced by noxious stimulation (28). The fact that the high dose of WIN 55,212–2 did not produce a significantly greater suppression of evoked activity than the low dose suggest that cannabinoid receptors may be saturated at 125 µg/kg (i.v.) of the cannabinoid.

The observed electrophysiological effects are consistent with receptor–mediated antinociceptive effects of cannabinoids that are observed in behavioral studies (see 4, 9, 10, see 14). A suppression of the limb withdrawal reflex to the mechanical stimulus used in the present experiments is also observed in lightly anesthetized rats after administration of WIN 55,212–2 (250µg/kg, i.v.), but not after administration of vehicle (29). These data, together with previous work demonstrating a cannabinoid–

induced suppression of pain reactivity to noxious mechanical stimuli (12), suggest that the observed inhibition of noxious stimulus–evoked activity of lumbar dorsal horn neurons by WIN 55,212–2 may have behavioral relevance. However, because general anesthetics may mask certain effects of cannabinoids in electrophysiological studies (30,31), some caution must be exercised in generalizing these results to the awake behaving animal.

Studies using antidromic activation have shown that WDR neurons project to the ventroposterolateral nucleus of the thalamus via the lateral spinothalamic tract (32,33). Perhaps the strongest evidence for the role of this pathway in pain neurotransmission is the well–localized sharp pain reported by humans upon electrical stimulation of this structure (34). The inhibition of spinal WDR neurons is consistent with our recent finding that WIN 55,212–2 produces marked inhibition of noxious stimulus–evoked activity of neurons in the ventroposterolateral nucleus of the thalamus (29). Taken together, these findings provide convincing evidence that cannabinoids modulate the activity of nociceptive neurons.

It is now well established that the nervous system actively modulates sensitivity to noxious stimuli via multiple neural substrates that serve to modify the efficiency of ascending pain tracts (see 35 for review). This modification of pain neurotransmission is mediated in part by the release of endogenous opioids (36, see 37 for review). However, nonopiate substrates for endogenous analgesia have been documented as well (38–40). Our findings raise the possibility that endogenous cannabinoids may be involved in nonopiate mechanisms of pain modulation (38,39). The circumstances under which endogenous cannabinoids exert such effects remain topics for future investigation.

Acknowledgements

The authors thank Drs. Richard C. Rogers, Gregory Holmes and Michael Beattie at The Ohio State University Medical School for advice on stabilizing the spinal cord for recording. We are grateful to Jonathon Crystal for his advice about the statistical analyses. W.J.M. is supported by the National Institute on Drug Abuse (DA05617–01). J.M.W. is grateful for the support of a Research Scientist Development Award from the National Institute of Mental Health (KO2–MH01083).

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SUPPRESSION OF NOXIOUS STIMULUS-EVOKED EXPRESSION OF FOS PROTEIN-LIKE IMMUNOREACTIVITY IN RAT SPINAL CORD BY A SELECTIVE CANNABINOID AGONIST

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Abstract In rats, cannabinoids inhibit behavioral responses to noxious stimulation with a potency and efficacy similar to that of morphine. However, because cannabinoids depress motor function, it has not been possible to state beyond any doubt that these effects were related to a dampening of noxious sensory input. Therefore, c-*fos* immunocytochemistry was used to explore the possibility that cannabinoids reduce behavioral responses to noxious stimuli by decreasing spinal processing of nociceptive inputs. Rats received systemic injections of the potent and selective cannabinoid agonist WIN 55,212-2, the receptor-inactive enantiomer WIN 55,212-3 or vehicle prior to observations in a model of tonic pain, the formalin test. As demonstrated previously, plantar injections of formalin led to lifting and licking of the injected paw, with two peaks of activity occurring at 5 and 30 min after injection. The cannabinoid agonist suppressed these pain responses and produced a reduction in mobility. Immunocytochemical processing of sections with an antibody to the Fos protein revealed that the cannabinoid markedly suppressed pain-evoked c-*fos* expression in the superficial and neck regions of the spinal dorsal horn, but not in the nucleus proprius. Decreased expression of c-*fos* also occurred in the ventral horn. The specificity of this effect and its probable mediation by cannabinoid receptors are suggested by three findings: (i) the suppression by the drug of both behavioral and immunocytochemical responses to pain was dose-dependent; (ii) neither the behavioral nor the immunocytochemical response to the noxious stimulus was significantly affected by the receptor-inactive enantiomer of the agonist; (iii) animals rendered tolerant to cannabinoids by repeated injections of the agonist showed reduced responses to the drug.

These findings suggest that cannabinoids inhibit the spinal processing of nociceptive stimuli and support the notion that endogenous cannabinoids may act naturally to modify pain transmission within the central nervous system.

Key words: cannabis, c-*fos*, pain.

The cloning of the cannabinoid receptor[34] and the discovery of anandamide, a putative endogenous cannabinoid receptor ligand,[6] opened a new avenue of cannabinoid research aimed at understanding the physiological and behavioral roles of endogenous cannabinergic neural systems.[5] The possibility that one role of endogenous cannabinoids is to modulate pain sensitivity was suggested by the many behavioral studies demonstrating analgesic effects of cannabinoids in rodents (e.g. Refs 1, 3, 7, 12, 19, 22, 23, 25 and 26). In these studies, animals showed profound reductions in motor responses to noxious stimuli of various modalities. These findings raise the question of whether the suppression of pain behavior observed after cannabinoid administration results from depression of the responsiveness of nociceptive neurons or from a direct action on the motor system.[22] However, little is known about the effects of cannabinoids on the responsiveness of nociceptive neurons. Recent work in our laboratory indicates that the selective cannabinoid agonist WIN 55,212-2 produces a rapid, reversible and enantioselective suppression of noxious stimulus-evoked activity in wide dynamic range neurons in rat lumbar dorsal horn.[13,14] These electrophysiological data suggest that cannabinoids modulate pain neurotransmission, but more research is needed to characterize the actions of cannabinoids on pain transmission in the spinal cord and brain.

Another approach to investigating the effects of cannabinoids on pain pathways is to study the effects of cannabinoids on c-*fos* expression evoked by noxious stimulation. Sensory stimuli lead to increased expression of the proto-oncogene c-*fos* in the CNS, and the anatomical distribution of immunoreactive neurons is dependent upon the modality of the stimulus.[2,14] Although c-*fos* is not a specific marker

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Abbreviations: WIN 55,212-2, (*R*)-4,5-dihydro-2-methyl-4-(4-morpholinylmethyl)-1-(1-naphthalenylcarbonyl)-6H-pyrrolo[3,2,1-ij]quinolin-6-one mesylate; WIN 55,212-3, inactive enantiomer of WIN 55,212-2.

for pain, noxious stimuli lead to expression of c-*fos* in both the superficial and deep layers of the dorsal horn,[2,9,14,31,35] areas known to be important for pain processing.[36] Furthermore, the prototypic narcotic analgesic morphine strongly suppresses noxious stimulus-evoked Fos-like immunoreactivity in the spinal cord.[31,32,35] Fos immunocytochemistry has also been employed in the study of antinociceptive actions of α_2-adrenoceptor agents,[27] *N*-methyl-D-aspartate receptor antagonists,[36] glucocorticoids[21] and acupuncture.[15] Fos immunocytochemistry has thus become an important tool for pain research, because suppression of c-*fos* expression can serve as an index of analgesia independent of motor responses.[31]

In this report, we describe the effects of the potent, enantioselective and conformationally restrained aminoalkylindole cannabinoid receptor agonist WIN 55,212-2[41] on c-*fos* expression evoked in the spinal cord by an injection of formalin in the paw. The formalin test,[4] a standard model of tonic pain, was used in the present study, because prolonged or repetitive noxious stimulation is necessary to reliably evoke expression of c-*fos* in the spinal cord.[2,35] In order to determine whether the changes in c-*fos* expression were mediated by actions at cannabinoid receptors, separate studies examined (i) different doses of the drug, (ii) the receptor-inactive enantiomer WIN 55,212-3 and (iii) the possibility that tolerance to cannabinoids would diminish the behavioral and neurochemical effects of the drug.

EXPERIMENTAL PROCEDURES

Drugs and chemicals

WIN 55,212-2 mesylate was purchased from Research Biochemicals Incorporated (Natick, MA). The inactive enantiomer WIN 55,212-3 mesylate was a gift from Dr Dean Haycock of Sterling-Winthrop Research Group (Rensselaer, NY). The drugs were dispersed in a vehicle containing emulphor, ethanol and saline (1:1:18) at a concentration of 10 mg/ml. The polyclonal rabbit Fos antibody (Ab-2) was purchased from Oncogene Science (Uniondale, NY). This antibody was raised in rabbit against a synthetic peptide fragment corresponding to residues 4–17 of human Fos protein.

Analgesia testing

The methods for analgesia testing were reviewed and approved by the Brown University Institutional Animal Care and Use Committee. Male Sprague Dawley rats (250–320 g) were obtained from Charles River Laboratories (Wilmington, MA) or our own breeding colony derived from Charles River stock. Because anesthesia leads to a restricted spinal distribution of Fos-like immunoreactive neurons in response to noxious stimuli,[31] and to correlate behavioral and neurochemical responses, the experiments were conducted in awake, freely-moving rats. Animals received a subcutaneous injection of 150 μl of dilute formalin (4% paraformaldehyde) in the plantar surface of the left hindpaw 10 min following i.p. injection of WIN 55,212-2 (5 mg/kg, $n = 5$, or 10 mg/kg, $n = 11$), the inactive enantiomer WIN 55,212-3 (10 mg/kg, $n = 5$) or vehicle ($n = 17$). A pain behavior rating was performed at 5 min intervals for 1 h. A score of 0 was recorded if the rat did not favor the injected paw during the interval, a score of 1 was recorded if the rat lifted his paw and a score of 2 was recorded if the rat licked his paw. Mean behavior scores were obtained for each 5 min interval. Two hours following administration of formalin, the experiment was terminated and the immunocytochemical procedures were performed.

Induction of tolerance

Rats ($n = 5$) received four i.p. injections of 15 mg/kg WIN 55,212-2, which were administered on the evening of Day 1, the morning and evening of Day 2, and the morning of Day 3. Twenty-four hours after the last injection, the rats were injected with 10 mg/kg of WIN 55,212-2 i.p., and behavioral and immunocytochemical procedures were conducted as described above. A separate group of animals ($n = 5$) received injections of vehicle at the same times during the tolerance-induction phase, except that the rats were injected with 10 mg/kg i.p. of WIN 55,212-2 on the test day. Comparisons were also made with naive animals ($n = 5$) receiving a single injection of vehicle on the test day.

Immunocytochemistry

Rats were deeply anesthetized by injections of pentobarbital (60 mg/kg, i.p.) and perfused with 100 ml of ice-cold heparinized (1000 U/ml) saline followed by 250–300 ml fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer) at approximately 3 ml/min. The lumbar–sacral segment of the spinal cord was removed, cleared of the covering sheath and postfixed in the fixative for 1–2 h. The spinal cord was then cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline overnight. The tissue was embedded in OCT compound at −20°C. Transverse sections (40 μm) of the spinal cord were cut using a cryostat. Alternate slices were collected in 0.1 M phosphate-buffered saline (floating), washed three times with the same buffer and pretreated with 3% normal rabbit serum for 1 h to block the non-specific binding. The sections were then incubated with rabbit polyclonal Fos protein antibody at a concentration of 1:100 for 40 h at 4°C. Fos-like immunoreactivity was visualized by the avidin biotin–peroxidase method,[12] using diaminobenzidine (0.05%) and H_2O_2 (0.01%) as the chromogens. The sections were mounted, air-dried and coverslipped. The specificity of the Fos immunostaining was checked by preabsorption of the antibody with a 10-fold excess of the peptide antigen.

Data analysis

Photomicrographs were taken of immunocytochemically stained spinal cord sections at ×100 magnification. The number of Fos-positive cells was counted on prints (approximately 11 × 16 cm) by an investigator who was blind to the experimental condition. Three sections from lumbar 4/5 determined by qualitative examination to contain the greatest number of labeled cells were selected from each rat. The number of labeled cells was counted and the average number of labeled cells was recorded. All cells demonstrating Fos-like immunoreactivity were counted as labeled regardless of label intensity. For each rat, the total number of cells was recorded as well as the subtotal in specific subdivisions of the spinal gray matter ipsilateral to the injected paw. Boundaries between laminae were marked on the photomicrographs by drawing lines in ink.[31] The subdivisions used were those defined by Presley *et al.*:[31] (i) the superficial laminae (laminae I and II), (ii) the nucleus proprius (laminae III and IV), (iii) the neck of the dorsal horn (laminae V and VI), and (iv) the ventral horn (laminae VII, VIII, IX and X).

Statistical analysis

Statistical comparison was carried out using analysis of variance. Significant treatment effects were isolated using individual *t*-tests. Because of the high degree of consistency in the behavioral response, all control animals were pooled into a single group and compared to the other treatment conditions by analysis of variance. For the

immunocytochemical experiments, each drug-treated control animal was paired with a vehicle-treated control. Control groups were constructed separately for each set of experiments from the matched pairs of animals, which were tested and processed for Fos immunocytochemistry at the same time. For all experiments, $P < 0.05$ was considered statistically significant.

RESULTS

*General features of c-*fos *expression*

The general features of c-*fos* expression in the spinal cord after intraplantar administration of dilute formalin were similar to those reported previously.[2,9,14,31,35] Neurons containing Fos-like immunoreactivity were easily identified by their darkly stained nuclei. Positive cells appeared in the ipsilateral dorsal horn and ventral horn, predominantly at lumbar 4/5 level (Figs 1A, 2). Approximately 45% of labeled neurons were localized to the superficial laminae (I and II). Lower levels were observed in the neck of the dorsal horn (laminae V and VI) and the adjacent ventral gray, constituting approximately 30 and 10% of all labeled cells, respectively. The rostrocaudal distribution of immunoreactive neurons in these two regions was more extensive than in the superficial laminae. Approximately 15% of immunoreactive neurons were localized to the nucleus proprius (laminae III and IV). Basal expression was extremely low in untreated rats. Likewise, c-*fos* expression was extremely low in the contralateral dorsal horn of treated rats.

Effects of acute administration of WIN 55,212-2

Administration of WIN 55,212-2 produced a significant suppression of pain-evoked c-*fos* expression in the lumbar spinal cord (Fig. 1B). This was shown by the significant decrease in the number of immunoreactive neurons in the superficial laminae ($F_{2,31} = 9.74$, $P < 0.001$) and the neck region ($F_{2,31} = 14.2$, $P < 0.0001$) compared to vehicle-treated controls (Fig. 2). The change was most prominent in the neck region, i.e. a decrease of 48% compared to the 28% decrease that was observed in the superficial dorsal horn. The nucleus proprius was unaffected ($F_{2,31} = 1.44$, NS), but a significant reduction of pain-evoked c-*fos* expression in the ventral horn was observed ($F_{2,31} = 7.41$, $P = 0.002$).

The effect of WIN 55,212-2 on pain-evoked c-*fos* expression was dose-dependent. Compared to the 5 mg/kg dose of the drug, the 10 mg/kg dose produced significantly greater suppression of the Fos response to noxious stimulation in the neck region (d.f. = 15, $t = 4.6$, $P < 0.05$) and in the ventral horn (d.f. = 15, $t = 6.72$, $P < 0.03$). The dose functions were not identical in the various regions studied. As shown in Fig. 2, the effect of the cannabinoid in the superficial laminae was maximal at 5 mg/kg, but this same dose produced an intermediate effect in the deep dorsal horn and was ineffective in the ventral horn.

Rats treated with the cannabinoid agonist showed reduced motor activity and a loss of licking and lifting of the injected paw during the 1 h following formalin injection (Fig. 3). Analysis of variance revealed marked differences between the drug-treated and control groups ($F = 121.23$, $P < 0.0001$). Animals treated with 5 mg/kg of the drug generally showed recovery of pain behavior (lifting of the paw) within 30 min after formalin injection (Fig. 3). By contrast, animals treated with 10 mg/kg WIN 55,212-2 showed significantly greater suppression of the response to formalin than rats treated with 5 mg/kg (d.f. = 14, $t = 11.6$, $P < 0.004$). Animals treated with 10 mg/kg of the agonist failed to respond to the noxious stimulus throughout the observation period and exhibited signs of catalepsy.

Lack of effect of the enantiomer WIN 55,212-3

Administration of WIN 55,212-3, the inactive enantiomer of WIN 55,212-2, failed to produce cannabinoid-like effects in either immunocytochemical or behavioral measures. After formalin injection, the rats showed levels of c-*fos* expression and pain-related behavior that were similar to the control rats. No significant differences were found between enantiomer and control in either the behavioural response (Fig. 4) or the levels of c-*fos* expression in any of the spinal areas examined (Fig. 1C, Table 1).

Cannabinoid tolerance

All animals that received repeated injections of WIN 55,212-2 showed a reduced analgesic effect compared to that observed after acute administration of WIN 55,212-2, i.e. animals exhibited lifting of the injected paw. However, complete tolerance, defined here as licking and lifting of the injected paw in a manner and extent that was indistinguishable from the control rats, was only observed in one animal. These data suggest that the tolerance procedures used in the present study effectively reduced the efficacy of the drug. In contrast to rats treated with vehicle, all five of the rats that were repeatedly injected with drug failed to exhibit catalepsy and immobility after i.p. injection of 10 mg/kg of WIN 55,212-2. These behavioral effects were confirmed by statistical analysis, which revealed that animals receiving repeated injections of the cannabinoid still exhibited a decreased response to the painful stimulus compared to control animals (d.f. = 25, $t = 27.5$, $P < 0.001$; Fig. 5). However, the analgesic response was significantly lower than that which occurred in animals that had not received repeated injections of the drug (d.f. = 14, $t = 42.4$, $P < 0.0001$). Thus, continuous exposure to WIN 55,212-2 resulted in a tolerance manifested by a loss of efficacy in behavioral measures, as demonstrated previously.[28,29]

Partial tolerance to the effect of cannabinoids on pain-evoked expression of Fos-like immunoreactivity also occurred following repeated administration of WIN 55,212-2 (Fig. 6) Although the tolerance

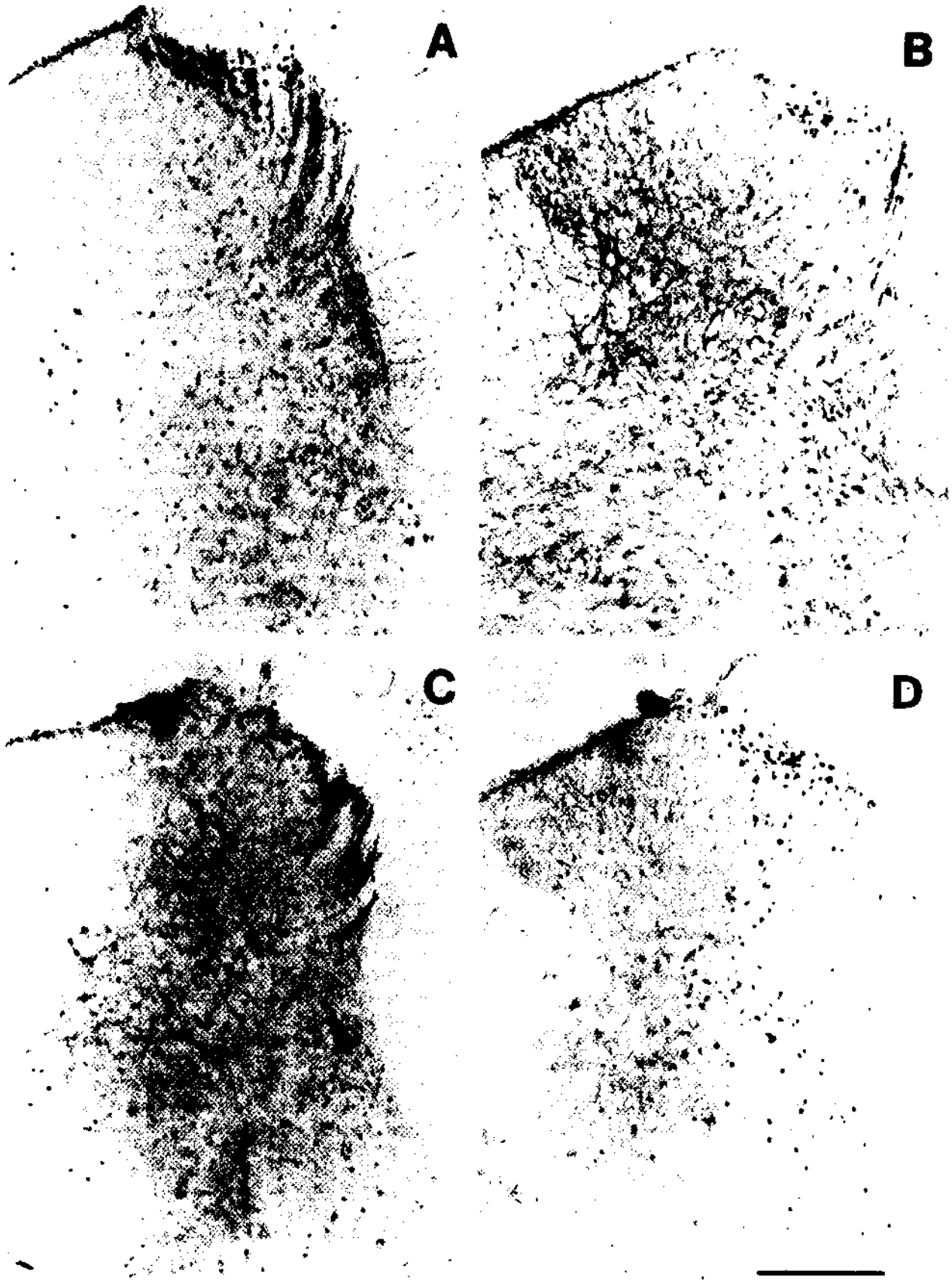

Fig. 1.

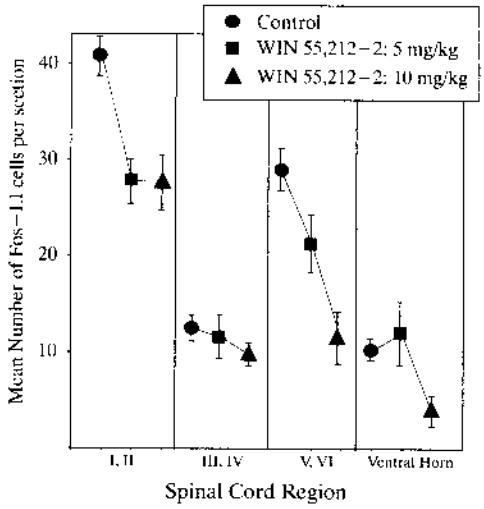

Fig. 2. Dose–response functions showing the number of neurons in various spinal cord laminae that exhibited pain-evoked Fos-like immunoreactivity in animals pretreated with vehicle or a cannabinoid agonist. The number of immunoreactive cells was first determined for each animal by calculating the mean for three sections qualitatively exhibiting the greatest number of labeled cells. Data shown represent mean number of labeled cells per section ± S.E.M. across subjects. Animals received an i.p. injection of either a vehicle control solution ($n = 17$) or WIN 55,212-2 (5 mg/kg, $n = 5$, or 10 mg/kg, $n = 12$) 10 min prior to formalin injection. Two hours later, the spinal cords were processed for immunocytochemistry. The number of immunoreactive neurons was significantly decreased by the drug in all laminae except III and IV.

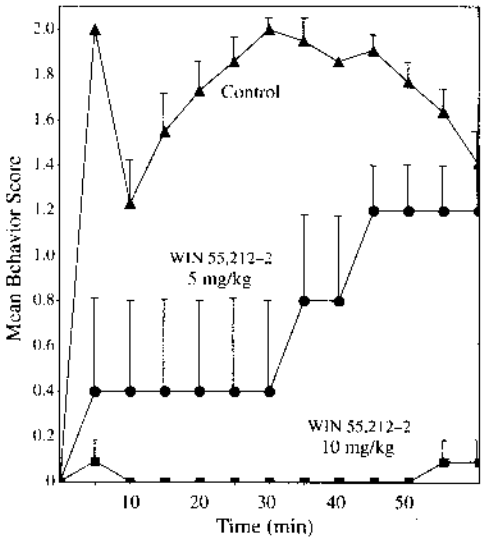

Fig. 3. Time course of the behavioral effects of intraplantar injection of formalin in rats following i.p. administration of vehicle ($n = 22$) or the potent cannabinoid agonist WIN 55,212-2 (5 mg/kg, $n = 5$, or 10 mg/kg, $n = 11$). Behavior was scored during the 1 h period following the injection of formalin, as described in the text. Control rats exhibited typical behaviors consisting of lifting and licking of the injected paw. Pain-related behavior in control rats exhibited a biphasic time course with peaks occurring during the first 5 min and around 30 min as shown. This behavior was suppressed especially during the first 30 min by 5 mg/kg WIN 55,212-2 and almost completely suppressed throughout the 1 h observation period by the 10 mg/kg dose of the drug. The vertical bars represent S.E.M.

regimen did not affect the ability of WIN 55,212-2 to decrease pain-evoked c-*fos* expression in the superficial dorsal horn, it did lead to a reduced effect in the neck region (d.f. = 15, $t = 9.09$, $P < 0.01$) and the ventral horn (d.f. = 15, $t = 12.6$, $P < 0.005$). These findings demonstrate that the long-term exposure to the cannabinoid agonist reduced its efficacy in the deep dorsal horn and the ventral horn.

DISCUSSION

The main finding of these studies was that the potent and selective cannabinoid agonist WIN 55,212-2 suppresses pain-evoked c-*fos* expression in the spinal dorsal horn. This effect was observed both in the superficial laminae and in the neck region (laminae V and VI) of the dorsal horn of the spinal cord, as shown by the significant decrease in the number of immunoreactive cells in these two regions. The suppression of Fos-like immunoreactivity was more pronounced in the neck region than in the superficial laminae, and failed to occur in nucleus proprius, a pattern similar to that observed after administration of morphine.[31,35]

Pharmacological specificity was established in the present work by examining dose-effects, receptor desensitization and an inactive enantiomer.[29] The results from all of these experiments support the hypothesis that the effects of cannabinoids on pain-evoked c-*fos* expression were mediated by cannabinoid receptors. Previous work demonstrated that repeated injections of cannabinoids produce tolerance to both the central and peripheral effects of the drug,[28] and cross-tolerance occurs with cannabinoids

Fig. 1. Photomicrographs depicting Fos-like immunoreactivity in the dorsal horn of rats that is induced by noxious stimulation. Following i.p. administration of a vehicle control solution or a dose of the potent cannabinoid agonist WIN 55,212-2, a 4% solution of paraformaldehyde was injected into the hindpaw and the animal's behavior was observed for 1 h, as described in the text. After a 2 h period the animals were killed and immunocytochemistry for Fos protein-like immunoreactivity was performed. (A) Example of a section obtained from a control (vehicle-treated) rat. (B) Example of a section from the dorsal horn of a rat treated with WIN 55,212-2 (10 mg/kg, i.p.). (C) Example of section from a rat treated with the inactive enantiomer WIN 55,212-3 (10 mg/kg, i.p.). (D) Example of a section obtained from an animal that was rendered tolerant to cannabinoids by repeated injections of WIN 55,212-2. Scale bar (lower right) = 100 μm.

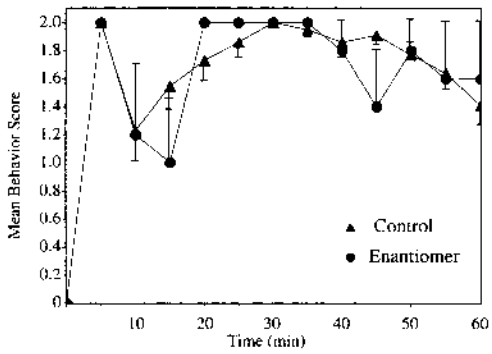

Fig. 4. Lack of effect of the inactive enantiomer WIN 55,212-3. Plantar injection of dilute formalin in rats caused pain-related behaviors such as licking and lifting of the paw (quantified as described in the text). The same significant increase in these behaviors occurred in rats treated with the inactive enantiomer (WIN 55,212-3) of the cannabinoid agonist (WIN 55,212-2) used in this study. As shown, the biphasic time-dependent behavioral effects of formalin injection are nearly identical for control ($n = 22$) and enantiomer-treated rats ($n = 5$), providing strong evidence for the receptor mediation of changes induced by the active compound.

having diverse chemical structures.[30] These data strongly suggest that cannabinoid receptors undergo biological adaptation in response to chronic occupation. We therefore hypothesized that continuous exposure to WIN 55,212-2 would decrease its ability to suppress Fos-like immunoreactivity evoked by intraplantar formalin. The partial tolerance we observed is consistent with previous behavioral research and supports the notion that the effects on c-*fos* expression were mediated by cannabinoid receptors.

Previous work demonstrated that cannabinoid receptors exhibit enantioselectivity.[20,25] Therefore, the observation that the receptor-inactive enantiomer WIN 55,212-3 failed to produce any effects in the present work adds further support to the notion that cannabinoid receptors mediated the observed effects. This observation, together with the partial tolerance and dose dependency of the effects, strongly suggest that the actions of WIN 55,212-2 in these experiments are both specific and receptor mediated.

As demonstrated with other cannabinoids,[17,34] WIN 55,212-2 did not decrease the swelling of the formalin-injected paw (data not shown). This finding, together with previous findings showing that direct central applications of cannabinoids produce analgesia,[19,23] suggest a central site of action of the drug in the present study. Because we used systemic injections, the site of action of the drug could be spinal, supraspinal or both. It is difficult to distinguish among these alternatives, because previous work demonstrated that cannabinoid analgesia involves both supraspinal and spinal sites of action.[19,23,33,37] Intraventricular[23] and intrathecal[19,37] administration of cannabinoids produce antinociception, and the antinociceptive effects of cannabinoids observed after intravenous administration[19] are attenuated following spinal transection. One site of action in spinal cord could be the superficial dorsal horn, where the cannabinoid receptor density is higher than the rest of the spinal gray[11] and where a significant suppression of c-*fos* expression was observed in the present study.

In the present study, the suppression of noxious stimulus-evoked c-*fos* expression was greater in the neck of the dorsal horn than in the superficial laminae. This same pattern of suppression was observed after systemic administration of morphine.[31] Furthermore, since the dose curves suggest that maximal efficacy was achieved at 5 mg/kg in the superficial dorsal horn, one may conclude that cannabinoids are not capable of fully suppressing c-*fos* expression in this area. It is thus possible that some neurons in the superficial laminae exhibit greater resistance to

Table 1. WIN 55,212-3 does not alter Fos-like immunoreactivity in rat lumbar spinal cord

Laminae	Control ($n = 5$)	Enantiomer ($n = 5$)
I, II	39.6 ± 2.0	38.1 ± 2.5
III, IV	12.2 ± 1.5	9.5 ± 1.3
V, VI	26.9 ± 4.3	24.1 ± 6.7
Ventral horn	8.4 ± 1.5	8.3 ± 3.8

Data are mean number of labeled cells per section ± S.E.M.; $P > 0.05$.

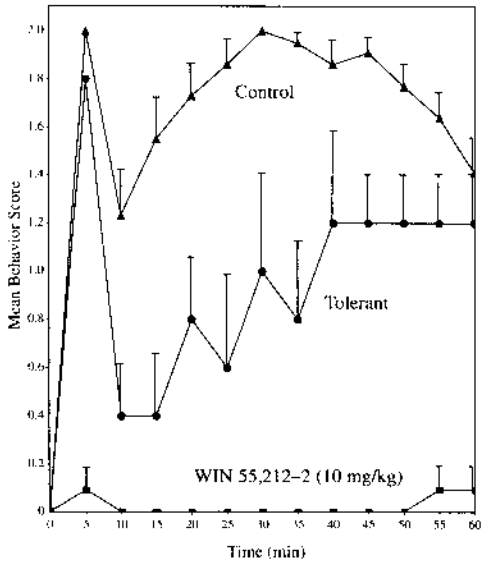

Fig. 5. Effect of WIN 55,212-2 on the time course of pain-related behaviors following plantar injections of dilute formalin in normal rats and rats rendered tolerant to cannabinnoids by repeated injections over a three day period. Control rats (▲, $n = 22$) exhibited the previously reported biphasic pain response characterized by marked peaks of paw licking at 5 and 30 min. The nearly complete suppression of this behavior by 10 mg/kg WIN 55,212-2 (■, $n = 11$) was markedly attenuated in rats subjected to the tolerance regimen (●, $n = 5$). Partial tolerance was indicated by the significant overall increase in pain-related behavior compared to normal rats receiving the cannabinoid agonist, but a decrease from rats that did not receive the drug. The vertical bars represent S.E.M.

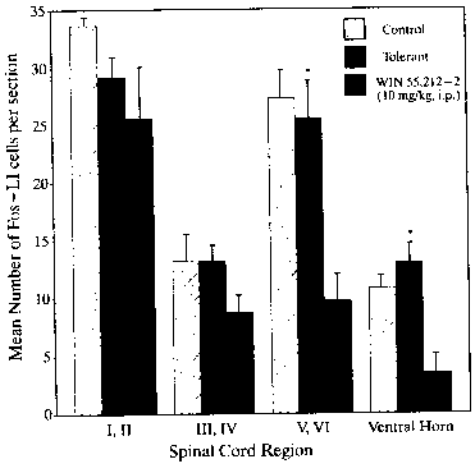

Fig. 6. Effect of WIN 55,212-2 on noxious stimulation-evoked expression of a Fos-like protein in various regions of the lumbar spinal cord in normal rats and rats rendered tolerant to cannabinoids by repeated injection of the potent cannabinoid agonist WIN 55,212-2 over a three day period (described in the text). As with the behavioral response, partial tolerance was observed. The cannabinoid agonist WIN 55,212 suppressed c-*fos* expression to a greater degree in normal rats than in rats rendered tolerant to cannabinoids. Control rats ($n = 5$) exhibited patterns of Fos-like immunoreactivity similar to that shown in Fig. 2. Asterisks denote a significant difference ($P < 0.05$) between animals receiving an acute injection of WIN 55,212-2 (10 mg/kg, i.p., $n = 5$) and animals rendered tolerant to the cannabinoid ($n = 5$). The vertical bars represent S.E.M.

analgesic agents. More work is needed to clarify the nature of these interactions.

The distribution of labeled neurons that were affected by the cannabinoid strongly suggests an action on nociceptive neurons. Jasmin *et al.*[15] demonstrated that the distribution of spinal Fos-like immunoreactivity following 1 h of walking on a rota-rod was significantly different from that found following noxious stimulation of the hindpaw. For example, motor behavior failed to induce the expression of Fos-like immunoreactivity in spinal cord areas that primarily mediate pain neurotransmission: the superficial dorsal horn, the outer substantia gelatinosa and the lateral, reticulated portion of lamina V. Indeed, non-noxious sensory stimulation also failed to produce labeling in these regions.[14] Therefore, the suppressive effects of WIN 55,212-2 in the superficial dorsal horn and other areas related to nociceptive processing provide a strong argument that cannabinoids modulate ascending nociceptive pathways via direct or indirect actions in the spinal dorsal horn. In this regard, it is important to note that some ventral horn neurons originating in the lumbar spinal cord project to the thalamus via the spinothalamic tract.[10] Therefore, the observed suppression of c-*fos* expression in neurons of the ventral horn may also reflect suppression of sensory pathways.

CONCLUSIONS

These experiments demonstrate a receptor-mediated action of cannabinoids on nociceptive neurons in the CNS. It would appear from these findings that one function of endogenous cannabinoids is to regulate pain sensitivity via modulation of spinal pain pathways. Nothing is known about the conditions which activate this novel non-opiate system or whether under natural conditions such modulation occurs. It would thus be of considerable interest to determine whether non-opiate forms of stimulation-produced analgesia or stress-induced analgesia involve endogenous cannabinoids.

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Results

Autoradiographic distribution of [^{3}H]WIN,55212–2 following ventricular administration

Examination of autoradiograms indicated that the distribution of [^{3}H] WIN55,212–2 was limited to periventricular structures. Dense labeling was found both rostral and caudal to the microinjection site. The structures in which significant labeling was observed included the septal area, the medial and lateral portions of the habenula, the anterior and posterior portions of the paraventricular thalamic nucleus, the periventricular hypothalamic area including the dorsomedial and ventromedial hypothalamic nuclei as well as the arcuate nucleus of the hypothalamus (Fig. 1). In addition, structures surrounding the aqueduct, including the entire periaqueductal gray and the dorsal raphe nucleus (Fig. 2), exhibited dense labeling following intracerebral ventricular (i.c.v.) administration.

Antinociceptive effects of WIN 55,212–2

The effects of i.c. microinjections of WIN55,212–2 into periventricular sites are illustrated in Fig. 2. There was no change in baseline tail–flick latencies following injections into the medial septal area (N=7), lHb (N=12), perihypothalamic area (N=7), and the ventrolateral periaqueductal gray (N=6). Microinjections of WIN55,212–2 into the arcuate nucleus (N=7) produced elevations in tail–flick latency during the test session; however, these effects were variable, often did not occur immediately following injection, and were not statistically significant. By contrast, microinjections into the dorsal or dorsolateral portion of the periaqueductal gray (mean ± s.e.m.; 18.3 ± 4.2); $F_{2,22} = 9.05$, $P = 0.001$) and into the dorsal raphe n. (26.6 ± 9.0; $F_{1,12} = 6.03$, $P = 0.03$) reduced nociceptive responding to noxious thermal stimuli.

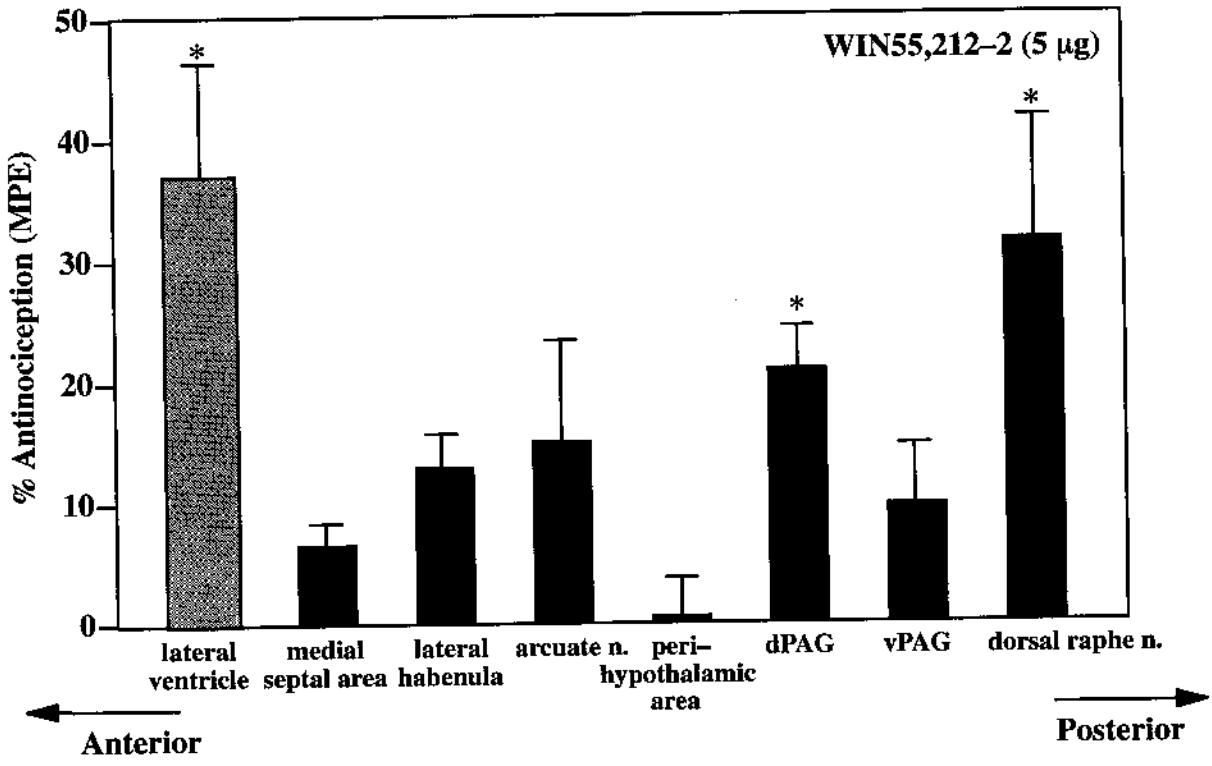

Fig. 2

Effects of a 5 µg dose of WIN55,212–2 microinjected into periventricular sites. Mean %MPE ± s.e.m. obtained for each site over first 15 min (6 tests) of 30 min test session. In no case did the antinociceptive effects from site injections exceed that produced by microinjections into the LV. Asterisks signifies $P < 0.05$ in post hoc analyses. Abbreviations: dPAG and vPAG; dorsolateral and ventrolateral portions of the periaqueductal gray.

The onset and time course of the cannabinoid–induced antinociception from microinjections into the lateral ventricle, the dorsolateral periaqueductal gray and the dorsal raphe is illustrated in Fig. 3. The onset of the increase in tail–flick latencies is slower after i.c.v. microinjection, compared with

the onset following direct i.c. microinjection into the dorsolateral periaqueductal gray and dorsal raphe. Analgesia reaches its peak in animals receiving i.c.v. injection (46 ± 6.7) at approximately 5 min post–injection, whereas the greatest degree of antinociception attained following dorsolateral periaqueductal gray and dorsal raphe microinjections (37 ± 5.4 and 45 ± 7.4, respectively) is almost immediately after injection. The antinociceptive actions of WIN55,212–2 in the dorsolateral periaqueductal gray and dorsal raphe never reached a level that was greater than that produced by i.c.v. microinjection of 5 μg. Although the antinociception was longer–lasting than that observed after direct site injections, there was no significant difference between magnitude or duration of these effects between the groups across time.

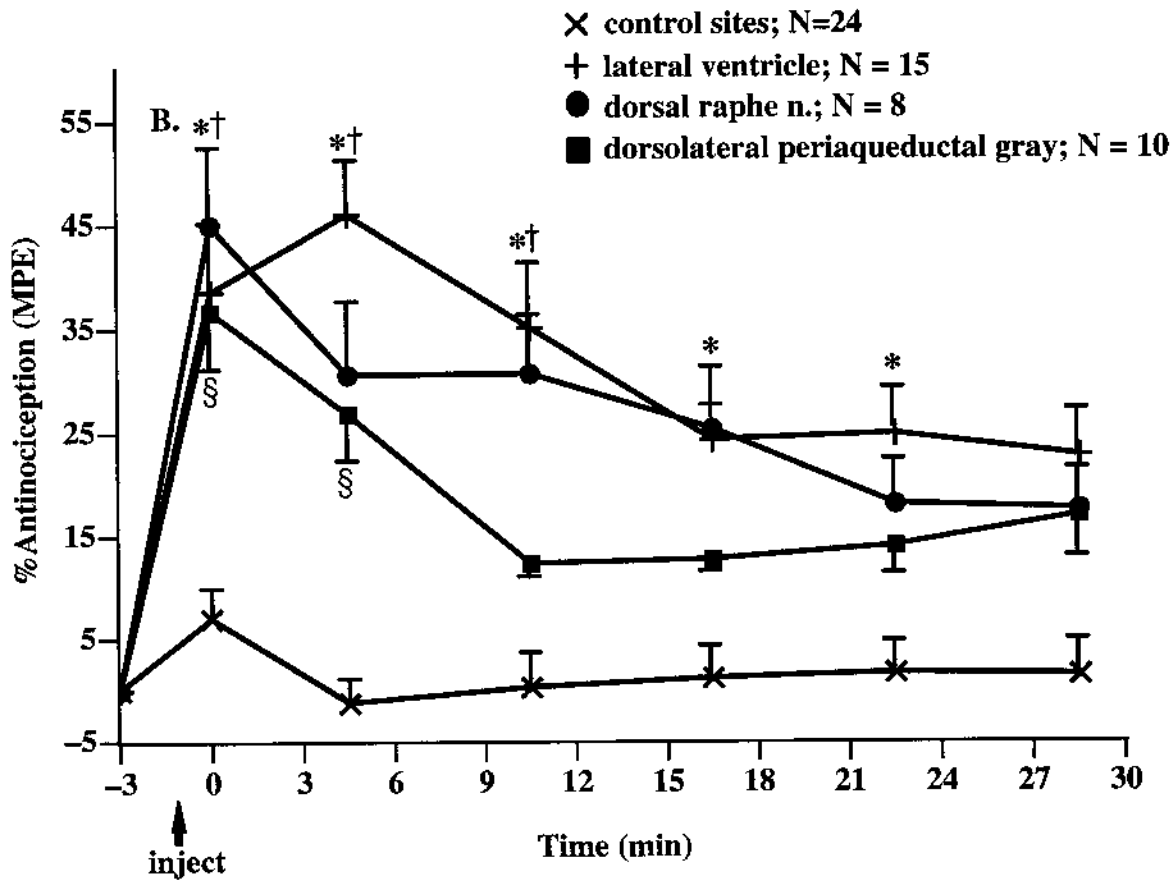

Fig 3.

Time course of i.c. microinjection of WIN55,212–2. Data are presented as mean ± s.e.m. at each time point. The onset of antinociception was immediate following direct site injection, as opposed to i.c.v. injection where peak effect did not occur until 5 min post injection. $P < 0.05$ (*ICV,†DR,§dPAG).

Discussion

The results of this study demonstrate that intraventricularly administered [^{3}H]WIN 55,212–2 is confined predominantly to periventricular structures during the peak of antinociception. Furthermore, direct i.c. microinjections of a low dose of the cannabinoid agonist, WIN55,212–2 into certain periventricular structures significantly decreases sensitivity to a noxious thermal stimulus in rats. Thus, significant elevations in tail–flick latencies were produced by microinjections into the dorsolateral periaqueductal gray and dorsal raphe, whereas injections into the medial septal area, lateral habenula, arcuate nucleus, perihypothalamic area, and ventrolateral periaqueductal gray failed to produce significant effects. The magnitude of the antinociceptive effects resulting from dorsolateral periaqueductal gray and dorsal raphe microinjection was approximately equal to the effects observed

following i.c.v. microinjection. Although the duration of the antinociceptive effects appeared to differ by site, these apparent differences were not significant.

In a previous report (1) two cannabinoid agonists (WIN55,212–2 and CP55,940) produced potent antinociceptive effects following i.c.v microinjection of 5 to 20 μg. Based on these findings, the lowest effective dose of WIN55,212–2 in that study (5 μg) was selected to examine particular brain sites, because direct injection into appropriate sites should yield more potent effects. Since microinjections in the dorsolateral periaqueductal gray and the dorsal raphe did not produce greater antinociceptive effects than i.c.v. microinjection of the same dose, one cannot reasonably conclude that the dorsolateral periaqueductal gray and dorsal raphe are the primary sites of action of cannabinoids in the brain. Conceivably, a principal site of action exists elsewhere and would mediate a significantly stronger effect than any observed in this study. Alternatively, it is possible that the approximately equianalgesic effects of direct site and i.c.v. microinjections are the result of additivity among the periventricular sites that are reached when the drug is injected in the ventricle. In spite of these limitations, the findings suggest that the dorsolateral periaqueductal gray and dorsal raphe are two sites of action of cannabinoids for the production of antinociceptive effects.

The latency to onset and the time course of the effects of dorsolateral periaqueductal gray and dorsal raphe microinjections differed from i.c.v. microinjections. The rapid onset and shorter time course of antinociception observed following dorsolateral periaqueductal gray and dorsal raphe microinjection make it highly unlikely that the observed effects were the result of diffusion to nearby sites or via the ventricular system to more distant locations. This conclusion is supported by the finding that microinjections into the dorsolateral periaqueductal gray and dorsal raphe produced an elevation in tail–flick latency, but injections into the neighboring ventrolateral periaqueductal gray had no effect.

The function of the periaqueductal gray and it's role in the endogenous modulation of nociceptive stimuli continues to be an area of considerable interest (see 35, for review). It is clear from this research that the periaqueductal gray possesses functionally distinct regions that support either opioid or non–opioid mediated circuits that modulate pain sensitivity (36). The findings that opiate antagonists often fail to or only partially reverse stimulation–produced analgesia elicited from the periaqueductal gray (29,37,38), and that i.c. microinjections of N–methyl D–aspartate (NMDA) into the dorsolateral periaqueductal gray produce a rapid increase in rat tail–flick latencies that is not antagonized by naloxone (39) are examples of the regional and neurochemical differences present within the periaqueductal gray. The results of the present study suggest that endogenous cannabinoids may act at cannabinoid receptors in these midbrain periventricular circuits to modulate pain sensitivity.

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AN EXAMINATION OF THE CENTRAL SITES OF ACTION OF CANNABINOID–INDUCED ANTINOCICEPTION IN THE RAT

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Summary

Microinjections of low doses of the potent and selective cannabinoids WIN 55,212–2 and CP 55,940 into the lateral ventricle produce long–lasting reduction in sensitivity to noxious thermal stimuli (1). To determine the central distribution of ventricularly administered WIN55,212–2, we microinjected an analgesic dose of the drug with [^{3}H]WIN55,212–2. At the peak time of antinociception, the radiolabeled drug was confined to periventricular sites throughout the brain. The contribution of particular periventricular structures to the antinociceptive effect was evaluated using intracerebral microinjection techniques and the tail–flick test. Guide cannulae were implanted above the following periventricular structures: the medial septal area, lateral habenlua, perihypothalamic area, arcuate nucleus of the hypothalamus, dorsal raphe nucleus and the dorsolateral and ventrolateral aspects of the periaqueductal gray. Microinjections of WIN55,212–2 (5 μg/0.5 μl) into the medial septal area, lateral habenula, perihypothalamic area, arcuate nucleus, and ventrolateral periaqueductal gray did not significantly affect tail–flick latencies. By contrast, microinjections of WIN55,212–2 into the dorsolateral periaqueductal gray and the dorsal raphe significantly elevated tail–flick latencies. The results of this study indicate that at least two periventricular structures within the brain are involved in cannabinoid antinociception.

The discovery of a G–protein coupled cannabinoid receptor (2,3) together with the identification of the putative endogenous cannabinoid ligand anandamide (4,5) and the partial purification of other putative ligands for the cannabinoid receptor (6,7) provide a strong argument for the existence of an endogenous cannabinoid neural system (see 8, for review). The following lines of evidence (1,9–18) suggest that one function of this novel system is to modulate pain sensitivity: (1) cannabinoids produce analgesia with nearly the same potency and efficacy as morphine in rodents; (2) the inactivity of the enantiomers of cannabinoids, the development of tolerance, and the strong correlation between cannabinoid receptor binding affinity and behavioral potency indicate that this effect is mediated by cannabinoid receptors; (3) the analgesic effects of cannabinoids are centrally mediated and have both spinal and supraspinal substrates, and; (4) cannabinoids inhibit nociceptive responses in wide dynamic range neurons in the spinal cord (see 19, this volume) and the thalamus (20), thus illustrating that cannabinoid effects on behavioral measures of pain are due at least in part to the inhibition of neurotransmission within spinothalamic nociceptive pathways. These findings suggest that endogenous cannabinoids may serve naturally to inhibit the processing of painful inputs.

Although cannabinoids have been shown to inhibit the transmission of noxious activity, little is known about the sites within the brain that mediate the antinociceptive effects of cannabinoids.

Since Tsou and Jang's (21) demonstration of the role of the periaqueductal gray in the analgesic effects of morphine, a variety of investigations using electrical stimulation and intracerebral (i.c.) microinjection techniques have revealed the presence of an extensive periventricular system that exerts powerful control over the processing of painful stimuli (22–27). Stimulation–produced analgesia is elicited by electrical stimulation of periventricular brain regions such as the medial septal area, intralaminar nucleus of the thalamus, dorsal raphe and periaqueductal gray (28). Although electrical stimulation as well as i.c. microinjection studies have focused on mechanisms of opiate analgesia, a sizable body of evidence suggests that non–opiate neurotransmitters or neuromodulators also play an important role in neural systems that modulate pain sensitivity (29–31). These findings suggest the possibility that the sites of action of cannabinoid analgesia may reveal components of a novel nonopiate, cannabinergic pain modulatory system. A site of considerable interest is the periaqueductal gray, which contains high concentrations of cannabinoid receptors (32) and was shown by Lichtman and Martin (14) to mediate antinociception following local microinjections of cannabinoids. To further elucidate the mechanisms of cannabinoid analgesia, we used the tail–flick test to examine the involvement a variety of periventricular brain nuclei in the modulation of nociceptive processing by cannabinoids.

Methods

Surgical procedures

Guide cannulae were implanted above targeted structures in male Sprague Dawley rats weighing 275 to 325 g under pentobarbital (50 mg/kg) anesthesia. The cannulae were made from 24 ga thinwall stainless steel hypodermic tubing (Small Parts Inc., Miami, Fl) which were beveled at the tip. The stereotaxic coordinates were based on modifications of the coordinates in the atlas of Paxinos and Watson (33) using zero points from bregma or lambda (anterior–posterior: AP), midline suture (lateral–medial: LM), and skull surface (dorsal–ventral: DV). The AP was referenced to bregma for the following structures (AP,LM,DV): medial septal area (+0.7, 0, 6.1), lateral ventricle (–1.0,–1.5,–4.3), arcuate nucleus (–2.5,–0.3,–10.1), perihypothalamic area (–3.0,–0.3,–9.0 to 9.8), lateral habenula (–3.6, –0.7,–5.7). The AP was referenced to lambda for the following structures: dorsolateral periaqueductal gray (+1.0, –0.6, –5.6), ventrolateral periaqueductal gray (+1.0, –0.6, –6.8), dorsal raphe (+1.0, –2.7, –7.9, with an 20° angle so that the cannula tract did not pass through aqueduct of Sylvius). To minimize damage, the DV were adjusted so that a 31 ga stainless steel injection needle from 1.0 to 5.0 mm beyond the tip of the guide cannulae depending on the depth of the structure. Dental acrylic and stainless steel screws were used to secure guide cannulae to the skull.

Drug preparation and administration

WIN 55,212–2 mesylate was purchased from Research Biochemicals Inc. (Natick, MA) and dissolved in 60% dimethylsulfoxide (DMSO). For the lateral ventricle injections, drugs were delivered at a dose of 5 μg/10 μl over 1 min. All other injections were delivered at a dose of 5 μg/0.5 μl over 72 s.

Autoradiography

Because intraventricular administration produces antinociception at low doses (1), studies of central sites of action of cannabinoids could be facilitated by knowing the brain distribution of intraventricularly administered cannabinoids. Therefore an autoradiographic study of the brain distribution of intraventricularly administered [^{3}H]WIN 55,212–2 was carried out. Rats (n=3) underwent surgery for placement of guide cannulae for i.c.v. microinjection. After a 3 to 5 day recovery period, a 10 μl solution containing 3.7 ng [^{3}H]WIN 55,212–2 (49.6 Ci/mMol; DuPont/New England Nuclear, Boston, MA) and 20 μg unlabeled WIN 55,212–2 in 60% DMSO was injected into the left lateral ventricle of each rat. Animals were then tested for antinociception using the tail–flick test. After 6 minutes, the approximate time to peak analgesia (1), animals were decapitated, their brains were removed, rapidly frozen, and stored at –80 °C. Twenty micron frozen coronal sections were thaw–mounted onto slides that were placed in X–ray cassettes with a sheet of LKB Ultrofilm and a set of tritium standards (American Radiochemicals Inc., St. Louis). Following an exposure time of 30 days, the film was developed using D–19 Kodak developer, and negative images were printed. Adjacent sections, stained with Cresyl violet, were used to identify structures. The autoradiograms and stained sections were digitized using an image analysis computer system (Imaging Research Inc., St. Catherines, Ontario, Canada).

Analgesia testing

Three to seven days after surgery, the antinociceptive effects of WIN 55,212–2 were measured using the tail–flick procedure of D'Amour and Smith (34). Each test consisted of a baseline period during which the latency to withdraw the tail from a radiant heat source was recorded every 3 min; a 10 s cut–off was employed to avoid tissue damage. After the establishment of stable tail–flick latencies (2.5 to 4.5 s), baseline latencies were recorded for a period of 15 min. Then, WIN 55,212–2 or a vehicle solution of DMSO was microinjected into the structure being tested. After the injection, tail–flick latencies were recorded every three minutes for at least 30 min. All experiments adhered to the guidelines on the study of pain in awake animals established by International Association for the Study of Pain and were approved by the Brown University Institutional Animal Care and Use Committee.

Histology

Animals were sacrificed by lethal injections of pentobarbital and were perfused transcardially with 0.9% saline and 10% formalin. Brains were removed, stored overnight in a 30% sucrose–formalin solution, and then frozen sections were obtained and stained with cresyl violet. These were examined under a microscope to identify location of each injection. Only animals with verified injection sites were included in the data analysis.

Data analysis

Antinociceptive effects of WIN 55,512–2 were determined by calculating the percent maximum possible effect (%MPE) derived from the following equation: (test latency – control latency)/(10 – control latency) X 100, where 10 represents the cut–off latency and the control latency equals the average of three baseline tests prior to injection. Control groups of animals receiving only vehicle injections were tested for structures where a mean %MPE of greater than 25 was obtained at some time point during the 30 min of post injection testing. For these brain regions, repeated measures ANOVA was used to test the significance of the injection of WIN55,212–2 compared to the control group. The Sheffe test was used to make post–hoc comparisons between groups, where appropriate. For time course analyses, an ANOVA was carried out on the control animals. Since there was no significant difference between sites in the control condition, these animals were pooled for post hoc comparisons.

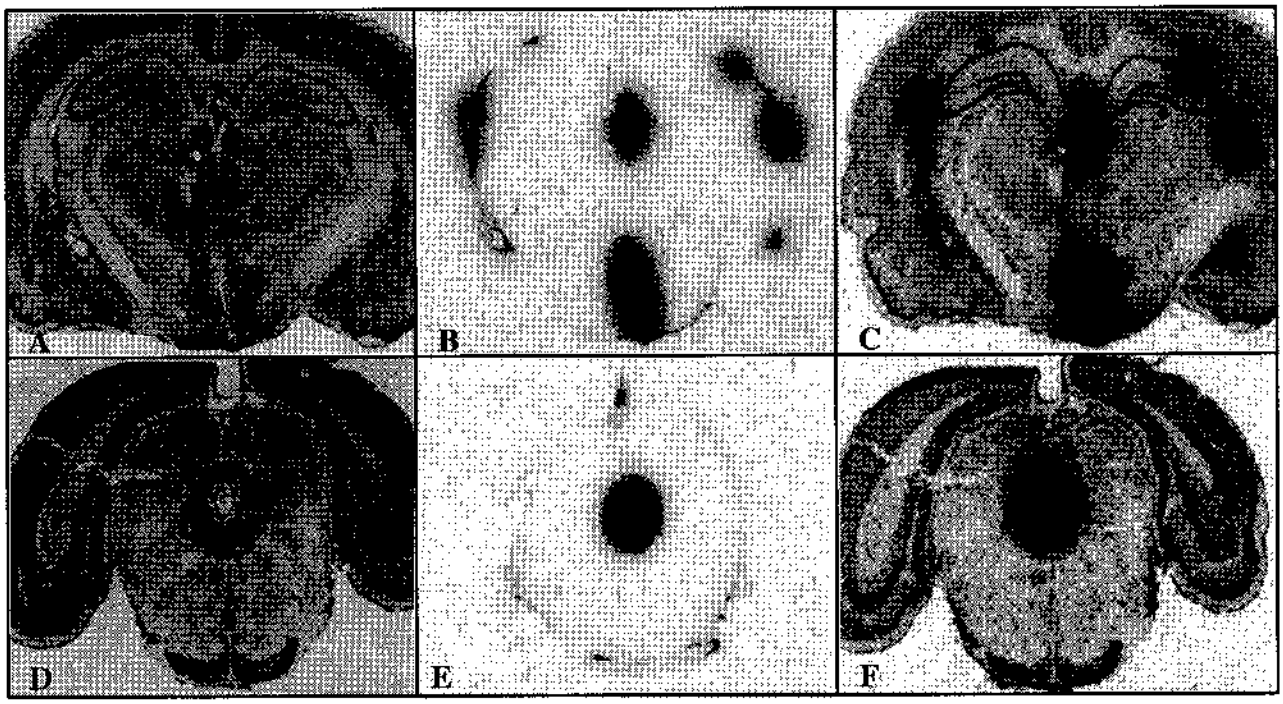

Fig. 1

Autoradiographic localization of [^{3}H]WIN 55,212–2 microinjected in the lateral ventricle as described in text. A–C: Coronal section through the diencephalon, D–F: Coronal section through the midbrain. A,D: nissl–stained sections, B,E: autoradiogram showing distribution of [^{3}H]WIN 55,212–2 (dark areas), C,F: Autoradiogram superimposed on nissl–stained section to aid in visualization of the distribution of radioisotope.

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Suppression of Noxious Stimulus-Evoked Activity in the Ventral Posterolateral Nucleus of the Thalamus by a Cannabinoid Agonist: Correlation between Electrophysiological and Antinociceptive Effects

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The CNS contains a putative cannabinergic neurotransmitter and an abundance of G-protein-coupled cannabinoid receptors. However, little is known about the function of this novel neurochemical system. Cannabinoid agonists produce antinociception in behavioral tests, suggesting the possibility that this system serves in part to modulate pain sensitivity. To explore this possibility, the effects of the cannabinoid agonist WIN 55,212-2 on nociceptive neurons in the ventroposterolateral (VPL) nucleus of the thalamus were examined in urethane-anesthetized rats. After identification of a nociresponsive neuron, a computer-controlled device delivered graded pressure stimuli to the contralateral hindpaw. WIN 55,212-2 (0.0625, 0.125, and 0.25 mg/kg, i.v.) suppressed noxious stimulus-evoked activity of VPL neurons in a dose-dependent and reversible manner. Noxious stimulus-evoked firing was affected more than spontaneous firing. These effects were apparently mediated by cannabinoid receptors, because the cannabinoid receptor-inactive enantiomer of the drug (WIN 55,212–3, 0.25 mg/kg) failed to alter the activity of this population of cells. Administration of morphine (0.5 mg/kg, i.v.) produced effects that were very similar to those produced by the cannabinoid. WIN 55,212-2 (0.25 mg/kg, i.v.) failed to alter the responses of non-nociceptive low-threshold mechanosensitive neurons in the VPL. WIN 55,212-2 produced antinociceptive effects with a potency and time course similar to that observed in the electrophysiological experiments, despite the differences in the anesthetic states of the animals used in these experiments. The antinociceptive and electrophysiological effects on VPL neurons outlasted the motor effects of the drug. Furthermore, the changes in nociceptive responding could not be attributed to changes in skin temperature. Taken together, these findings suggest that cannabinoids decrease nociceptive neurotransmission at the level of the thalamus and that one function of endogenous cannabinoids may be to modulate pain sensitivity.

Key words: cannabinoid analgesia; tetrahydrocannabinol; anandamide; thalamus; nociception; rat

The identification of specific G-protein-coupled cannabinoid receptors (Howlett et al., 1990) and the discovery of anandamide (Devane et al., 1992), a putative ligand for these receptors, provide strong evidence for an endogenous cannabinergic neural system (for review, see Pertwee, 1993). These discoveries are the foundation for new research aimed at understanding the functions of endogenous cannabinoids. Although much is known about the pharmacology of cannabinoids, little is known about their actions on particular neural systems, and no definitive statements can be made about the functions of endogenous cannabinoids.

The high levels of cannabinoid receptors in the CNS suggest that endogenous cannabinoids are a major class of neuromodulators. Studies of the distribution of cannabinoid receptors revealed that they occur in concentrations that equal or exceed those of the most plentiful neurotransmitter receptors known (Herkenham et al., 1991; Herkenham, 1995). With regard to the present investigations of the role of cannabinoids in pain modulation, it is notable that the concentration of cannabinoid receptors in spinal cord (Herkenham et al., 1991) is 10 to 50 times higher than the level of opiate receptors (Faull and Villiger, 1987; Besse et al., 1991). The high concentration of cannabinoid receptors in specific brain areas accounts for the powerful effects of cannabinoids on behavior and suggests that endogenous cannabinoids are a major neurochemical system in the brain.

Although there are no direct data on the functions of endogenous cannabinoids, administration of their synthetic counterparts suggests that endogenous cannabinoids modulate pain sensitivity. Exogenous cannabinoids reduce responsiveness to noxious thermal stimuli (Buxbaum, 1972; Sofia, 1973; Bloom et al., 1977; Jacob et al., 1981; Yaksh, 1981; Lichtman and Martin, 1991a,b), mechanical stimuli (Sofia et al., 1973), and chemical stimuli (Moss and Johnson, 1980) in rats and mice, with a potency and efficacy similar to that of morphine (Buxbaum, 1972; Bloom, 1977; Jacob et al., 1981). However, these compounds also suppress motor function (Loewe, 1946; Gough and Olley, 1977; Ueki, 1980) and decrease neurotransmission in the output pathways of the basal ganglia (Miller and Walker, 1995), raising questions about the interpretation of results from behavioral tests of pain sensitivity (Cartmell et al., 1991). Several recent studies suggest that cannabinoids suppress nociceptive processing at the level of the spinal cord (Lichtman et al., 1991a,b; Hohmann et al., 1995; Tsou et al.,

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1996); however, the extent to which these effects are conserved throughout ascending sensory pathways is not known.

The spinothalamic tract plays an integral role in the transmission of nociceptive information from the spinal to supraspinal level (Mitchell and Hellon, 1977; Guilbaud et al., 1980; Peschanski et al., 1980a,b, 1983) (for review, see Willis, 1984). Originating primarily in the dorsal horn of the spinal cord, spinothalamic tract neurons terminate in several thalamic nuclei including the ventroposterolateral (VPL) nucleus (Lund and Webster, 1967; McAllister and Wells, 1981; Peschanski et al., 1983; Peschanski and Besson, 1984). The VPL nucleus receives inputs from spinal wide dynamic range neurons, which encode the strength of noxious and non-noxious stimuli (Mendell, 1966; Giesler et al., 1976) (see also Price, 1988). This specificity is maintained at the level of the VPL, at which somatotopically arranged neurons with relatively small receptive fields represent the location and intensity of noxious somatic stimuli (Peschanski et al., 1980a,b, 1983; Guilbaud et al., 1980, 1987). Furthermore, morphine suppresses the responses of VPL neurons to noxious stimuli (Hill and Pepper, 1978; Benoist et al., 1983), an important demonstration in view of the strategic location of the VPL within pain-processing circuitry.

The cannabinoid agonist WIN 55,212-2 was used throughout the experiments reported herein. The potency and selectivity of this compound and its ability to produce cannabinoid receptor-mediated behavioral and electrophysiological effects have been documented using both *in vitro* and *in vivo* approaches (Compton et al., 1992; D'Ambra et al., 1992; Jansen et al., 1992; Pertwee et al., 1993; Felder et al., 1995). Here, we describe experiments that demonstrate the following. (1) A cannabinoid agonist inhibits the activity of nociceptive neurons but not mechanosensitive neurons in the VPL of anesthetized rats in a manner similar to morphine. (2) There is a strong relationship between the electrophysiological and antinociceptive effects of the cannabinoid. (3) The cannabinoid's electrophysiological and antinociceptive actions can be dissociated from its effects on thermoregulation and motor function. Together these findings demonstrate that cannabinoids selectively inhibit nociceptive neurotransmission in rat spinothalamic tract neurons and suggest a possible function of a cannabinergic receptor system in the modulation of pain sensitivity.

MATERIALS AND METHODS

Drug preparation and administration. WIN 55,212-2 (Research Biochemicals International, Natick, MA) and WIN 55,212–3 (a gift from Dean A. Haycock, Sterling Research Group, Rensselaer, NY) were dissolved in a 1:1:18 mixture of ethanol: emulphor (Alkamuls EL-620, Rhone-Poulenc, Cranbury, NJ): saline. Morphine sulfate (Mallincrodt, Paris, KY) was dissolved in saline at a concentration of 0.5 mg/ml. WIN 55,212-2 was prepared in concentrations of 0.0625, 0.125, and 0.25 mg/ml. Drugs were administered in a volume of 1 ml/kg through the lateral tail vein.

Electrophysiological methods

Surgical preparation. For all electrophysiological experiments, male Sprague Dawley rats weighing 250–400 gm were anesthetized with urethane (1.5 g/kg, i.p., supplemented as required) and placed in a stereotaxic frame. Body temperature was monitored and maintained at 37°C using an automated heating pad. The cortex above the VPL was exposed, the dura mater was excised, and the brain was kept moist with 0.9% NaCl.

Electrode preparation and recording. Single-barrel glass micropipettes were pulled in a Narashige PE2 puller; tips were broken back to ~1 μm diameter. The electrode was filled with a saturated solution of fast green dye in 2 M NaCl. Electrode penetrations were made in the region of the VPL (−3.2 mm posterior, −3.6 mm lateral, and −4.9 to 5.4 mm ventral from bregma) based on the atlas of Paxinos and Watson (1986). On isolation of a spontaneously active single neuron, its response to light brushing with a camel hair brush of the contralateral and ipsilateral hindpaws was examined. If the neuron was responsive to the stimulus, then its response to light pinching with forceps and joint movement was determined. Neurons that responded to brushing and pressure, but not to joint movement, were then tested for their responses to a graded pressure stimulus applied to the receptive field. Data were collected for 1 sec before stimulus onset, during the 5 sec of the stimulus application, and for an additional 5 sec after termination of the stimulus. In all cases, drugs were tested on only one cell at one dose per animal.

Histology. At the end of each experiment, fast green dye was ejected by passing 30 μA through the electrode (tip negative) for 20 min to mark the location of the recording site. Animals were perfused transcardially with 0.9% NaCl, followed by 10% formalin. Brains were removed and stored overnight in a 30% sucrose–formalin solution. Frozen sections (40 mm) were mounted, stained with neutral red, and examined microscopically to localize the recording site. Data from a particular subject were included in the study only if histological examination revealed that the recording was obtained from a neuron within the boundaries of the VPL.

Administration of noxious pressure. A computer-controlled miniature air cylinder (described by Hohmann et al., 1995) was used to administer the pressure stimulus. The stimulus was 5 sec in duration, rising continuously from zero pressure to a peak of 4.6 kg/cm^2 over a 3 sec period and held for 2 sec.

Experimental procedure. Baseline responses to the noxious pressure stimulus were determined by applying the stimulus 10 times at 1 min intervals. The drug or vehicle was then injected intravenously, and the stimulus was applied at 1 min intervals for 10 min. To follow the recovery from the effects of the drug, a 10 min rest period was allowed; then the stimulus was delivered every 2 min until the responses returned to within 20% of baseline.

Data acquisition. The output of the preamplifier was connected to an electronic circuit that produced a logic pulse for each action potential. The output was passed to a computer, which stored the time of occurrence of each action potential (0.1 msec accuracy) and produced a graphical display of the data as they were acquired.

Classification of nociresponsive neurons. Stimulus–response functions were calculated from pretreatment baseline data by plotting the firing rate against the mean applied pressure during the increasing portion of the stimulus (0–3 sec). Preliminary analyses indicated that the stimulus–response functions were logarithmic rather than linear. Therefore, the values for firing rate and pressure were subjected to logarithmic transformation followed by linear regression analysis. Only neurons that exhibited stimulus–response functions with a slope of at least 0.2 and an r value of at least 0.5 were classified as wide dynamic range neurons and included in the study.

Examination of responses of non-nociceptive mechanosensitive neurons to non-noxious stimuli after WIN 55,212-2. Non-nociceptive mechanosensitive neurons (n = 5) were recorded in the VPL using methods based on those described by others (Angel and Clark 1975; Dong et al., 1978; Peschanski et al., 1981; Miletic and Coffield, 1989; Montagne-Clavel and Oliveras, 1995). These neurons usually exhibited little or no spontaneous activity; therefore, a search stimulus (light tapping with a wooden probe) was used to identify candidate neurons. Once isolated, the receptive field of the neuron was mapped on the plantar surface of the contralateral hindpaw using an insect pin, and the region that yielded maximal responses was marked in ink. Activity was evoked in these cells using a light-touch stimulus, which consisted of gently tapping the receptive field with a rounded wooden probe (3 mm diameter). Tapping the skin within the receptive field of the neuron produced reliable and reproducible activation of mechanosensitive neurons. These neurons were characterized as non-nociceptive based on the lack of a greater response to the noxious pressure stimulus (4.6 kg/cm^2) than to the light-touch stimulus. The light-touch stimulus was applied at the same intervals and for the same duration as the pressure stimulus used to evoke activity in wide dynamic range neurons in the VPL. After baseline responses were established, the effect of the high dose of WIN 55,212-2 (0.25 mg/kg) on activity evoked in these neurons by the non-noxious stimulus was examined.

Data analysis. As noted above, all electrophysiological data were stored as the time of occurrence of each action potential. These data were transformed into firing rates (mean number of action potentials/duration of the interval) for successive intervals before and after administration of the stimulus using computer programs written by the investigators on a Hewlett-Packard 9000/720 workstation. These data were used to construct the peristimulus time histograms (PSTHs) shown in the figures. Similar techniques were used to determine the mean stimulus pressure at different times, which were combined with the calculated firing rates to

construct stimulus–response functions. These data were transferred to an IBM mainframe for statistical analysis using BMDP Statistical Software (Los Angeles, CA). ANOVA and appropriate *post hoc* tests were used to analyze treatment effects. The Greenhouse-Geiser (1959) correction was applied to interaction terms containing a repeated factor.

The effects of various drug treatments on stimulus–response functions of nociceptive neurons were determined by calculating the mean response for each drug condition and performing a linear regression on the $\log_{10}$-transformed mean firing rates against the $\log_{10}$-transformed mean pressures using the method of least squares. Estimation of 95% confidence intervals for slope was determined using the method described by Goldstein (1964). The significance of the correlation coefficients was determined by ANOVA. Differences among the slopes of the mean stimulus–response functions were assessed using the method described by Edwards (1984), and the p value was adjusted to account for multiple comparisons by the Bonferroni method (Myers, 1972).

The duration of the electrophysiological effects was calculated as the interval during which responses deviated from predrug levels by >20%.

Behavioral methods

Antinociceptive effects of WIN 55,212-2: mechanical stimulation. A separate experiment was performed to examine the effect of WIN 55,212-2 on the behavioral response to the noxious pressure stimulus. In this experiment, the threshold of the withdrawal reflex to the computer-controlled mechanical pressure stimulus was determined. Because of the nature of the apparatus, it was necessary to lightly anesthetize rats (n = 12) for this experiment. This was accomplished by intraperitoneal injection of urethane (1 gm/kg), which produced a reduction in motor tone without suppressing nociceptive withdrawal or corneal reflexes. A hindpaw was placed in the pressure device, and the noxious stimulus used in the electrophysiological experiments was applied; electrophysiological responses were not recorded during this procedure. Throughout the period of stimulation, the pressure within the air cylinder was digitized (10 samples/sec) by a computer for later determination of the pressure at which a nociceptive withdrawal reflex occurred. When a withdrawal reflex occurred (judged by a sudden and vigorous withdrawal flexion of the hindlimb), the stimulus was immediately removed, the pressure at which the response occurred was recorded, and a 3 min interval was allowed to pass before the next test. After a stable baseline was established, either WIN 55,212-2 (0.25 mg/kg, i.v.) or vehicle was administered, and the rat was tested at 3 min intervals for the next 60 min.

Antinociceptive effects of WIN 55,212-2: thermal stimulation. A second experiment was performed to further examine the relationship between the effects of the drug on behavioral and electrophysiological responses to noxious stimuli. This experiment used awake animals (n = 20) and an established measure of pain sensitivity, that is, the tail flick test of D'Amour and Smith (1941). Initially, the radiant heat source was adjusted to produce tail-flick latencies in the range of 2.5–4.5 sec. Then the latency to withdraw the tail from the noxious thermal stimulus was recorded every 3 min for 15 min. WIN 55,212-2 (0, 0.0625, 0.125, 0.25 mg/kg) was administered intravenously via the lateral tail vein, and testing resumed for the following 45 min or until latencies returned to within 20% of baseline. Time–effect curves were constructed as the percent maximal possible effect (%MPE) derived from the following equation:

$$\%\,\mathrm{MPE} = \frac{\text{test latency} - \text{control latency}}{\text{cut-off latency} - \text{control latency}} \times 100,$$

where the control latency equaled the mean of three predrug baseline tests, and the cut-off latency was 10 sec.

Measurement of tail and paw temperature. It has been reported that changes in skin temperature can lead to artifactual changes in apparent pain sensitivity (Tjolsen et al., 1989). Therefore, the effects of the vehicle and WIN 55,212-2 on skin temperature were examined in two experiments, one under conditions similar to those used in the electrophysiological experiments and another under conditions similar to those used in the behavioral experiments. In the first experiment, sham-operated rats (n = 3) were anesthetized with urethane, positioned on a feedback-controlled heating pad, and secured in a stereotaxic frame, as described for the electrophysiology experiments. A craniotomy was performed over the VPL, and the dura mater was excised. After ~1.5 hr of acclimatization to the heating pad, skin temperature was measured by means of a copper–constantan type T thermocouple probe (diameter = 1.2 mm, Teflon insulated) (Omega Engineering, Stamford, CT), which was fastened to the hindpaw with adhesive tape. Baseline readings of skin temperature were recorded at 3 min intervals for 15 min before intravenous administration of WIN 55,212-2 (0.25 mg/kg) and continued at 3 min intervals for the subsequent 60 min.

To examine further the possibility that the drug altered nociceptive responses by changing skin temperature, tail skin temperature was recorded in awake animals at 3 min intervals for 15 min before and 120 min after administration of either WIN 55,212-2 (0.25 mg/kg, i.v.) or the vehicle (n = 13). The procedure for measuring temperature was the same as described above, except that the thermoprobe was attached to the ventral surface of the tail where the thermal stimulus is normally applied for tail-flick testing.

Effects of WIN 55,212-2 on motor function. To examine the relationship between the antinociceptive and motor effects of the drug, motor function was assessed using two different measures, and the time course of the effects of WIN 55,212-2 was recorded. The first study used the rotorod procedure, a sensitive measure of motor coordination (Dunham and Miya, 1957; Kinnard and Carr, 1957). A second study, which was performed in a separate group of rats, examined the cataleptic effects of WIN 55,212-2.

A 6 cm rotorod treadmill (UGO Basile model 7700, Stoelting, Chicago, IL) was set to rotate at a constant speed of 10 rpm. Rats (n = 16) were trained to run continuously for 2 min during two training sessions separated by 1–2 hr. After this criterion was achieved, animals received injections of either WIN 55,212-2 (0.25 mg/kg, i.v.) or vehicle. Animals were tested for their ability to remain on the rotorod at 5 postinjection times (5, 10, 20, 30, and 60 min). If a fall occurred during the test session, the animal was immediately returned to the rotorod, and the mean duration on the treadmill was calculated for the two attempts.

Catalepsy was measured using a bar test similar to that described by Pertwee and Wickens (1991). Each rat (n = 6) was placed with both forelegs over a horizontal stainless steel bar (diameter = 0.5 cm) 9 cm above a Plexiglas base. The latency to descend from the bar was used as the index of catalepsy. The test was performed before injection of WIN 55,212-2 (0.25 mg/kg, i.v.) and at 3 min intervals for 30 min thereafter. A maximum descent latency of 60 sec was allowed.

Data analysis. ANOVA was used to assess changes in nociceptive responding, skin temperature, and motor function. As with the electrophysiological studies, the duration of the drug effects for the measures described above (withdrawal from noxious pressure, tail flick, catalepsy, rotorod) was determined by calculating the time during which responding deviated from the mean predrug response by >20%. The data from the experiments on catalepsy were analyzed by the nonparametric sign test, because of floor and ceiling effects. $p < 0.05$ was considered statistically significant in all experiments.

The experiments reported herein were approved by the Brown University Institutional Animal Care and Use Committee.

RESULTS

A total of 32 nociceptive and 5 non-nociceptive neurons were recorded in the VPL. As shown in Figure 1, neurons were found in the region of VPL observed previously to contain neurons with receptive fields on the contralateral hindpaw (Angel and Clark, 1975).

Characterization of nociresponsive VPL neurons

The nociresponsive neurons included in this study fired spontaneously at a mean rate of 4.6 ± 0.6 Hz (SEM) and responded to the increasing intensity of the pressure stimulus with increases in firing rate (Fig. 2*A*). The peak mean firing rate of all nociceptive neurons recorded in the VPL (measured before drug treatment) was 14.6 ± 0.6 Hz, which occurred during maximum stimulus pressure. A repeated-measures ANOVA was performed on all 32 nociceptive cells for the 10 predrug baseline trials. Data were extracted from 20 successive time intervals, during which stimulus strength was continuously increasing. This analysis revealed that firing rate increased as a function of pressure ($F_{(19,494)} = 33.74$, $p < 0.00005$). There were no significant differences in the evoked response across baseline trials or among the drug groups for either preinjection spontaneous firing rate or stimulus-evoked firing rate.

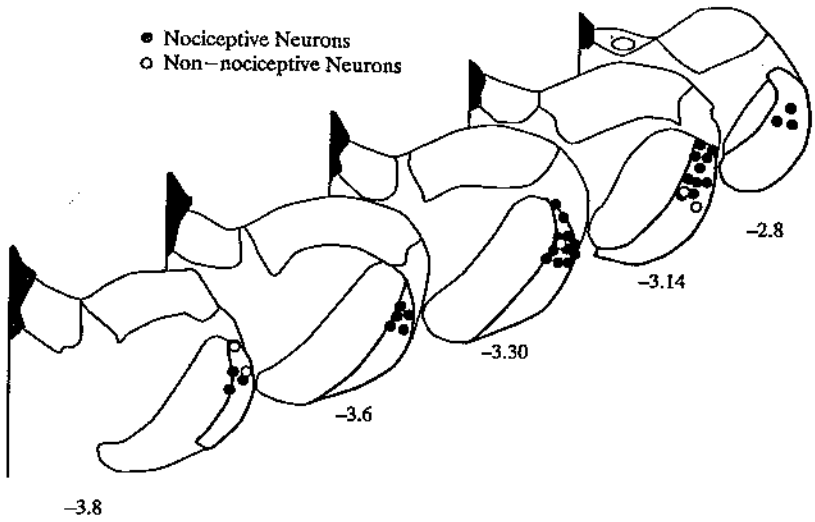

Figure 1. Reconstruction of the anatomical locations of neurons recorded in this study. Sections through the right diencephalon were redrawn according to the atlas of Paxinos and Watson (1986). *Heavy black lines* outline the VPL nucleus of the thalamus. *Filled circles* show the location of nociceptive neurons, whereas *open circles* show the location of non-nociceptive neurons included in the study.

The mean slope of the preinjection (log–log) stimulus–response function of the 32 nociresponsive neurons examined was 0.84 (n = 32, r = 0.990) (Fig. 2*B*). This population of neurons may be classified as wide dynamic range type, because each cell showed a graded response over a wide range of stimulus intensities including noxious levels.

Lack of effect of vehicle and the cannabinoid receptor-inactive enantiomer WIN 55,212-3

Neither the vehicle nor WIN 55,212-3, the cannabinoid receptor-inactive enantiomer, altered the responses of the nociceptive neurons in the VPL to the noxious pressure stimulus (Fig. 3*A*). Repeated-measures ANOVA was performed to compare the mean response with 10 presentations of the stimulus (at various pressure levels including zero pressure during pre- and poststimulus periods) before and after injection of enantiomer or vehicle. Separate analyses failed to reveal any effects of either the vehicle or WIN 55,212-3.

Effects of WIN 55,212-2 on nociceptive neurons

ANOVA revealed that WIN 55,212-2 (0.0625, 0.125, 0.25 mg/kg, i.v.) decreased both spontaneous and noxious stimulus-evoked activity ($F_{(3,19)}$ = 12.88, $p < 0.0001$) (Fig. 3*B*,*C*). As shown in Figure 4, there were dose-dependent differences in the times that the drug significantly suppressed nociceptive stimulus-evoked activity.

Although WIN 55,212-2 decreased both spontaneous and evoked firing rates, the drug produced a greater effect on evoked firing (Fig. 5). ANOVA compared the rate of spontaneous firing after the vehicle and various doses of WIN 55,212 during the (1 sec) prestimulation period to the firing rate during the last (most noxious) second of stimulation. The significant interaction between drug treatment and stimulation condition in this analysis revealed a larger effect of the drug on noxious stimulus-evoked firing than on spontaneous firing ($F_{(3,19)}$ = 11.41, p = 0.0002) (Fig. 5).

WIN 55,212-2 produced dose-dependent changes in the slopes of the stimulus–response functions (Fig. 6). The slope of the stimulus–response function after injection of the lowest dose of the drug was not significantly different from that observed after the vehicle. However, the slopes of the mean stimulus–response functions obtained after the doses of 0.125 and 0.25 mg/kg WIN 55,212-2 were more shallow than those obtained after the vehicle ($p < 0.05$ for both comparisons). The slopes of the mean stimulus–response functions after the two higher doses did not differ from each other. At the highest dose of the drug (0.25 mg/kg), the slope stimulus–response function was only 0.1 ($p > 0.05$), and its 95% confidence limits included zero (i.e., a horizontal line), indicating that the neurons were unable to encode stimulus strength with increasing firing rates.

Morphine (0.5 mg/kg, i.v.) also decreased the responsiveness of neurons in VPL ($F_{(1,4)}$ = 13.9, p = 0.02) (Fig. 7). Like the cannabinoid, morphine produced a marked downward shift of the stimulus–response function. As shown in Figure 7, the effect of morphine (0.5 mg/kg) was very similar to that produced by 0.0625 mg/kg WIN 55,212. The slopes of the stimulus–response functions for these two treatments did not differ.

Lack of effect of WIN 55,212-2 on non-nociceptive mechanosensitive neurons in VPL

A matched-pairs t test was used to compare firing evoked in non-nociceptive cells by the non-noxious stimulus with that evoked by the noxious pressure stimulus. This analysis failed to reveal a significant difference between firing rate during non-noxious and noxious stimulation [T_4 = 1.34, $p > 0.05$, nonsignificant (ns); mean firing rate ± SEM: 4.1 ± 1.1 vs 2.6 ± 0.8 Hz for non-noxious and noxious levels of evoked activity, respectively]. The cannabinoid failed to suppress activity evoked by the non-noxious tap stimulus ($F_{(1,4)}$ = 1.24, p = 0.33, ns; mean firing rate ± SEM: 4.1 ± 1.1 vs 4.6 ± 1.0 Hz for pre- and postinjection mean levels of evoked activity, respectively) (see Fig. 10). In fact, the drug appeared to increase the signal-to-noise ratio of some cells (e.g., Fig. 8); i.e., background firing slowed, and the cells exhibited a sharper response to the tap stimulus.

Lack of effect of WIN 55,212-2 on paw temperature in anesthetized rats

Repeated-measures ANOVA conducted on the skin temperature readings obtained over time from the plantar surfaces of the hindpaws of animals treated with WIN 55,212-2 (0.25 mg/kg, i.v.) (n = 3) failed to reveal any drug effects on skin temperature. The overall change in mean pre- and postinjection skin temperature was only 0.6°C (mean ± SEM: 31.7 ± 0.3 vs 31.1 ± 0.3°C for pre- and postinjection, respectively).

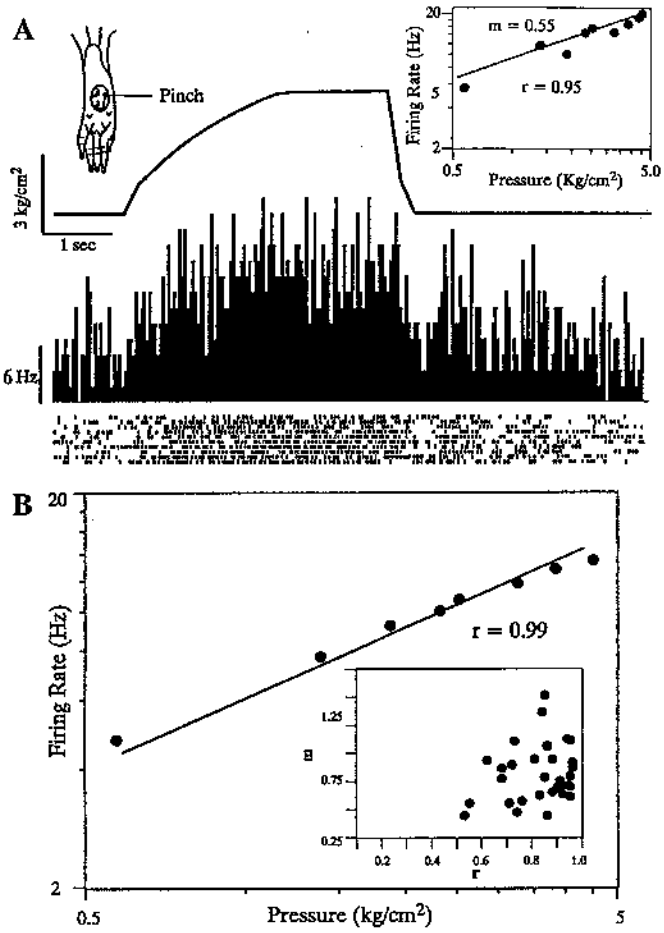

Figure 2. *A*, Example of a PSTH for a single VPL neuron before drug treatment. *Top left*, Site of application of the noxious pressure stimulus. *Top center*, Level of the applied pressure in register with the PSTH below. *Top right*, The stimulus–response function (pressure vs firing rate) of this cell. *Bottom*, Raster plot showing firing pattern of neuron. A *single dot* represents the occurrence of a single action potential; *rows* represent successive applications of the stimulus. The increased density of dots during the stimulus illustrates the increased firing rate of the neuron. *B*, Mean predrug stimulus–response functions for all nociceptive neurons included in the study ($n = 32$). The data used in the regression were derived by averaging the preinjection stimulus-evoked firing rate during the graded portion of the stimulus to obtain estimates of the mean firing rate at eight (mean) levels of pressure. The logarithms of the mean firing rates and pressures were subjected to linear regression. This yielded a slope of 0.84 for the mean stimulus–response function and a correlation coefficient of 0.99. *Inset*, Scatter plot of slope of predrug stimulus–response function versus correlation coefficient for all nociceptive neurons used in the study. All neurons exhibited a correlation coefficient of at least 0.5; the slopes of the $\log_{10}$ transforms of stimulus–response functions in untreated animals ranged from 0.2 to 1.25.

Tail skin temperature changes in awake rats after injections of WIN 55,212-2 and vehicle

Repeated-measures ANOVA on the pre- and postinjection measures of tail skin temperature in awake rats revealed neither a significant overall difference between the effects of vehicle and WIN 55,212-2 nor a significant interaction effect (drug treatment across measurement times). However, there was a significant effect of testing time ($n = 13$) ($F_{(38,418)} = 3.13, p = 0.03$) attributable to a short-lived but consistent increase in temperature 3 min after injection (mean increase = 2.8°C). The lack of any significant differences or interactions involving drug groups, together with the rapid onset (3 min) and brief duration of the effect (return to within 0.87°C of mean baseline temperature within 3 min), suggests that this was a nonspecific effect of the intravenous injection in the tail vein.

Relationship between antinociceptive and electrophysiological effects

To determine the magnitude and duration of the effects of WIN 55,212-2 (0.25 mg/kg, i.v.) on paw withdrawal, a repeated-measures ANOVA, blocked by time (12 min/block), was performed. Consistent with previous findings (Hohmann et al., 1995), baseline withdrawal responses occurred at a mean pressure of 3.0 ± 0.3 kg/cm² in sedated rats. WIN 55,212-2 increased the pressure required to elicit a nociceptive paw-withdrawal reflex to 4.0 ± 0.3 kg/cm² ($F_{(5,50)} = 3.00, p < 0.05$). Ten minutes after drug administration, three of six animals failed to respond to the pressure stimulus before it was terminated at its maximal pressure of 4.6 kg/cm². Antinociception was observed in this test for >30 min (Table 1). Thus, the duration of the antinociceptive effect was very similar to the duration of the change in responsiveness of nociceptive neurons in the VPL (Fig. 9*A*). WIN 55,212-2 (0.0625 to 0.5 mg/kg, i.v.) also produced dose-dependent elevations in tail-flick latencies (Fig. 9*B*). Furthermore, a comparison of the effect of WIN 55,212-2 on firing rate and tail-flick latencies revealed a strong correlation ($r = 0.99$) between the inhibition of noxious stimulus-evoked activity at different doses of the drug and the increase in tail-flick latencies.

Motor effects of WIN 55,212-2 in awake animals

As expected from previous research (Loewe, 1946; Gough and Olley, 1977; Ueki, 1980), WIN 55,212-2 (0.25 mg/kg, i.v.) induced motor impairment in both tests of motor function. WIN 55,212-2 (0.25 mg/kg, i.v.) produced a significant decrease in running time in the rotorod test ($F_{(4,56)} = 4.25, p < 0.01$). Likewise, four of six animals treated with the drug showed maximum effects (60 sec cutoff latency) in the test of catalepsy, and all animals showed increased descent latency compared with the predrug injection ($p = 0.016$).

The main finding of interest in these experiments was the shorter duration of the effect of the drug on motor function (catalepsy and rotorod) than on responses to noxious stimulation (paw withdrawal, tail flick, and noxious stimulus-evoked firing).

Table 1. Comparison of the time course of the 0.25 mg/kg dose of WIN 55,212-2 on motor, nociceptive, and electrophysiological activity

Measure	Minutes to recovery to within 20% of baseline value (mean ± SEM)	Anesthesia
Catalepsy	4.5 ± 1.5	None
Rotorod	13 ± 5.6	None
Tail flick	28 ± 2.1[a,b]	None
Noxious stimulus-evoked neuronal activity	35 ± 7.6[a]	Surgical
Paw withdrawal	38 ± 8.5[a,b]	Sedation

ANOVA revealed a significant difference between the mean recovery times for each measure. Comparisons between measures were determined by Bonferroni *post hoc* comparison using pooled variance.

[a] Time course significantly different ($p < 0.05$, Newman–Keuls test) from rotorod and catalepsy.

[b] Time course not significantly different from cannabinoid-induced-inhibition of noxious stimulus-evoked neuronal activity.

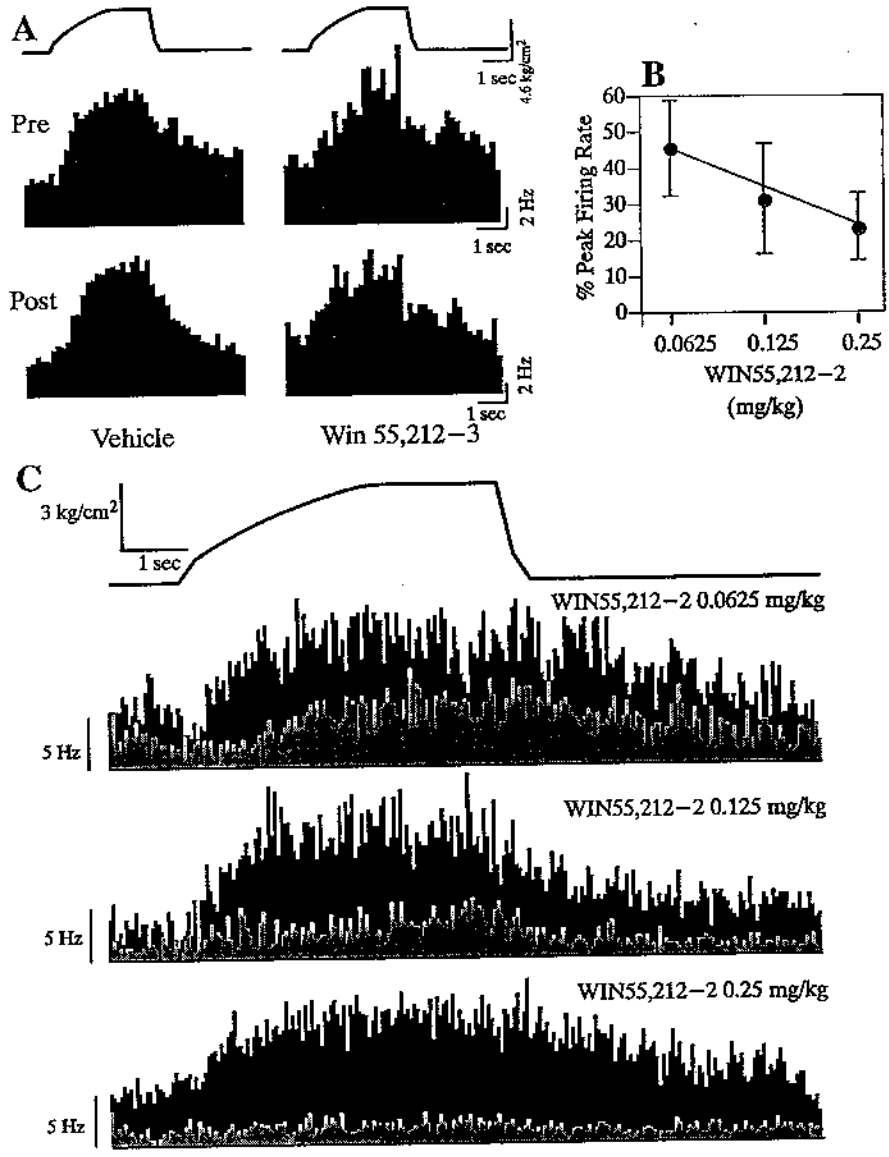

Figure 3. *A*, Spontaneous and evoked firing before and after administration of the vehicle ($n = 6$) or the cannabinoid receptor-inactive compound WIN 55,212-3 ($n = 4$). *Top*, Level of the applied pressure in register with the PSTHs below. *Middle* and *bottom*, Average of 10 predrug (*middle*) and 10 postdrug (*bottom*) firing rate histograms. Neither the vehicle nor the inactive enantiomer WIN 55,212-3 produced an effect on spontaneous or evoked firing. *B*, Inhibition of peak evoked-activity by various doses of WIN 55,212-2. *C*, Dose-dependent decreases in the responsiveness of VPL neurons to a noxious pressure stimulus after administration of the cannabinoid agonist WIN 55,212-2. *Top center*, *Line* over the histograms showing the pressure applied to the paw at various times. *Bottom three histograms*, *Black histograms* represent the group mean response during 10 min before drug administration; *gray histograms* represent the mean response during 10 min after administration WIN 55,212-2.

One-way ANOVA revealed significant differences in the duration of the effects of WIN 55,212-2 on the various measures, which were determined for each animal as the amount of time during which responses deviated by >20% from the individual's mean predrug response ($F_{(4,27)} = 6.00, p = 0.0014$) (Fig. 10). Comparisons between all pairs of means using the Newman–Keuls *post hoc* test revealed that the duration of the drug effect did not differ between the measures of motor function (catalepsy and rotorod). Likewise, the duration of the drug effect did not differ among responses to different types of noxious stimulation (tail flick, paw withdrawal, electrophysiology). However, the duration of the effect of the drug on each measure of motor function was shorter than the effect on each measure of responsiveness to noxious stimuli (Table 1) ($p < 0.05$ for all comparisons). These differences in time course were independent of the state of anesthesia, because the duration of drug-induced changes in sensory responses were not significantly different in awake (tail flick), sedated (paw withdrawal), and deeply anesthetized (electrophysiology) rats.

DISCUSSION

The cannabinoid receptor agonist WIN 55,212-2 inhibited stimulus-evoked activity of nociceptive neurons in the VPL. It appears that the drug had a selective effect on nociceptive neurons, because it produced greater inhibition of noxious stimulus-evoked firing than spontaneous firing and failed to alter the activity of non-nociceptive mechanosensitive neurons in the VPL. It appears that these effects were mediated by cannabinoid receptors, because they were potent, dose-dependent, reversible, and not produced by the receptor-inactive enantiomer or the vehicle.

Previous studies from our laboratory have provided evidence for a role of cannabinoids in the processing of nociceptive information. For example, systemic administration of WIN 55,212-2 suppressed both noxious stimulus-evoked expression of c-fos in the spinal dorsal horn and pain-related behavior (Tsou et al., 1996). The role of cannabinoid receptors was suggested by the lack of effect in animals rendered tolerant to cannabinoids and by the lack of effect of the receptor-inactive enantiomer WIN 55,212-3. A separate study showed that WIN 55,212-2 selectively

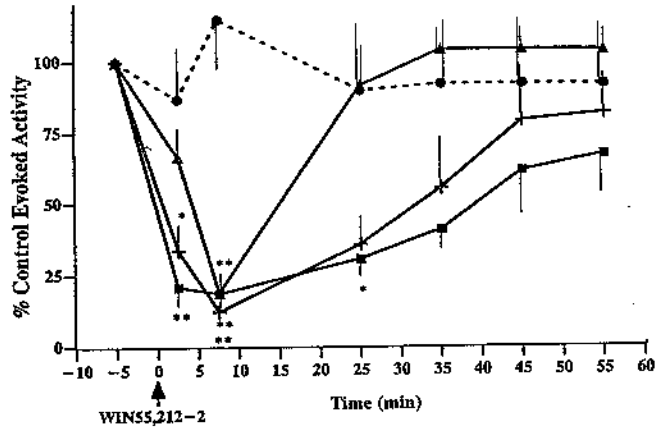

Figure 4. Evoked firing over time before and after intravenous administration of the vehicle or a dose of WIN 55,212-2. Vehicle (*filled circles*) injections had no effect on noxious stimulus-evoked activity. The cannabinoid agonist produced marked dose-dependent differences in evoked firing during the later periods: 0.0625 mg/kg (*filled triangles*), 0.125 mg/kg (*plus signs*), 0.25 mg/kg (*filled squares*) WIN 55,212-2. *Asterisks*, Significantly different from control: $*p < 0.05$; $**p < 0.01$ (Dunnet test). See text for additional details on the statistical analysis of this experiment.

inhibited noxious stimulus-evoked responses of wide dynamic range neurons in the dorsal horn of the spinal cord without affecting the responses of non-nociceptive neurons (Hohmann et al., 1995). The finding in this study that similar effects occur in the thalamus supports our previous work and suggests that at least some of the dorsal horn neurons recorded previously were spinothalamic tract neurons.

As expected from previous research (Tsou and Chang, 1964; Calvillo et al., 1974; Kitahata et al., 1974; Hill and Pepper, 1978; Benoist et al., 1983; Homma et al., 1983), morphine suppressed noxious stimulus-evoked firing in VPL. A comparison of WIN 55,212-2 with morphine indicates that the effects of these compounds were very similar. Both WIN 55,212-2 and morphine reduced non-noxious and noxious stimulus-evoked activity in VPL

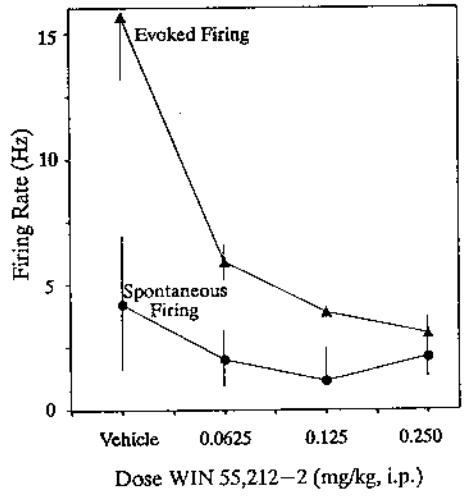

Figure 5. Effect of various doses of WIN 55,212-2 on spontaneous and noxious stimulus-evoked activity. Firing rate (averaged over 10 stimulus presentations after drug injection) during the 1 sec preceding the stimulus (*Spontaneous Firing*) and the last (most noxious) 1 sec of stimulation. ANOVA revealed a significantly greater effect on evoked compared with spontaneous firing. *Vertical lines* represent SEMs.

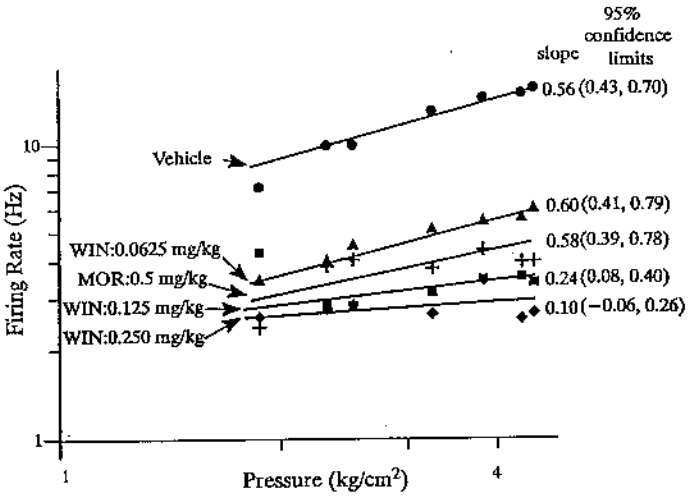

Figure 6. Mean stimulus–response functions after administration of vehicle (*filled circles*), WIN 55,212-2 [0.0625 mg/kg (*filled triangles*), 0.125 mg/kg (*filled squares*), or 0.25 mg/kg (*filled diamonds*)] or morphine (*plus signs*). The lowest dose of the drug (0.0625 mg/kg) reduced the overall firing but did not alter the slope of the stimulus–response function. Morphine (0.5 mg/kg) showed a similar effect. Significant decreases in slope occurred at higher doses of WIN 55,212-2 (0.125 and 0.25 mg/kg). Postinjection slope values and confidence limits for estimation of β were determined as described in Materials and Methods.

neurons, and both compounds produced a similar maximum effect: nearly complete inhibition of noxious stimulus-evoked firing. The similar efficacy of the two classes of compounds observed in this study supports previous behavioral studies that found comparable analgesic efficacy of the two classes of compounds (Buxbaum, 1972; Bloom, 1977; Jacob et al., 1981). Although cannabinoids and opiates produce similar effects on nociception, there is little evidence for a direct interaction of cannabinoids with opiate receptors (for review, see Martin, 1986). Nonetheless, it does appear that the two systems may share some neural substrates at either a cellular level or via common actions on neurochemicals known to modulate pain perception (Gascon and Bensemana, 1975; Welch et al., 1995).

The suppressive effect of WIN 55,212-2 on noxious stimulus-evoked activity does not represent an anesthetic effect of the drug,

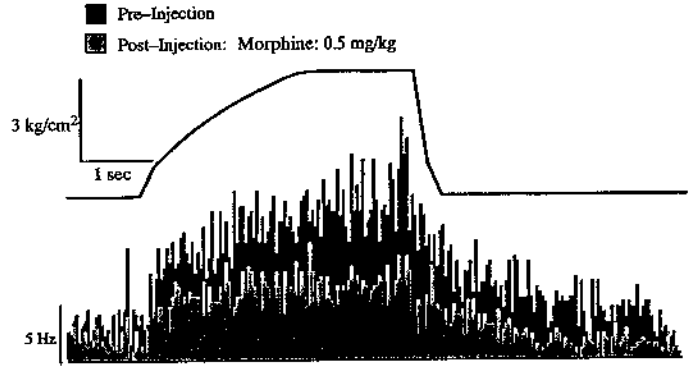

Figure 7. PSTH illustrating the effect of morphine (0.5 mg/kg, i.v.) on stimulus-evoked activity in the VPL. *Top center*, *Line* over the histograms showing the pressure applied to the paw at various times. *Bottom*, *Black* histograms represent the group mean response during 10 min before drug administration; *gray* histograms represent the mean response during 10 min after administration of morphine. As shown, morphine markedly reduced stimulus-evoked activity.

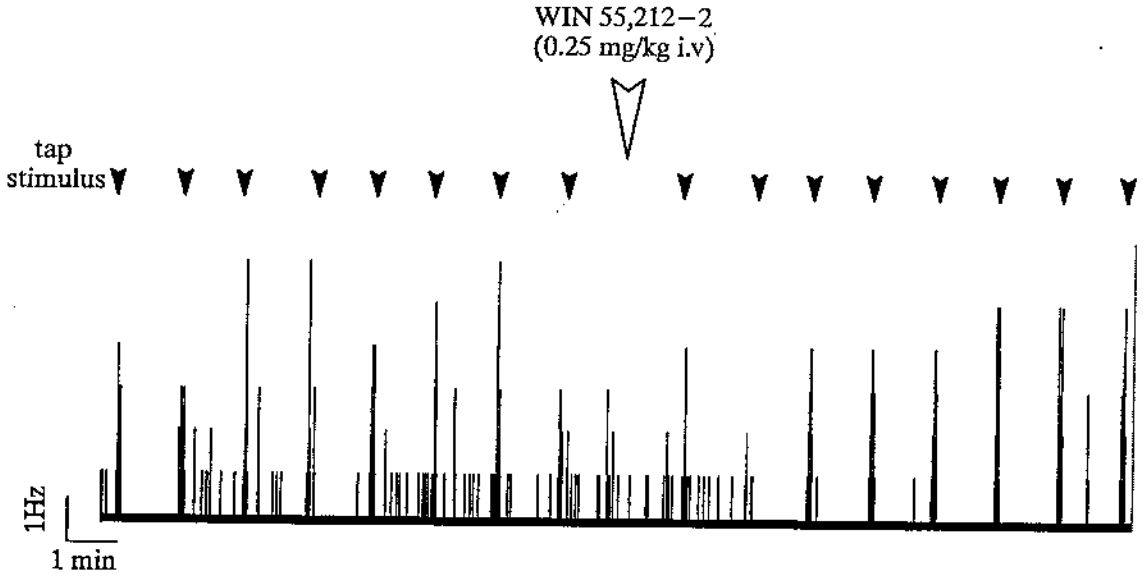

Figure 8. Firing rate histogram of mechanosensitive non-nociceptive neuron before and after administration (*open triangle*) of WIN 55,212-2 (0.25 mg/kg, i.v.). The cannabinoid agonist did not significantly reduce the responses of mechanosensitive neurons to tap stimuli (*filled triangles*) administered every minute. Note the reduction in spontaneous activity after the drug and the more robust response to the stimulus in this cell after the drug.

because it failed to alter the evoked activity of non-nociceptive mechanoreceptive neurons in the same area. Furthermore, the cannabinoid agonist had a greater effect on evoked firing than on spontaneous firing. The lack of effect of the drug on non-nociceptive mechanosensitive neurons in the VPL is consistent with the previous failure of a cannabinoid to affect non-nociceptive mechanosensitive neurons in the spinal dorsal horn (Hohmann et al., 1995). These findings suggest that the effect of the cannabinoid on nociceptive neurons is selective.

The drug-induced decrease in electrophysiological and behavioral sensitivity to noxious stimuli outlasted the impairment of motor function, providing evidence for a dissociation between cannabinoid-induced changes in motor and pain-related behavior. Thus, profound analgesia and suppression of electrophysiological responses to noxious stimuli occurred during periods when motor impairment was minimal. Because the potency of WIN 55,212-2 in tests of analgesia is much greater than its potency in tests of motor function (Abood and Martin, 1992), one would expect the analgesic effect to outlast the motor impairment. These findings provide a strong basis for the conclusion that the actions of WIN 55,212-2 on pain and movement are mediated by separate processes and suggest that the decreased behavioral responsiveness to noxious stimuli does not result merely from a disruption of motor function. This conclusion is supported by studies that demonstrated antinociceptive actions of cannabinoids in a test (vocalization to shock) that does not require a gross motor response (Ferri et al., 1981, 1986).

The effects observed in these experiments cannot be accounted for by changes in skin temperature, because skin temperature was unchanged by the drug under the conditions used in the electrophysiological experiments. Presumably, the automatic heating circuitry prevented any such effects. In the behavioral experiments, the change in tail-flick latency was not attributable to a change in tail temperature, because the small change observed lasted for only one-tenth the duration of the change in tail-flick latency and could not be attributed to the drug. Moreover, the increase in tail temperature observed would be expected to lead to an artifactual decrease in tail-flick latency (an apparent hyperalgesic state) (Tjolsen et al., 1989), an effect opposite to the observed increase.

The relationship between the electrophysiological, antinociceptive, and motor effects of WIN 55,212-2 also cannot be accounted for by the level of anesthesia used in the experiments. No significant differences were found among the time courses of the changes in tail-flick latency (awake), paw-withdrawal latency (sedation), or the electrophysiological effects (surgical anesthesia). However, the duration of the effect on the tail-flick reflex (awake), paw-withdrawal reflex (sedation), and evoked firing (surgical anesthesia) was significantly greater than the duration of the effects on rotorod performance (awake) and catalepsy (awake). If the differences in time course were the result of anesthesia, one would expect catalepsy, rotorod, and tail flick to be similar; paw withdrawal intermediate; and noxious stimulus-evoked firing the longest. This clearly did not occur. Thus, the shorter duration of the cannabinoid effect on motor behavior compared with nociceptive responsiveness cannot be accounted for by a faster clearance of the drug during the waking state. These findings demonstrate that the differences in the time course of motor impairment compared with behavioral antinociception and thalamic electrophysiology reflect actions at a neural systems level rather than merely an artifact of anesthesia.

The site of action of WIN 55,212-2 was not investigated in this study; however, this is an important question for future investigations. Previous behavioral studies suggested that cannabinoid receptor-mediated antinociception is mediated by both spinal and supraspinal sites (Yaksh, 1981; Lichtman and Martin, 1991; Smith and Martin, 1991; Martin et al., 1993), consistent with the presence of cannabinoid receptors in brain and spinal areas that modulate the transmission of nociceptive information (e.g., the spinal dorsal horn and periaqueductal gray) (Herkenham et al., 1991). Recent work from this laboratory demonstrated that microinjections of a low dose of WIN 55,212-2 into the periaqueductal gray or dorsal raphe nucleus elevate tail-flick latencies (Martin et al., 1995). Kayser and co-workers (1983) reported that microinjections of morphine into these areas also depress noci-

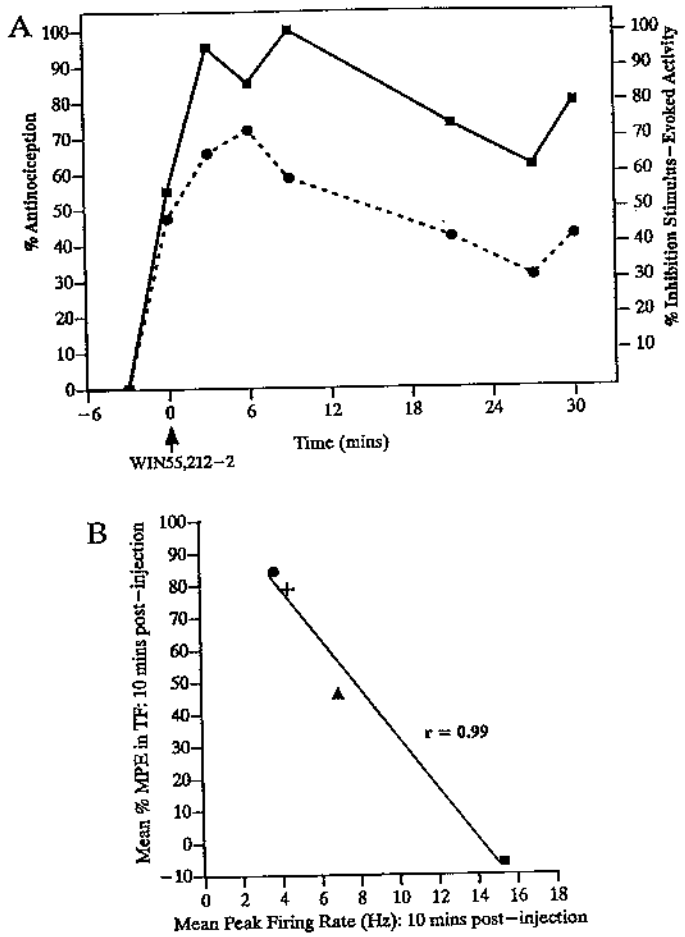

Figure 9. *A*, Time course of the antinociceptive and electrophysiological effects of WIN 55,212-2 (0.25 mg/kg, i.v.). Antinociception was assessed as the pressure at which rats (lightly anesthetized) exhibited a withdrawal response to a mechanical stimulus that increased in intensity over time, as described in the text. Data are presented as percent antinociception. For electrophysiology experiments (in separate animals under surgical anesthesia), the same mechanical stimulus was used to apply pressure to the contralateral hindpaw, whereas stimulus-evoked activity was recorded from individual neurons in the VPL. The effects of WIN 55,212-2 are presented as percent inhibition of stimulus-evoked activity relative to preinjection values. Note that the inhibition of paw withdrawal (*filled circles*) (as percent antinociception) parallels the inhibition of stimulus-evoked activity (*filled squares*) in VPL neurons. *B*, The relationship between inhibition of noxious-stimulus-evoked activity and inhibition of tail-flick reflex were determined using regression analysis. For each procedure, vehicle (*filled square*) or WIN 55,212-2 [0.0625 mg/kg (*filled triangle*), 0.125 mg/kg (*plus sign*), or 0.25 mg/kg (*filled circle*)] was administered to separate groups of animals. Tail-flick data (awake animals) are presented as mean %MPE during the 10 min after injection. Electrophysiology data are presented as mean peak firing rate during the 10 min after injection. The high correlation ($r = 0.99$) is indicative of a relationship between the behavioral and the electrophysiological responses.

ceptive responses of ventrobasal thalamic neurons. Thus, the periaqueductal gray and dorsal raphe are potential mediators of the effects we observed.

In summary, the results of this study demonstrate that cannabinoids reduce sensory transmission within an important ascending nociceptive pathway. These effects were mediated by cannabinoid receptors and followed a time course that was indistinguishable from that of the analgesic effects of the drug but different from that of the motor effects. The alteration in the

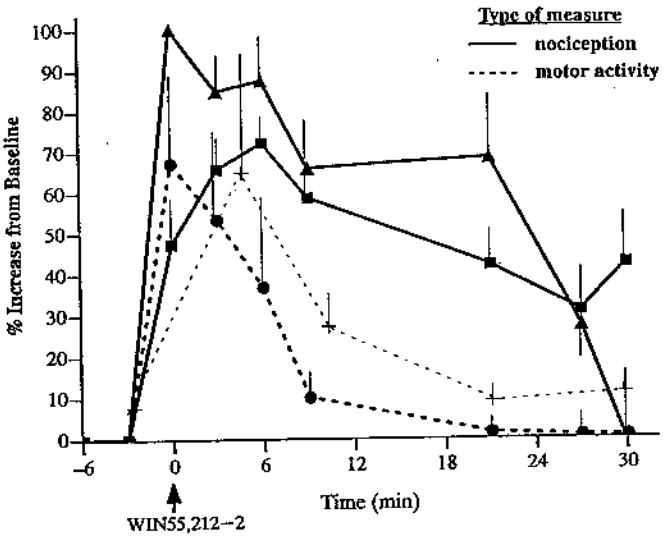

Figure 10. Effect of WIN 55,212-2 (0.25 mg/kg, i.v.) on two measures of nociception (*solid lines*) and two measures of motor activity (*dashed lines*). WIN 55,212-2 impaired motor function in tests of both ataxia (*plus signs*) and catalepsy (*filled circles*). However, the antinociceptive effects produced by WIN 55,212-2 in the paw-withdrawal (*filled squares*) and tail-flick (*filled triangles*) tests significantly outlasted the impairment of motor function, which suggests mediation of these effects by separate processes. Data are presented as percent increase from baseline.

stimulus–response functions of nociceptive neurons produced by WIN 55,212-2 was very similar to that produced by morphine, a powerful narcotic analgesic. These findings suggest a possible role of the endogenous cannabinoid anandamide in pain modulation. If this substance serves as a cannabinoid neurotransmitter, it would appear that one of its functions is to modulate pain transmission by decreasing the sensory responsiveness of neurons within the spinothalamic pathway. The circumstances under which anandamide is released and its site(s) of action remain important topics for future investigations.

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