EFFECTS OF NEUROTOXIC DESTRUCTION OF DESCENDING NORADRENERGIC PATHWAYS ON CANNABINOID ANTINOCICEPTION IN MODELS OF ACUTE AND TONIC PAIN SENSITIVITY

by

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(Under the Direction of Andrea G. Hohmann)

ABSTRACT

The effects of neurotoxic destruction of descending catecholaminergic projections on cannabinoid antinociception were examined in models of acute and tonic pain sensitivity. Intrathecal 6-hydroxydopamine depleted spinal norepinephrine (by ~85%) without altering levels of dopamine or serotonin. In the tail-flick test, the antinociceptive effect of WIN55,212-2 observed in lesioned rats was attenuated relative to sham-operated rats. WIN55,212-2 suppressed tonic pain behavior in the formalin test in sham-operated rats during phase 2 (15-60 min). In lesioned rats, WIN55,212-2 suppressed pain behavior during phase 1 (0-9.9 min) and phase 2A (10-39.9 min) but not during phase 2B (40-60 min). WIN55,212-2 suppressed formalin-evoked Fos protein expression in the lumbar dorsal horn of sham-operated rats but not in lesioned rats. These data suggest that cannabinoids produce antinociception, in part, by modulating descending noradrenergic systems and support a differential involvement of noradrenergic projections to the spinal cord in cannabinoid modulation of acute versus tonic pain sensitivity.

INDEX WORDS: anandamide, nociception, Fos, formalin test

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DEDICATION

This thesis is dedicated to my parents and grandfather, Maria S. Cardenas, Jose G. Gutierrez, and Jose G. Gutierrez (Abuelito). Without their support I would not be the person that I am today. To my “abuelito”, thanks for always being kind and loving.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1: INTRODUCTION

Cannabinoids produce antinociception through spinal and supraspinal mechanisms. The potency of cannabinoids in producing antinociception correlates with the ability of cannabinoids to suppress noxious stimulus-evoked activity in pain-sensitive neurons (Hohmann, Tsou, & Walker, 1998; Martin, Hohmann, & Walker, 1996; Meng, Manning, Martin, & Fields, 1998). In the tail-flick test, antinociception induced by the cannabinoid agonist Δ^9-tetrahydrocannabinol (Δ^9-THC) is inhibited by spinal administration of the a_2-noradrenergic receptor antagonist yohimbine (Lichtman & Martin, 1991a). By contrast, the nonspecific serotonin receptor antagonist methysergide did not alter cannabinoid antinociception in this model of acute pain (Lichtman & Martin, 1991a). These data implicate the involvement of a spinal a_2-adrenergic link in cannabinoid antinociception, and are consistent with the hypothesis that cannabinoids modulate descending noradrenergic projections, in part, to suppress acute pain.

The antinociceptive (Lichtman & Martin, 1991b) and electrophysiological (Hohmann, Tsou, & Walker, 1999a) effects of systemically administered cannabinoids are attenuated following spinal transection. These studies are consistent with direct evidence implicating a role for supraspinal sites in cannabinoid modulation of nociception (Martin, Tsou, & Walker, 1998; Meng et al., 1998; Monhemius, Azami, Green, & Roberts, 2001; Walker, Huang, Strangman, Tsou, & Sanudo-Pena, 1999). These supraspinal sites include neural substrates also known to mediate mu opioid analgesia (Fields & Basbaum, 1999). Lesions of the dorsolateral funiculus (DLF)

virtually eliminate the antinociceptive effect of morphine in the tail-flick test. In addition, DLF lesions produce concentration-dependent changes in the behavioral response to formalin in a rat model of tonic pain, the formalin test (Abbott, Hong, & Franklin, 1996). Intraplantar administration of formalin induces a biphasic pattern of pain-related behavior. The biphasic response is characterized by an early acute period (Phase 1), a brief quiescent period, and a second phase of sustained "tonic" pain behavior (Phase 2). Neurotoxic destruction of descending noradrenergic pathways has also been reported to alter morphine antinociception in the formalin test (Martin, Gupta, Loo, Rohde, & Basbaum, 1999).

C-*fos* immunocytochemistry has been used to examine the laminar distribution of spinal neurons modulated by cannabinoids in animal models of tissue injury and inflammation (Hohmann, Tsou, & Walker, 1999b; Martin, Loo, & Basbaum, 1999; Nackley, Makriyannis, & Hohmann, 2003; Nackley, Suplita II, & Hohmann, 2003; Tsou et al., 1996). Though not a specific marker for pain, noxious stimuli induce expression of the c-*fos* proto-oncogene in both the superficial and deep layers of the dorsal horn (Bullitt, 1990; Hunt, Pini, & Evan, 1987; Presley, Menetrey, Levine, & Basbaum, 1990)–neural sites implicated in the processing of nociceptive information. Previous studies have demonstrated that cannabinoids suppress tonic pain behavior and Fos protein expression evoked by intraplantar administration of formalin (Hohmann et al., 1999b; Tsou et al., 1996). Thus, quantitative estimates of Fos-like immunoreactive (FLI) cells, evoked by noxious peripheral stimulation, (Hunt et al., 1987) represent an index of neuronal activation that is independent of motor responses typically measured in behavioral assessments of antinociception.

The present studies were conducted to examine the contribution of descending noradrenergic projections to the spinal cord to cannabinoid antinociception in models of acute and tonic pain sensitivity. Sensitivity to acute and tonic pain was assessed in the tail-flick and formalin tests, respectively. Cannabinoid modulation of formalin-evoked Fos protein expression was examined in sham-operated rats and in rats treated intrathecally with the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) to deplete spinal norepinephrine. Cannabinoid modulation of formalin-evoked neuronal activity was evaluated at the level of the spinal dorsal horn in the same animals used in the behavioral assessments of cannabinoid antinociception.

CHAPTER 2: MATERIALS AND METHODS

Subjects

Seventy-one male Sprague-Dawley rats (300-350g Charles River Laboratories, Wilmington, MA) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983).

Surgery

Intrathecal (i.t.) catheters (PE-10 tubing; Clay Adams, Sparks, MD) were surgically implanted (Yaksh & Rudy, 1976) 3 to 5 days prior to performing 6-hydroxydopamine (6-OHDA) or sham lesions. Rats that showed signs of motor dysfunction were eliminated from the experiment.

Drug preparation and administration

Chemicals (6-OHDA, pargyline and WIN55,212-2) were obtained from Sigma Aldrich (St. Louis, MO). The catecholamine neurotoxin 6-OHDA (20 µg) was dissolved in 0.9% saline containing 0.1% ascorbic acid and delivered intrathecally in a volume of 10 µl. Pargyline (20 mg/kg) was dissolved in saline and administered intraperitoneally (i.p.) 10 min prior to intrathecal administration of 6-OHDA (Archer, Jonsson, Minor, & Post, 1986; Janss, Jones, & Gebhart, 1987; Post et al., 1987). WIN55,212-2 (5 or 10 mg/kg i.p.) was dissolved in vehicle containing emulphor, ethanol and saline (1:1:8 and 1:1:18) in the tail-flick and formalin tests, respectively.

HPLC monoamine assays

Spinal cord and brain levels of monoamines were analyzed using a modified method of Soares (Soares et al., 1999). High performance liquid chromatography (HPLC) with electrochemical detection was used to quantify norepinephrine (NE), serotonin (5-HT), and dopamine (DA) in lumbar spinal cord and brain of lesioned and sham-operated rats. Spinal cord and whole brain samples were homogenized in mobile phase (0.1 M NaH_2PO_4, 0.2 mM 1-octanesuflonic acid, 0.1 mM EDTA, in 5% acetonitrile, pH 3.1) and centrifuged (1000g at 4ºC for 10 min). The supernatant (600 µl) was combined with 3,4-dihydroxy-benzylamine (0.4 ng/20 µl DHBA), the internal standard. The eluate (70 µl) was directly injected onto the separation column (Phenomenex Synergi 4µ MAX-RP; 150 x 4.6 mm). A refrigerated autosampler (Waters 717-plus, Milford, MA) and a pump (Waters 510, Milford, MA) were used in conjunction with a pulsed electrochemical detector (Waters 464). The electrochemical detector functioned with a glassy carbon working electrode set at 5 nA with respect to an Ag/AgCl reference electrode. Chromatograms were monitored, recorded and analyzed with Millennium 32 software (version 3.2, 1999; Millipore, Milford, MA). Peaks for NE, DA, and 5-HT were quantified by height and compared to daily standard lines fitted by regression analysis to a series of four to five standards analyzed throughout the day. Correlation coefficients for the daily standard lines always exceeded .98.

Assessment of acute pain sensitivity

Withdrawal responses to thermal stimulation of the tail were assessed using the tail-flick test (D'Amour & Smith, 1941). A 10-sec cut off latency was used to prevent tissue damage. Rats were habituated to restraining tubes for 15 min prior to testing. After

establishing stable baseline responses to thermal stimulation of the tail, the cannabinoid agonist WIN55,212-2 (5 or 10 mg/kg i.p.) or vehicle was administered to sham-operated and lesioned rats. Tail-flick latencies were assessed every 4 min for 1 h and then at 10 min intervals for an additional 30 min. Rats were killed by decapitation immediately after testing. Lumbar spinal cords and brains were extracted to quantify depletion of NE induced by intrathecal administration of 6-OHDA

Assessment of tonic pain sensitivity

The formalin test was used to examine the behavioral response to a persistent noxious stimulus in 6-OHDA-lesioned and sham-operated rats. Rats were acclimated to the test apparatus twice before the actual test day. Each rat was placed in an elevated clear plexiglass chamber with a 45° mirror placed underneath to allow for an unobstructed viewing of the rats' paws. WIN55,212-2 (5 mg/kg i.p.) or vehicle was administered 10 min prior to a unilateral intraplantar injection of formalin (2.5%, 50 µl). Pain behavior was quantified in 5-min bins over 60 min using a QBASIC program developed by Dr. K.B.J. Franklin (McGill University, Montreal, Canada). Weighted pain scores were calculated to represent the time spent licking, lifting and favoring the injected paw for each bin (Abbott, Franklin, & Westbrook, 1995; Coderre, Fundytus, McKenna, Dalal, & Melzack, 1993; Dubuisson & Dennis, 1977) where a score of 0 = normal weight bearing of the injected paw or no pain, 1 = favoring or resting the injected paw lightly on the floor, 2 = lifting the injected paw and 3 = licking or biting of the injected paw (Dubuisson & Dennis, 1977). This method of pain rating has been validated as superior to any single measure (Abbott et al., 1995; Coderre et al., 1993). For the purpose of data analysis, the second phase was further subdivided into phase 2A (10-39.9 min) and phase

2B (40-60 min), representing the onset and offset of the late phase, respectively, as described previously (Malmberg & Yaksh, 1992).

Immunocytochemistry

Two hours following intraplantar administration of formalin, rats were deeply anesthetized with sodium pentobarbital (65 mg/kg i.p.). Rats were perfused intracardially with ice-cold 0.1M phosphate-buffered saline (PBS) followed by fixative (10% formalin in 0.1M phosphate buffer) at a rate of 20 ml/min. The lumbar-sacral segment of the spinal cord was removed, cryoprotected and embedded in matrix at -21ºC. Transverse sections (40 µm) of the spinal cord were cut at the L4-L5 level using a cryostat. Alternate floating sections were collected in 0.1M PBS. The sections were washed twice with the same buffer and immersed in 0.3% H_2O_2 (30 min). Sections were pretreated with 3% normal goat serum (2 h) to block non-specific binding and then incubated with rabbit polyclonal Fos protein antibody (1:10,000 for 48 h at 0ºC). Fos-like immunoreactivity was visualized by the avidin-biotin-peroxidase method, (Hsu, Raine, & Fanger, 1981) using diaminobenzidine (0.05%) as the chromagen. The sections were mounted onto gelatin-subbed slides, air-dried and coverslipped. Spinal cord sections from the different experimental conditions were processed concurrently to control for variability in staining intensity across experiments. The specificity of the immunostaining was verified by preabsorbtion of the antibody with the peptide antigen and by omission of the primary antibody from the immunostaining protocols. Our control experiments have previously demonstrated that the placement of the intrathecal catheter alone failed to induce Fos in the absence of intraplantar formalin (Hohmann et al., 1999b).

Quantification of Fos-like immunoreactive cells

Three sections from L4-L5 qualitatively exhibiting the greatest number of labeled cells were selected from each rat. The selection of sections and quantification of the number of Fos-like immunoreactive (FLI) cells was performed by an investigator blind to the experimental condition (Nackley, Makriyannis et al., 2003; Nackley, Suplita II et al., 2003; Tsou et al., 1996). All cells expressing Fos-like immunoreactivity were counted, regardless of staining intensity. For each rat, the total number of cells was recorded as well as the subtotal in four cytoarchitectonically-defined subdivisions of the spinal gray matter. The subdivisions used were the superficial laminae (laminae I and II), the nucleus proprius (laminae III and IV), the neck of the dorsal horn (laminae V and VI) and the ventral horn (laminae, VII, VIII, IX, and X). The number of FLI cells in each subdivision was counted twice to ensure accuracy. Using these methods, means of our separate determinations typically differ by less than 2% (Nackley, Makriyannis et al., 2003; Nackley, Suplita II et al., 2003).

Statistical analysis

Behavioral data were analyzed by repeated measures Analysis of Variance (ANOVA) and Tukey-Kramer post hoc tests. The Greenhouse-Geisser correction was applied to all repeated factors (Greenhouse & Geisser, 1959). ANOVA was subsequently used to evaluate experimental differences in tonic pain behavior during phase 1 (0-9.9 min) as well as during each component of the late phase of formalin-induced pain behavior, i.e. phase 2A (10-39.9 min) and phase 2B (40-60 min) (Malmberg & Yaksh, 1992). The area under the curve (AUC), representing the distribution of pain behavior evoked by intraplantar formalin (Ruda, Ling, Hohmann, Peng, & Tachibana, 2000), was

calculated for each rat and subjected to ANOVA. The AUC thus provided an index of the total amount of formalin-induced pain behavior observed over the entire 60-min observation interval. Planned comparisons were used to assess the statistical significance of experimental differences in the total number of FLI neurons as well as the different subtotals in each spinal cord region. $P < 0.05$ was considered to be statistically significant.

CHAPTER 3: RESULTS

Assessment of monoamine levels

Intrathecal administration of 6-OHDA induced a selective depletion of NE ($F_{1, 38} = 52.79$, $P < 0.0002$) in rat lumbar spinal cord without affecting levels of DA or 5-HT. Brain levels of NE, DA and 5-HT did not differ in sham-operated or lesioned rats (Table 1).

Effects of WIN55,212-2 on acute pain sensitivity

Tail-flick latencies did not differ in sham-operated and lesioned rats prior to administration of WIN55,212-2 or vehicle. WIN55,212-2 produced a characteristic antinociceptive effect in the tail-flick test ($F_{2, 33} = 29.552$, $P < 0.0002$). ANOVA revealed that post-injection tail-flick latencies were lower in lesioned rats relative to sham-operated rats ($F_{1, 33} = 9.706$, $P < 0.004$; Fig.1A). WIN55,212-2-induced antinociception was attenuated in 6-OHDA-lesioned rats relative to sham-operated rats ($F_{2, 33} = 4.278$, $P < 0.03$). By contrast, tail-flick latencies in sham-operated and lesioned rats receiving vehicle did not differ from each other. Therefore, the two vehicle groups were pooled to form a single control group for all subsequent analyses.

Tail-flick latencies in sham-operated and lesioned rats receiving WIN55,212-2 differed from the control condition ($F_{4,34} = 19.564$, $P < 0.002$) and this effect was time dependent ($F_{36,306} = 2.449$, $P < 0.002$). In sham-operated rats, WIN55,212-2 produced dose-dependent antinociception relative to control conditions ($P < 0.003$ for all comparisons). Tail-flick latencies were lower in lesioned rats receiving the low or high

dose of WIN55,212-2 relative to sham-operated rats receiving the same doses of WIN55,212-2 ($P < 0.05$ for all comparisons). Tail-flick withdrawal latencies were greater in sham-operated rats receiving the high dose of the cannabinoid agonist (10 mg/kg i.p.) relative to all other groups ($P < 0.05$); (Fig.1B).

Effects of WIN55,212-2 on tonic pain sensitivity

Formalin-induced pain behavior, assessed using the weighted scores method, did not differ in sham-operated and lesioned rats receiving vehicle (Mean weighted pain score ± SEM: 1.78 ± 0.056 vs. 1.70 ± 0.055 for sham and lesioned rats). Moreover, the AUC of pain behavior was similar between groups (Mean AUC ± SEM: 103.0 ± 4.2 vs. 98.6 ± 6.06 weighted pain score-min for sham-operated and lesioned rats receiving the vehicle, respectively); therefore, the vehicle groups were pooled into a single control group for all subsequent statistical analyses.

Formalin-induced weighted pain scores were greater in the vehicle-treated control animals compared to sham-operated or lesioned rats receiving WIN55,212-2 ($F_{2, 29} = 9.007$, $P < 0.0010$; $P < 0.002$ for each comparison; Fig. 2A). The time course of pain behavior induced by intraplantar formalin differed as a function of group ($F_{22, 319} = 3.098$, $P < 0.004$). Group differences in formalin-induced pain behavior were observed during phase 1 ($F_{2,29} = 7.365$, $P < 0.003$) as well as phase 2A ($F_{2,29} = 11.185$, $P < 0.0004$) and phase 2B ($F_{2,29} = 3.996$, $P < 0.03$). In sham-operated rats, WIN55,212-2 suppressed formalin-evoked pain behavior during both phase 2A ($P < 0.007$) and phase 2B ($P < 0.01$) relative to control conditions. By contrast, in lesioned rats, WIN55,212-2 suppressed pain behavior relative to control conditions during phase 2A ($P < 0.0002$) but not during phase 2B (Fig. 2B). Phase 1 pain behavior was also greater in control rats

compared to lesioned rats receiving WIN55,212-2 ($P < 0.0008$). Four rats (2 sham-operated and 2 lesioned rats) receiving WIN55,212-2 showed no licking behavior throughout the observation interval. In addition, a profound hypoactivity was noted by visual inspection in one sham-operated rat treated with the cannabinoid.

The AUC of pain behavior was greater in the control group compared to sham-operated or lesioned rats receiving WIN55,212-2 ($F_{2,29}= 9.26$, $P < 0.0009$, $P < 0.004$ for each comparison; Fig. 2C). The AUC of pain behavior did not differ in sham-operated and lesioned rats receiving WIN55,212-2, suggesting that the total amount of pain behavior observed was similar between groups.

Effects of WIN55,212-2 on formalin-evoked Fos protein expression in sham-operated and lesioned rats

Levels of formalin-evoked Fos protein expression did not differ in sham-operated and lesioned rats receiving vehicle (Table 2); therefore the vehicle groups were pooled into a single control group for subsequent analyses. WIN55,212-2 suppressed the total number of cells expressing formalin-evoked Fos-LI in the lumbar spinal cord of sham-operated rats ($P < 0.02$) but no suppression was observed in lesioned animals (Fig 3A). In sham-operated rats, WIN55,212-2 suppressed formalin-evoked Fos-LI in the superficial ($P < 0.02$) and neck region ($P < 0.03$) of the dorsal horn and in the nucleus proprius ($P < 0.03$) relative to control conditions. By contrast, WIN55,212-2 failed to suppress the number of formalin-evoked FLI cells in 6-OHDA-lesioned rats in any spinal cord region. In the superficial dorsal horn, the suppressive effect of the cannabinoid on formalin-evoked Fos protein expression was attenuated following destruction of descending noradrenergic projections; in lamina I and II, the number of formalin-evoked FLI cells

was greater in lesioned rats receiving WIN55,212-2 relative to sham-operated rats receiving the same dose of the cannabinoid ($P < 0.05$; Fig. 3B). Example photomicrographs depict formalin-evoked Fos protein expression in rat lumbar dorsal horn in sham-operated and lesioned rats receiving vehicle or WIN55,212-2 (Fig. 4).

Table 1

Norepinephrine (NE), dopamine (DA) and serotonin (5-HT) levels (ng/μg) in lumbar spinal cord and whole brains samples derived from sham-operated and 6-OHDA (20 μg i.t.) lesioned rats.

		NE	DA	5-HT
Spinal Cord	Sham	0.40 ± 0.04	0.02 ± 0.01	0.46 ± 0.02
	Lesion	0.06 ± 0.02***	0.02 ± 0.01	0.44 ± 0.04
Brain	Sham	0.51 ± 0.02	0.73 ± 0.03	0.52 ± 0.03
	Lesion	0.50 ± 0.02	0.73 ± 0.03	0.48 ± 0.02

*** Significant difference from sham-operated group, $P < 0.001$.

Table 2

Laminar distribution of FLI cells in sham-operated and lesioned rats receiving vehicle

Condition	I, II	III, IV	V, VI	Ventral	Total
Sham-veh	93.38 ± 8.26	29.06 ± 3.74	97.38 ± 11.42	14.81 ± 3.73	234.63 ± 22.75
Les-veh	85.21 ± 7.14	26.33 ± 5.43	89.21 ± 10.64	13.38 ± 3.73	214.13 ± 24.88

Data are Mean number of cells ± SEM.

Figure 1. (A) Time course of WIN55,212-2-induced antinociception in 6-OHDA-lesioned (20 µg i.t.) and sham-operated rats in the tail-flick test. Tail-flick latencies observed in sham-operated (Sh) and lesioned (Les) rats following administration of vehicle or WIN55,212-2 (5 or 10 mg/kg, i.p.). The antinociceptive effect of WIN55,212-2 was attenuated following depletion of spinal NE. $N = 6 - 7$ per group. (B) Mean tail-flick latencies for the entire observation interval shown in A. Sham-operated and lesioned rats receiving vehicle did not differ from each other and were pooled to form a single control group. $^{**}P < 0.01$, $^{*}P < 0.05$, different from Control, Les-WIN 5 mg/kg, and Sh-WIN 10 mg/kg. $^{\#}P < 0.05$, different from Sh-WIN 5 mg/kg, Sh-WIN 10 mg/kg, and Les-WIN 10 mg/kg. $^{xx}P < 0.05$, different from all groups.

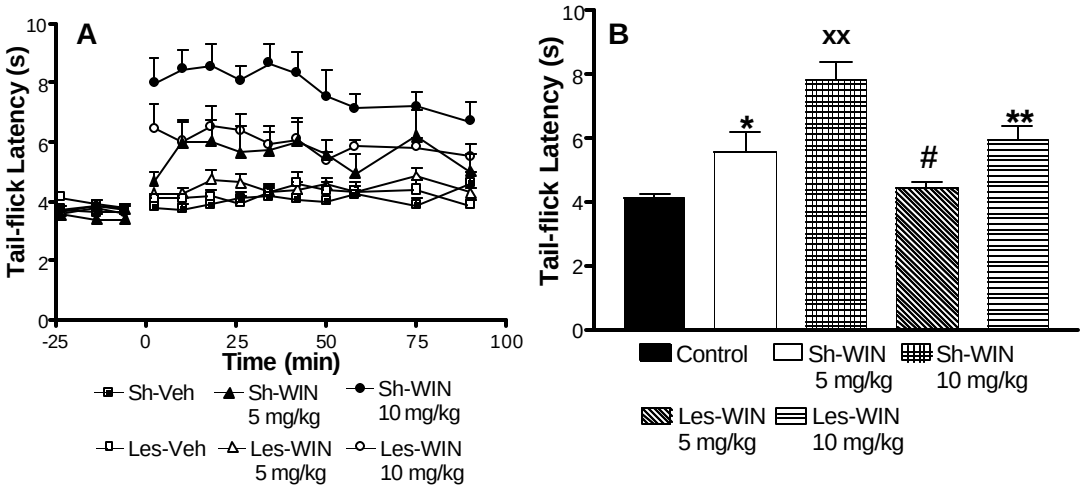


Figure 1: Effects of WIN55,212-2 on acute thermal pain in 6-OHDA-lesioned and sham-operated animals

Figure 2 (A). Time course of formalin-induced pain behavior in 6-OHDA-lesioned and sham-operated rats receiving WIN55,212-2 (WIN; 5 mg/kg i.p.) or vehicle (Veh). (B) WIN55,212-2 suppressed pain behavior in sham-operated rats during phase 2A and phase 2B of the formalin test but not during phase 1. WIN55,212-2 suppressed pain behavior in lesioned animals during phase 1 and phase 2A but not during phase 2B. Sham-operated and lesioned rats receiving vehicle did not differ from each other and were pooled to form a single control group. (C) Mean area under the curve (AUC) of pain behavior in control rats and sham-operated and lesioned rats receiving WIN55,212-2. The AUC was greater in the control condition compared to sham-operated or lesioned rats receiving WIN55,212-2; the AUC in these latter groups did not differ from each other. $N = 8$ per group. $^{**}P < 0.01$, $^{*}P < 0.05$, different from Control.

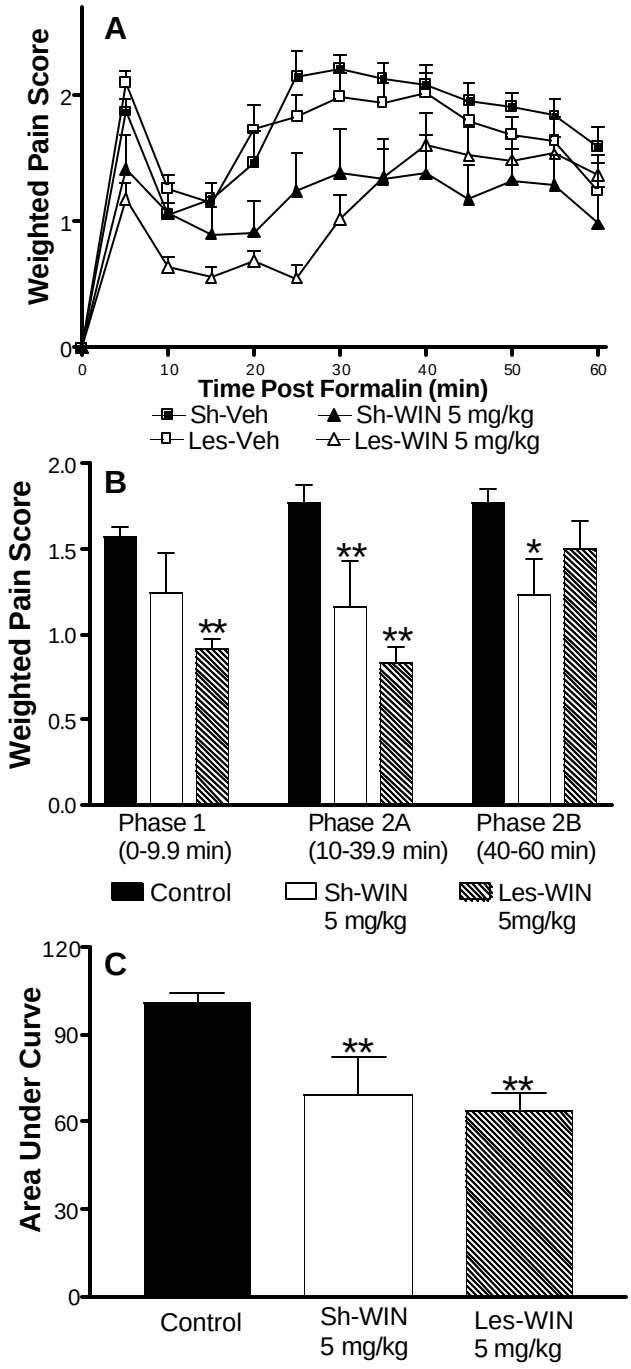
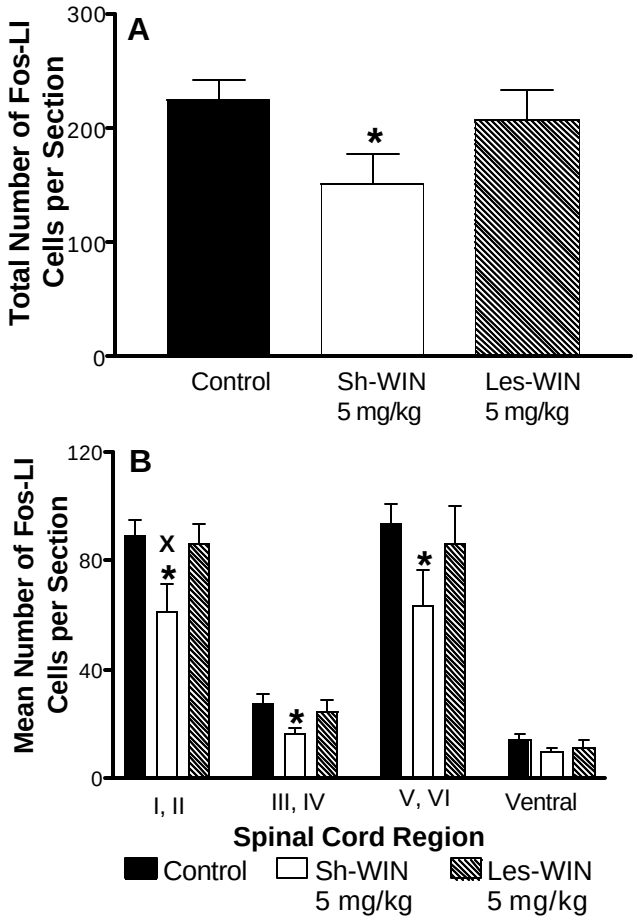


Figure 2: Effects of WIN55,212-2 on Formalin evoked pain behavior in sham-operated and lesioned rats

Figure 3. (A) WIN55,212-2 suppressed the total number of formalin-evoked FLI cells in the lumbar dorsal horn of sham-operated rats but no suppression was observed in 6-OHDA (20 μg i.t.)-lesioned rats. Sham-operated and lesioned rats receiving vehicle did not differ from each other and were pooled to form a single control group. (B) Laminar distribution of formalin-evoked FLI cells in sham-operated and lesioned rats receiving WIN55,212-2 (5 mg/kg i.p.) or vehicle. WIN55,212-2 suppressed formalin-evoked Fos-LI in the dorsal horn of sham-operated rats but not in lesioned rats. $N = 8$ per group. $^{*}P < 0.05$, different from Control. $^{x}P < 0.05$, different from Control and Les-WIN 5 mg/kg.



Effects of WIN55,212-2 on formalin evoked Fos protein expression in sham-operated and lesioned rats

Figure 4. Example photomicrographs show formalin-evoked Fos protein expression in the lumbar spinal cord of sham-operated (Top: A, B) and lesioned (Bottom: C, D) rats receiving vehicle (Left: A,C) or 5 mg/kg i.p. WIN55,212-2 (Right: B, D). The scale bar equals 100 μm.

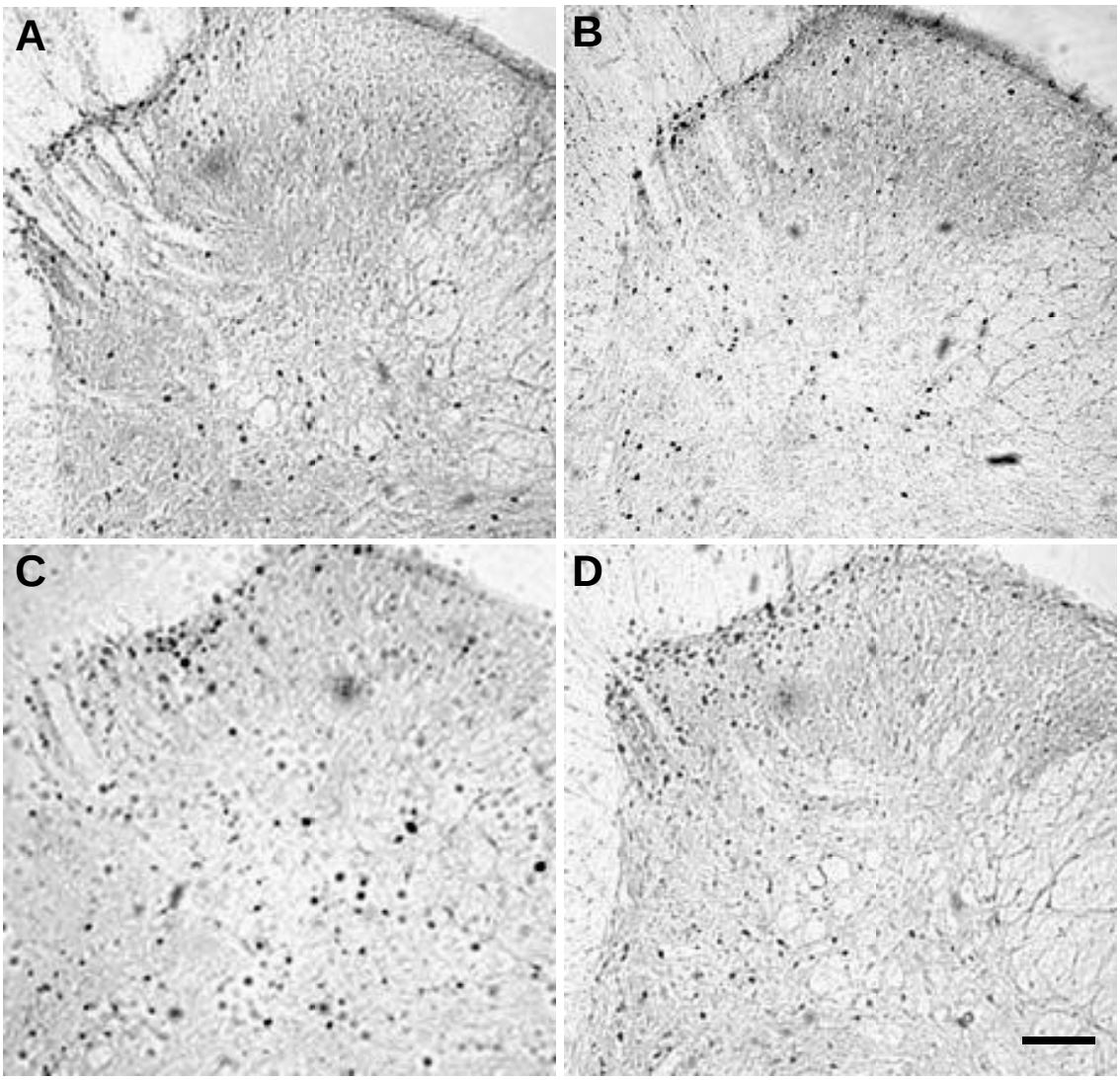


Figure 4: Example photomicrographs show Formalin-evoked Fos protein expression in the lumbar dorsal horn of sham-operated and lesioned-rats

CHAPTER 4: DISSCUSSION

The present studies were conducted to assess the effects of neurotoxic destruction of spinally projecting noradrenergic pathways on cannabinoid antinociception in rat models of acute and tonic pain sensitivity. In our study, intrathecal administration of 6-OHDA selectively depleted NE in the rat lumbar spinal cord without affecting levels of DA or 5-HT. These observations are consistent with other published reports. (Archer et al., 1987) The selectivity of this intrathecal neurotoxin treatment for depleting spinal NE was verified by high performance liquid chromatography (HPLC). Moreover, brain levels of NE, DA, and 5-HT did not differ in sham-operated and lesioned rats, indicating that the intrathecally administered neurotoxin did not diffuse to supraspinal levels to induce nonspecific effects. By contrast, we have observed nonspecific depletions of 5-HT following intrathecal administration of the noradrenergic neurotoxin DSP-4 (unpublished data), consistent with other reports documenting the lack of specificity of DSP-4 following systemic administration (Archer et al., 1987; Hallman & Jonsson, 1984). Our data are consistent with anatomical demonstrations of bulbospinal noradrenergic projections to the dorsal horn that originate in the locus coeruleus/subcoeruleus and the A7 and A5 cell groups of the pons (Clark & Proudfit, 1993; Fritschy & Grzanna, 1991; Kwiat & Basbaum, 1992; Lyons, Fritschy, & Grzanna, 1989).

The cannabinoid agonist WIN55,212-2 produced dose-dependent increases in tail-flick latencies in sham-operated rats, consistent with other published reports showing that cannabinoids are antinociceptive in models of acute pain sensitivity (Fox et al., 2001;

Martin et al., 1991; Smith & Martin, 1992; Welch, Thomas, & Patrick, 1995). By contrast, the antinociceptive effect of WIN55,212-2 in the tail-flick test was markedly attenuated following neurotoxic destruction of descending noradrenergic pathways. In fact, a dose of WIN55,212-2 (5 mg/kg, i.p.) that produced antinociception in sham-operated rats was ineffective in rats exhibiting an 85% depletion of spinal NE; tail-flick latencies observed in 6-OHDA-lesioned rats receiving a behaviorally effective dose of WIN55,212-2 did not differ from latencies observed in rats receiving the vehicle. By contrast, thermal nociceptive thresholds were similar in lesioned or sham-operated rats receiving the vehicle.

The attenuation of antinociception observed in 6-OHDA-lesioned rats in the tail-flick test was partially surmounted by increasing the dose of the cannabinoid agonist. The high dose of WIN55,212-2 (10 mg/kg, i.p.), administered to lesioned rats, produced antinociception similar to that observed in sham-operated rats receiving the low dose of WIN55,212-2 (5mg/kg, i.p.). Our data are consistent with the observation that inhibition of spinal a_2-adrenoceptors attenuates cannabinoid antinociception (Lichtman & Martin, 1991a). These data collectively suggest that cannabinoids produce antinociception, in part, by modulating descending noradrenergic systems. Furthermore, microinjection of WIN55,212-2 to the brainstem noradrenergic nucleus A5 produces antinociception in the tail-flick test, (Martin, Coffin et al., 1999) providing further support for the contribution of a descending noradrenergic system to cannabinoid antinociception.

Intrathecal administration of 6-OHDA has been used to assess the role of descending noradrenergic pathways in mediating inhibition of the tail flick reflex produced by focal electrical stimulation in the lateral reticular nucleus (LRN) and the

locus coeruleus-subcoereulus (LC/SC) (Janss et al., 1987). The LRN and LC/SC overlap the A_6 and A_1 noradrenergic regions that have been implicated in descending modulation of spinal nociceptive transmission (Janss et al., 1987). Similar to results observed in our study, spinal NE depletion did not alter baseline tail-flick latencies. In this latter study, the stimulation threshold for inhibiting the tail-flick reflex from either brainstem structure did not differ in 6-OHDA and sham-operated animals (Janss et al., 1987).

In the formalin test, WIN55,212-2 produced antinociception in sham-operated rats and suppressed formalin-evoked Fos protein expression in the lumbar dorsal horn. These data are consistent with previous observations of cannabinoid-induced suppressions of pain behavior as well as formalin-evoked c-*Fos* expression in the lumbar dorsal horn in otherwise untreated rats (Hohmann et al., 1999b; Tsou et al., 1996). The timecourse of cannabinoid antinociception in the formalin test differed in sham-operated and lesioned rats; in lesioned rats, WIN55,212-2 suppressed tonic pain behavior during phase 1 and phase 2A of the formalin test but not during phase 2B whereas in sham-operated rats, WIN55,212-2 produced antinociception during the entire late phase (phase 2A and 2B) of nociceptive responding. Despite these temporal differences in the antinociceptive effects of WIN55,212-2, the total amount of pain behavior, as revealed by the AUC analyses, was similar in sham-operated and lesioned rats receiving the cannabinoid. Thus, differences in the dependent measure used to assess formalin pain may affect the interpretation of cannabinoid effects on formalin-evoked pain behavior in sham-operated and lesioned rats.

We used the avidin-biotin-peroxidase method (Hsu et al., 1981) to visualize noxious stimulus-evoked Fos protein-like immunoreactivity in lumbar spinal cord

sections of sham-operated and lesioned rats that were evaluated behaviorally in the formalin test. These studies provide a useful supplement to the behavioral studies because the effects of cannabinoid administration on formalin-evoked neuronal activity can be evaluated at the level of the spinal dorsal horn independent of the suppressive effects of cannabinoids on motor activity (Fox et al., 2001; Martin et al., 1991). In sham-operated rats, WIN55,212-2 suppressed the total number of formalin-evoked FLI cells. Suppression of FLI was observed in the superficial laminae, nucleus propius, and neck region of the spinal dorsal horn, consistent with previous work demonstrating suppression of formalin evoked Fos protein expression in the lumbar dorsal horn of intact rats by an equivalent dose of WIN55,212-2 (Tsou et al., 1996). By contrast, no suppression of formalin-evoked Fos-LI was observed in lesioned rats in any spinal cord region. In the superficial dorsal horn, the suppressive effect of WIN55,212-2 on formalin-evoked Fos-LI was attenuated following depletion of spinal NE; greater numbers of formalin-evoked Fos-LI cells were observed in lamina I and II of lesioned rats relative to sham-operated rats receiving the same dose of the cannabinoid.

In the present study, sham-operated and lesioned rats receiving the vehicle exhibited similar levels of formalin-evoked Fos-LI and pain behavior. The behavioral data are consistent with other studies (Fasmer, Berge, Tveiten, & Hole, 1986; Sawynok, Reid, & Doak, 1995) demonstrating similar levels of formalin-evoked pain behavior in sham-operated and 6-OHDA (i.t.) lesioned rats at 11-14 days post lesion. At this delay, depletions of spinal NE were comparable to that observed in our study (Fasmer et al., 1986; Sawynok et al., 1995). By contrast, hypoalgesia has been observed in the formalin test at earlier time points (e.g. 3-6 days post-lesion), (Tjolsen, Berge, & Hole, 1991) even

within the same laboratory, (Fasmer et al., 1986) and may reflect transient increases in transmitter turnover in the remaining noradrenergic fibers and/or supersensitivity of postsynaptic receptors.

Following destruction of descending noradrenergic pathways, a disparity was apparent in the effects of cannabinoids on formalin-evoked FLI and pain behavior. In lesioned animals, a suppression of pain-related behavior was observed in the absence of a suppression of formalin-evoked Fos-LI. In fact, in the superficial lamina, the suppressive effect of the cannabinoid on formalin-evoked Fos protein expression was significantly attenuated relative to sham-operated rats. The absence of a cannabinoid-induced suppression of formalin-evoked FLI in lesioned rats may reflect the restricted time-course of cannabinoid antinociception observed in the lesioned animals (i.e. the absence of suppression of pain behavior during phase 2B) and/or the influence of motor suppressive effects of cannabinoids on the behavioral assessment of tonic pain behavior– motor suppression could mask an attenuation of nociceptive processing revealed in the immunocytochemical studies and be interpreted as antinociception in the behavioral studies. Consistent with the hypothesis, WIN55,212-2 exhibits an ED_{50} of 0.87 and 2.61 mg/kg s.c. in disrupting motor performance in the rotarod test of hypoactivity and ring test of catalepsy, respectively (Fox et al., 2001). However, the dose of WIN55,212-2 (5 mg/kg i.p.) used to assess tonic pain sensitivity failed to produce antinociception in 6-OHDA (20 µg i.t)-lesioned rats in the tail-flick test. Thus, WIN55,212-2-induced-hypoactivity is unable to account for the failure to observe antinociception in 6-OHDA-lesioned animals in our assessments of acute pain sensitivity. The assessment of cannabinoid modulation of formalin-evoked Fos protein expression in the same animals

evaluated for cannabinoid antinociception thus provides a useful indirect marker of antinociception, because it is independent of motor responses (Jasmin, Gogas, Ahlgren, Levine, & Basbaum, 1994). Our data confirm that tonic pain mechanisms are differentially regulated from acute pain mechanisms and suggest that descending noradrenergic systems play a preferential role in cannabinoid modulation of acute nociception.

Our findings contrast with the effects of intrathecal administration of the noradrenergic immunotoxin anti-dopamine β-hydroxylase-saporin (anti-DβH-saporin) on morphine antinociception in the formalin test (Martin, Gupta et al., 1999). Toxin-treated animals exhibited a modest enhancement of morphine-antinociception during phase 1 of the formalin test. In this study, formalin-evoked Fos-LI did not differ in morphine-treated rats that received either anti-DβH-saporin or vehicle intrathecally (Martin, Gupta et al., 1999). In otherwise untreated rats, anti-DβH-saporin treatment also produced a modest decrease in phase 2 pain behavior as well as formalin-evoked Fos protein expression in lamina V and VI with no changes in other spinal laminae.

Several methodological differences could explain the different results obtained in the two studies. First, Martin and colleagues (Martin, Gupta et al., 1999) administered an immunotoxin intrathecally to deplete spinal NE; this treatment produced extensive reductions in dopamine β-hydroxylase (DβH) immunoreactivity in LC and A5 as well as in the lumbar spinal cord, based upon qualitative evaluation. By contrast, in our work, the neurotoxin 6-OHDA was administered intrathecally to destroy descending noradrenergic projections to the spinal cord and HPLC was used to quantify monoamine depletion; in our study, NE was depleted at spinal but not at supraspinal levels in the absence of

changes in DA or 5-HT. Second, different concentrations of formalin were employed in the two studies. Third, Martin and colleagues assessed licking and lifting behavior during phase 1 and phase 2 whereas we used the weighted pain score method of Dubuisson and Dennis (Dubuisson & Dennis, 1977) to quantify the time course and distribution of pain behavior. Lesions of descending noradrenergic pathways have been reported to shift the nociceptive response from active forms of pain-related behavior such as licking to simpler forms of behavior– factors that could contribute to differences in the observed behavioral results (Tjolsen et al., 1991). Finally, we examined the effects of cannabinoids on formalin-induced pain behavior in 6-OHDA-lesioned and sham-operated rats in the same study whereas Martin and colleagues (Martin, Gupta et al., 1999) examined the effects of anti-DβH-saporin treatment on formalin-induced pain behavior in separate studies conducted either in the absence or presence of morphine.

In conclusion, the present data demonstrate that cannabinoid antinociception is attenuated following neurotoxic destruction of descending noradrenergic pathways. This attenuation was especially prominent in an assessment of acute thermal pain sensitivity. By contrast, similar levels of pain behavior were observed in sham-operated and lesioned rats receiving the vehicle in tests of both acute and tonic pain sensitivity, suggesting that noradrenergic projections to the spinal cord are not tonically regulating nociceptive responding under the conditions examined here. Our data also support the hypothesis that cannabinoids differentially regulate descending noradrenergic projections to produce antinociception in models of acute and tonic pain.

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