ACTIVATION OF CANNABINOID CB₁ AND CB₂ RECEPTORS SUPPRESSES PAINFUL PERIPHERAL NEUROPATHY EVOKED BY THE CHEMOTHERAPEUTIC AGENT VINCristine

by

ELIZABETH JOCELYN RAHN

(Under the Direction of Andrea G. Hohmann)

ABSTRACT

Chemotherapeutic treatment with vincristine induces severe side-effects including neuropathic pain. The present study was conducted to evaluate the efficacy of cannabinoids in suppressing vincristine-induced behavioral sensitization to mechanical stimulation (tactile allodynia). WIN55,212-2 (0.75-2.5 mg/kg i.p.), a potent cannabinoid agonist, induced a dose-dependent suppression of mechanical hypersensitivity in vincristine-treated rats. By contrast, WIN55,212-3, the receptor inactive enantiomer of WIN55,212-2, did not alter mechanical withdrawal thresholds relative to vehicle. The CB₁ antagonist SR141716 (2.5 mg/kg i.p.) and CB₂ antagonist SR144528 (2.5 mg/kg i.p.) blocked the anti-allodonic effects of WIN55,212-2. The CB₂ selective agonist AM1241 (2.5 mg/kg i.p.) also suppressed vincristine-induced mechanical hypersensitivity, and this effect was blocked by the CB₂ but not the CB₁ antagonist. By contrast, the opiate analgesic morphine (2.5 mg/kg i.p.), did not alter vincristine-induced mechanical hypersensitivity relative to control conditions. The present results provide evidence that cannabinoids suppress chemotherapy-evoked neuropathy through activation of both CB₁ and CB₂ receptors.

INDEX WORDS: Neuropathic Pain, Allodynia, Cancer, Chemotherapy, CB₁ and CB₂
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DEDICATION

I would like to dedicate this thesis in loving memory to Murray.
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CHAPTER 1: INTRODUCTION

Painful peripheral neuropathy evoked by chemotherapeutic treatment has been studied by researchers and physicians for many years now although most patients are unaware of the condition’s existence prior to undergoing chemotherapy treatment (for review see Cata et al., 2006). The choice of chemotherapeutic agent, dose schedule, type of cancer being treated, and presence of concomitant medical problems all affect the severity of chemotherapeutic neuropathy (Bacon et al. 2003; Cata et al. 2006b; Polomano and Bennett 2001; Sandler et al. 1969). The most commonly used classes of chemotherapeutic agents, including the vinca alkaloids (e.g. vincristine), taxol-derived (e.g. paclitaxel), and platinum-derived (e.g. cisplatin) agents have all been linked to the development of chemotherapeutic neuropathy.

Vincristine has been postulated to induce anti-tumor effects through alteration of cytoskeletal structure and disorientation of microtubules (Tanner et al. 1998; Topp et al. 2000). Substantial neurofilament accumulation in cell bodies and proximal axons have also been observed following chemotherapeutic treatment. This increase might disrupt axonal transport ultimately leading to parasthesias and dysesthesias in the periphery where results of axonal transport interference would initially be evident (Topp et al. 2000). More recently, however, chemotherapy-evoked painful neuropathy has been observed in the absence of morphological damage at the electron microscopic level, suggesting that the observed neurotoxicity is not dependent upon microtubule disruption (Dougherty et al. 2004; Flatters and Bennett 2006; Polomano et al. 2001). Bennett and colleagues have
recently proposed that painful peripheral neuropathy observed following paclitaxel or vincristine administration results from abnormal elevations of cytosolic calcium (Ca\(^{2+}\)) levels that is attributable to atypical mitochondrial function and a dysregulation of cellular calcium homeostasis (Flatters and Bennett 2006; Siau and Bennett 2006).

Vincristine-induced neuropathic pain can limit the dosing and duration of potentially life-saving anti-cancer treatment (Jackson et al. 1988). Although aspirin, ibuprofen, and celebrox are commonly prescribed to patients experiencing chemotherapy-evoked neuropathy, these prophylactic interventions show little efficacy in treating the accompanying painful neuropathy (Lynch et al. 2004). The absence of confirmed prophylactic treatments for chemotherapy-evoked neuropathic pain makes the identification of effective alternative analgesics an urgent medical need (Siau and Bennett 2006).

Cannabinoids—drugs that share the same molecular target as \(\Delta^9\)-tetrahydrocannabinol, the psychoactive ingredient in cannabis—are efficacious in suppressing painful neuropathy evoked in animal models of traumatic nerve injury (Bridges et al. 2001; Fox et al. 2001; Herzberg et al. 1997; Ibrahim et al. 2003; LaBuda and Little 2005). Cannabinoids suppress neuropathic pain behavior through activation of both CB\(_1\) and CB\(_2\) receptor subtypes. CB\(_1\) receptors are the predominant subtype expressed in the central nervous system (CNS) (Zimmer et al. 1999). CB\(_2\) receptors are prevalent in the periphery (Buckley et al. 2000; Munro et al. 1993), but are also expressed at low levels in the CNS (Beltramo et al. 2006; Van Sickle et al. 2005). Pascual and colleagues recently reported that WIN55,212-2, a mixed cannabinoid agonist, suppressed paclitaxel-induced neuropathic pain through a mechanism that is dependent upon CB\(_1\)
receptor activation (Pascual et al. 2005). The mechanisms underlying the development of painful peripheral neuropathies induced by distinct chemotherapeutic agents remain poorly understood (for review see Cata et al. 2006b). Dissimilar neuropathic pain symptoms may be induced by chemotherapeutic drugs of different classes (Bacon et al. 2003; Cata et al. 2006a; Cata et al. 2006b; Cella et al. 2003; Flatters and Bennett 2004; Polomano and Bennett 2001; Sandler et al. 1969), and such syndromes, in turn, may respond differently to pharmacological treatments (Flatters and Bennett 2004). The present study was conducted to evaluate the efficacy of cannabinoids for the treatment of vincristine-induced painful peripheral neuropathy. We used the mixed CB1/CB2 agonist WIN55,212-2 and the CB2-selective agonist AM1241 to investigate the contribution of both CB1 and CB2 receptors to cannabinoid modulation of chemotherapy-evoked painful peripheral neuropathy.
CHAPTER 2: MATERIALS AND METHODS

Subjects

One hundred and forty-four adult male Sprague-Dawley rats (223-353 g; Harlan, Indianapolis, IN) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann 1983).

Drugs and Chemicals

Vincristine sulfate was obtained from Tocris Cookson (Ellisville, MO). WIN55,212-2 (WIN-2), a potent CB₁/CB₂ agonist, WIN55,212-3 (WIN-3), the inactive enantiomer of WIN55,212-2, and morphine sulfate were purchased from Sigma Aldrich (St. Louis, MO). AM1241, a potent CB₂-selective agonist, was synthesized in the laboratory of Alexandros Makriyannis. SR141716, a CB₁-selective antagonist, and SR144528, a CB₂-selective antagonist, were provided by NIDA. All drugs were dissolved in a vehicle of 10% ethanol, 10% emulfur, and 80% saline. Drug or vehicle was administered in a volume of 1 ml/kg bodyweight with one exception. In experiments where antagonists were coadministered with AM1241, due to limits in solubility, the total injection volume was 1.5 ml/kg. Weights were recorded daily.

General Experimental Methods

Drug effects were evaluated using a single stimulus modality to prevent stimulus sensitization. Baseline responses to mechanical or thermal stimulation of the hindpaw
were established on day zero. Rats subsequently received once daily injections of either vincristine sulfate (0.1 ml/kg/day i.p.) or saline (1 ml/kg/day i.p.) over 12 days, immediately following behavioral testing. The injection paradigm consisted of five once daily injections, followed by a two day interval where no injections were administered, followed by five subsequent once daily injections, as described previously (Weng et al. 2003). In all studies, the experimenter was blinded to the drug condition.

Assessment of Tactile Allodynia

Tactile allodynia was assessed using a digital Electrovonfrey Anesthesiometer (IITC model Alemo 2290-4; Woodland Hills, CA) equipped with a rigid tip. Rats were placed underneath inverted plastic cages and positioned on an elevated mesh platform. Rats were allowed to habituate to the chamber for 10 - 15 min prior to testing. Stimulation was applied to the midplantar region of the hind paw through the floor of the mesh platform. Withdrawal thresholds to punctate mechanical stimulation were measured in duplicate for each paw. Mechanical stimulation was terminated upon paw withdrawal; consequently, there was no upper threshold limit set for termination of a trial. Mechanical thresholds were evaluated before and 24 h following every injection of vincristine or saline. On the test day (day 12), baseline mechanical withdrawal thresholds were assessed and effects of pharmacological manipulations were evaluated (approximately 24 h following the terminal injection of vincristine or saline).

Following assessment of baseline mechanical withdrawal thresholds (on day 12), vincristine-treated animals received intraperitoneal injections of WIN55,212-2 (0.75 mg/kg, 1.5 mg/kg, or 2.5 mg/kg; n = 8 per group) or vehicle (n = 8). A separate group received WIN55,212-3 (2.5 mg/kg i.p.), the receptor-inactive enantiomer of WIN55,212-
2 (n = 8). Comparisons were made with separate groups receiving either the CB₂ selective agonist AM1241 (2.5 mg/kg i.p. n = 8) or the opiate agonist morphine (2.5 mg/kg i.p.). The morphine dose was selected based upon its ability to suppress hyperalgesia in the spinal nerve ligation model of neuropathic pain (LaBuda and Little 2005), and its ability to induce antinociception in the tail-flick test. To determine pharmacological specificity, groups received intraperitoneal injections of either WIN55,212-2 (2.5 mg/kg) coadministered with either SR141617 (2.5 mg/kg; n = 8) or SR144528 (2.5 mg/kg; n = 8), AM1241 (2.5 mg/kg) coadministered with either SR141617 (2.5 mg/kg; n = 8) or SR144528 (2.5 mg/kg; n = 8) or either antagonist administered alone (n = 8 per group).

In all studies, mechanical withdrawal thresholds were evaluated (on day 12) approximately 24 h following the terminal injection of vincristine. Paw withdrawal thresholds were measured before (baseline) and at 30 and 60 minutes post injection of drug or vehicle. To evaluate the possible resolution of vincristine-induced painful peripheral neuropathy, vincristine-treated rats receiving vehicle were additionally evaluated for the presence of tactile allodynia 31 days following the terminal injection of vincristine.

Assessment of Thermal Hyperalgesia

Paw withdrawal latencies to radiant heat were measured in duplicate for each paw using the Hargreaves test (Hargreaves et al. 1988) and a commercially available plantar stimulation unit (IITC model 33; Woodland Hills, CA). Rats were placed underneath inverted plastic cages positioned on an elevated glass platform. Rats were allowed to habituate to the apparatus for 10 - 15 min prior to testing. Radiant heat was presented to the midplantar region of the hind paw through the floor of the glass platform. Stimulation
was terminated upon paw withdrawal or after 20 s to prevent tissue damage. Thermal withdrawal latencies are reported as the mean of duplicate determinations averaged across paws. Thermal paw withdrawal latencies were assessed prior to injection of vincristine or saline (day 0) and on days 3, 6, 9, and 12. Thermal hyperalgesia was assessed in groups receiving ten daily injection of either vincristine (n = 12) or saline (n = 6) over 12 days. Animals receiving vincristine were subsequently tested for the presence of tactile allodynia (on day 12) using methods described above.

**Catalepsy Testing**

Catalepsy testing was performed on test day 12 using a modification of the bar test (Martin et al., 1996; Pertwee and Wickens, 1991) in rats previously evaluated for thermal hyperalgesia (as described above). Rats were returned to their home cages for at least 30 min following assessment of thermal hyperalgesia, prior to initiation of baseline catalepsy assessment. Animals were placed on a stainless steel bar suspended 9 cm above a flat platform; forepaws were suspended over the bar and hindpaws were in contact with the table as described by Martin et al. (1996). Baseline measurements of catalepsy were assessed in vincristine rats 2 h following termination of thermal testing. Catalepsy was reassessed in vincristine-treated animals receiving either vehicle (n = 6) or WIN55,212-2 (2.5 mg/kg i.p., n = 6). A separate group of vincristine-treated animals which did not undergo thermal testing received AM1241 (2.5 mg/kg i.p., n = 6). Two groups of otherwise naive animals received either WIN55,212-2 (2.5 mg/kg i.p., n = 6) or WIN55,212-2 (10 mg/kg i.p., n = 6). Latency to limb movement was measured in all groups 30, 45, and 60 min post drug injection.
Statistical Analyses

Data were analyzed using analysis of variance (ANOVA) for repeated measures and ANOVA as appropriate. The Greenhouse-Geisser correction was applied to all repeated factors. Paired t-tests were used to compare post-drug thresholds with pre-vincristine (baseline) thresholds. Post hoc comparisons were performed using Fisher’s PLSD test. \( P < 0.05 \) was considered statistically significant.
CHAPTER 3: RESULTS

General Results

Body weight did not differ between groups prior to administration of vincristine or saline. Consistent weight gain was observed over the injection time course in saline-treated animals ($F_{1,40} = 41.515, P < .0002$; Fig. 1A). By contrast, an absence of weight gain was observed in vincristine-treated groups at all post-injection intervals ($F_{11,440} = 23.32, P < 0.0002; P < 0.001$ for each comparison). Fig. 1A presents quantitative data on changes in body weight over the course of vincristine or saline treatment for groups shown in Fig. 1B and 3. Similar weight trends were observed in all other studies (data not shown).

In all studies, responses to mechanical and thermal stimuli did not differ between right and left paws for any group ($P > 0.36; P > 0.90$, respectively); therefore, withdrawal thresholds are presented as the mean of duplicate measurements, averaged across paws. In all studies, vincristine decreased paw withdrawal thresholds to mechanical stimulation ($P < 0.0002$ for all experiments; Fig. 1B).

Assessment of Tactile Allodynia Following Systemic Administration of WIN55,212-2

Prior to administration of vincristine in a subset of groups differences in paw withdrawal thresholds were detected ($F_{3,28} = 5.104, P < .006$, Fig 3). However, there were no differences in paw withdrawal thresholds following vincristine treatment prior to drug or vehicle administration ($P > .46$). In vincristine-treated rats, WIN55,212-2 induced a dose-dependent increase in mechanical withdrawal thresholds relative to vehicle ($F_{3,28} =$
5.141, \( P < 0.006 \), Fig. 3). Post hoc analyses confirmed that the high dose of WIN55,212-2 (2.5 mg/kg i.p.) produced the maximal suppression of tactile allodynia, and outlasted the effects of the middle (1.5 mg/kg i.p.) and low (0.75 mg/kg i.p.) doses \((P < .02 \text{ for all comparisons})\). The high dose of WIN55,212-2 effectively normalized mechanical withdrawal thresholds relative to pre-vincristine levels (one tailed t-test, \( P = 0.059 \)).

Baseline differences in paw withdrawal thresholds were detected for animals receiving vehicle and WIN55,212-3 \((F_{2,21} = 4.080, P < .04; P < .02; \text{Fig. 4A})\). However, following vincristine treatment and prior to drug or vehicle administration no differences in paw withdrawal thresholds were detected \((P > .78)\). The WIN55,212-2-induced increase in mechanical withdrawal thresholds was receptor-mediated \((F_{2,21} = 17.78, P < .0002; \text{Fig. 4A})\); WIN55,212-2 (2.5 mg/kg, i.p.) suppressed tactile allodynia relative to treatment with vehicle or WIN55,212-3 (2.5 mg/kg i.p.), the receptor-inactive enantiomer of WIN55,212-2 \((P < 0.0002 \text{ for each comparison})\). Mechanical withdrawal thresholds in WIN55,212-3-treated animals did not differ from vehicle at any time point.

**Pharmacological Specificity**

In vincristine-treated rats, administration of the CB1-selective antagonist SR141716 (2.5 mg/kg i.p.) or the CB2-selective antagonist SR144528 (2.5 mg/kg i.p.) did not alter paw withdrawal thresholds relative to vehicle \((F_{2,21} = .252, P > .77, \text{Fig 4B})\). Both antagonists blocked the suppression of vincristine-evoked tactile allodynia induced by WIN55,212-2 \((F_{3,28} = 5.79, P < 0.004; P < 0.05 \text{ for each comparison; Fig. 4C})\) and this blockade was time dependent \((F_{6,56} = 9.51, P < 0.0002)\). Post hoc comparisons failed to reveal a differential blockade of the anti-allodynic effects of WIN55,212-2 with either antagonist. Paw withdrawal thresholds were higher in groups receiving WIN55,212-2
alone compared to either antagonist coadministration group. Partial and complete blockade of the WIN55,212-2-induced attenuation of vincristine-induced tactile allodynia was observed at 30 and 60 min post-injection, respectively ($P < 0.05$ for each comparison; Fig 4C).

Assessment of Tactile Allodynia Following Systemic Administration of AM1241

WIN55,212-2 suppressed vincristine-evoked tactile allodynia ($F_{3,28} = 9.83, P < 0.0002$; Fig 5A) relative to treatment with either vehicle, the CB$_2$ agonist AM1241 or the opiate analgesic morphine ($P < 0.003$ for each comparison). The time course of the antiallodynic effects was differentially effected by the experimental treatments ($F_{6,56} = 5.35, P < 0.003$). Post hoc analyses confirmed that both AM1241 (2.5 mg/kg i.p.) and WIN55,212-2 suppressed tactile allodynia relative to vehicle or morphine and this suppression was maximal at 30 min post-injection ($P < .05$ for all comparisons; Fig. 5A). However, the anti-allodynic effect of WIN55,212-2 was greater ($P < 0.008$) and of longer duration than that induced by AM1241 (Fig. 5A). By 60 min post-injection, AM1241 no longer suppressed tactile allodynia relative to vehicle. Paw withdrawal thresholds in groups receiving morphine (2.5 mg/kg i.p.) did not differ from vehicle at any time point.

The AM1241-induced suppression of vincristine-induced mechanical hypersensitivity was similar to that induced by the low and middle doses of WIN55,212-2 (0.75 and 1.5 mg/kg i.p.); thresholds were elevated at 30 min post injection and returned to vehicle levels by 60 min post-injection ($P < .04$ for all comparisons; Fig. 5B).

The AM1241-induced suppression of tactile allodynia was mediated by CB$_2$ receptors ($F_{2,21} = 8.58, P < .002$, Fig. 5C). The anti-allodynic effects of AM1241 were blocked by the CB$_2$ antagonist SR144528 ($P < .003$), but not by the CB$_1$ antagonist
SR141716. Paw withdrawal thresholds were lower in groups receiving AM1241 coadministered with SR144528 compared to groups receiving AM1241 alone ($P < 0.003$) or coadministered with SR141716 ($P < 0.002$).

Assessment of Thermal Hyperalgesia in Vincristine-treated Animals

Paw withdrawals latencies to thermal stimulation did not differ in vincristine and saline-treated groups at any post-injection interval (Fig. 2A). Nonetheless, the same vincristine-treated group exhibited robust tactile allodynia when compared with their saline-treated counterparts 24 h following the terminal injection of vincristine ($F_{1,16} = 26.36, P < .0002$, Fig. 2B).

Assessment of Catalepsy

Doses of WIN55,212-2 (2.5 mg/kg i.p.) and AM1241 (2.5 mg/kg i.p.) that suppressed vincristine-evoked tactile allodynia were compared with a dose of WIN55,212-2 (10 mg/kg i.p.) known to impair motor activity (Fig. 6). WIN55,212-2 (10 mg/kg i.p.) induced catalepsy in the bar test ($F_{4,25} = 4.34, P < 0.01$, Fig. 6) relative to control conditions. Post hoc analyses revealed that catalepsy was greater in groups receiving the high dose of WIN55,212-2 compared to all other groups ($P < .05$ for all comparisons). Neither WIN55,212-2 nor AM1241, administered at doses that suppressed vincristine-evoked tactile allodynia, suppressed motor activity in the bar test (Fig. 6).
Figure 1. A. An absence of normal weight gain was observed in groups treated with the chemotherapeutic agent vincristine relative to saline-treated controls. B. Time course of vincristine-induced tactile allodynia, as demonstrated by a lowering of the threshold for paw withdrawal to punctuate mechanical stimulation. ** $P < 0.0002$ different from control conditions by ANOVA and Fisher's PLSD post hoc test.
Figure 1: Effects of vincristine on body weight and the development of mechanical hypersensitivity
Figure 2A. Vincristine did not induce thermal hyperalgesia relative to the control condition. B. The same vincristine-treated animals showed robust tactile allodynia, defined as a lowered threshold for paw withdrawal to punctuate mechanical stimulation. ** $P < 0.0002$ by ANOVA. $N = 6-12$ per group.
Figure 2: Effects of vincristine on thermal paw withdrawal latencies
Figure 3. The CB1/CB2 agonist, WIN55,212-2 (2.5, 1.5, and 0.75 mg/kg i.p.), induced a dose-dependent suppression of vincristine-induced tactile allodynia. * $P < 0.04$ different from the middle and low dose of WIN55,212-2 (1.5 and 0.75 mg/kg i.p.), # $P < 0.005$ different from vehicle, X $P < 0.02$ different from WIN55,212-2 (1.5 mg/kg i.p.). $N = 8$ per group.
Figure 3: Effects of the mixed CB$_1$/CB$_2$ agonist WIN55,212-2 on vincristine-induced mechanical hypersensitivity
Figure 4A. WIN55,212-2 (2.5 mg/kg i.p.) suppressed vincristine-evoked tactile allodynia relative to the receptor-inactive enantiomer WIN55,212-3 (2.5 mg/kg i.p.) or vehicle. B. The CB$_1$ antagonist, SR141716 (2.5 mg/kg i.p.) and the CB$_2$ antagonist SR144528 (2.5 mg/kg i.p.) did not alter vincristine-induced tactile allodynia relative to vehicle. C. WIN55,212-2 increased mechanical withdrawal thresholds relative to all other groups. SR141716 and SR144528 induced a time-dependent blockade of the anti-allodynic effects of WIN55,212-2. ** $P < 0.0005$ different from vehicle and WIN55,212-3, * $P < 0.02$ different from all groups, X $P < .02$ different vehicle, and # $P < 0.04$ different from all groups by ANOVA and Fisher’s PLSD post hoc test. $N = 8$ per group.
Figure 4: Pharmacological specificity of the WIN55,212-2 mediated suppression of vincristine-induced mechanical hypersensitivity
Fig. 5A. WIN55,212-2 (2.5 mg/kg i.p.) induced the maximal suppression of vincristine-evoked tactile allodynia relative to all other groups. The anti-allodynic effect of WIN55,212-2 (2.5 mg/kg i.p.) outlasted that of AM1241 (2.5 mg/kg i.p.). B. AM1241 and WIN55,212-2 (1.5 and 0.75 mg/kg i.p.) produce a similar suppression of vincristine-induced mechanical hypersensitivity. C. The AM1241-induced suppression of vincristine-induced hypersensitivity was blocked by the CB₂ antagonist SR144528 (2.5 mg/kg i.p.) but not by the CB₁ antagonist SR141716 (2.5 mg/kg i.p.). * \( P < 0.03 \) different from vehicle and morphine, ** \( P < 0.005 \), # \( P < 0.008 \), and X \( P < 0.04 \) different from all groups, by ANOVA and Fisher’s PLSD post hoc test. \( N = 8 \) per group.
Figure 5: Effects of the CB₂ selective agonist AM1241 on vincristine-induced mechanical hypersensitivity
Figure 6. Anti-allodynic doses of AM1241 and WIN55,212-2 failed to produce cataleptic behavior in vincristine-treated rats. WIN55,212-2 (10 mg/kg i.p.) induced catalepsy at all post-injection time points in otherwise naive rats. * $P < 0.05$ different from all groups by ANOVA and Fisher’s PLSD post hoc test. $N = 6$ per group.
Figure 6: Effects of cannabinoids on catalepsy in vincristine-treated and control rats
CHAPTER 4: DISCUSSION

The present studies demonstrate that activation of both cannabinoid CB1 and CB2 receptor subtypes attenuates vincristine-induced painful peripheral neuropathy. Using the vincristine injection paradigm employed here, animals remained in relatively good health, as characterized by the absence of mortality observed with higher dosing paradigms (Authier et al. 1999; Authier et al. 2003a). Vincristine treatment induced a failure of normal weight gain relative to saline-treated controls, similar to other published reports (Weng et al. 2003). A small percentage of animals (<5%) in our study exhibited gastrointestinal bleeding during the later stages of the experiment (i.e., days 5 – 12). Gastrointestinal tract bleeding is a common problem for cancer patients undergoing chemotherapeutic treatment (Jackson et al. 1988; Ozcan et al. 2003; Sandler et al. 1969; Tolstoi 2002). Weng et al. (2003) reported no similar symptoms and normal stool for animals receiving the same vincristine dosing paradigm used here. This difference may be attributed to the relatively low frequency of symptom occurrence in our study and the fact that the present behavioral pharmacology experiments utilized roughly three times the number of subjects compared to the electrophysiological study conducted by Weng et al. (2003). Vincristine-induced hypersensitivity to mechanical stimulation developed by day 3 post-injection, similar to other published reports (e.g., Authier et al. 2003b), reaching its lowest levels on day 7 and remaining stable until day 12 (drug evaluation day). Previous studies similarly report that vincristine-induced tactile allodynia typically is maximal by day 8 post-injection (Nozaki-Taguchi et al. 2001; Weng et al. 2003).
Changes in mechanical withdrawal thresholds in vincristine-treated animals cannot be attributed to the development of sensitization to repeated testing because tactile allodynia was absent in groups receiving saline in lieu of vincristine. By 31 days following the terminal injection of vincristine, tactile allodynia had completely resolved in vincristine-treated animals, and normal weight gain was observed (data not shown). A lack of recovery from vincristine-induced tactile allodynia has been reported in the literature with other dosing paradigms (Nozaki-Taguchi et al. 2001).

In our study, thermal hyperalgesia was notably absent in vincristine-treated rats that nonetheless exhibited robust tactile allodynia. By contrast, paclitaxel has been shown to induce thermal hyperalgesia, which may be absent in vincristine and cisplatin models of chemotherapeutic neuropathy (Authier et al. 2003a; Authier et al. 2003b; Cata et al. 2006a; Lynch et al. 2004; Nozaki-Taguchi et al. 2001; Weng et al. 2003). Thermal hyperalgesia is mediated primarily by unmyleniated fibers (C fibers; Yeomans and Proudfit 1996), which show no evidence of degeneration following vincristine administration (Cata et al. 2006b). Nonetheless, vincristine administration may induce both cold allodynia (Lynch et al. 2004) and cold hyperalgesia (Authier et al. 2003b). These observations are consistent with clinical reports indicating that vincristine patients have significantly altered reactions to presentation of cold stimuli, so much so that many actually avoid contact with their own freezers and refrigerators (Cata et al. 2006b).

Upregulation of neuropeptide Y (NPY) in medium and large diameter cells of the dorsal root ganglion has been postulated to underlie the development of tactile allodynia following spinal nerve ligation. It is noteworthy, therefore, that unilateral injection of NPY into the nucleus gracilis– the dorsal root entry zone of myelinated primary afferents
to the spinal cord– in otherwise naive rats also induces a reversible ipsilateral tactile allodynia in the absence of thermal hyperalgesia (Ossipov et al. 2002). It is unclear whether similar changes in the neurochemical phenotypes of dorsal root ganglion cells accompany the development of vincristine-evoked tactile allodynia.

Activation of either cannabinoid CB1 or CB2 receptor subtypes suppressed the maintenance of vincristine-evoked tactile allodynia. It is possible that CB1-mediated anti-allodynic efficacy outlasts that associated with activation of CB2. At 60 min post injection, the CB2-selective agonist AM1241 no longer suppressed vincristine-evoked tactile allodynia, whereas the mixed CB1/CB2 antagonist WIN55,212-2 remained effective. This hypothesis could also explain the time-dependence of the antagonist blockade of WIN55,212-2-induced suppression of vincristine-evoked mechanical hypersensitivity. The antiallodynic effects of WIN55,212-2 were partially blocked by either the CB1 or CB2 antagonist at 30 min post-injection, but either antagonist (administered alone) completely blocked the anti-allodynic effects of WIN55,212-2 by 60 min post-drug, when AM1241 no longer exhibited anti-allodynic efficacy. Our data also raise the possibility that targeting multiple analgesic mechanisms simultaneously may induce synergistic antinociceptive effects.

In our study, WIN55,212-2 induced a dose-dependent suppression of vincristine-induced mechanical hypersensitivity. WIN55,212-2 (2.5 mg/kg i.p.) normalized mechanical withdrawal thresholds relative to pre-vincristine levels without inducing a reliable antinociceptive effect. By contrast, a lower dose of WIN55,212-2 (1.5 mg/kg i.p.) was sufficient to reverse both tactile alldynia and thermal hyperalgesia in a paclitaxel model of chemotherapy-induced neuropathy (Pascual et al. 2005). In this latter study, the
maximally effective dose of WIN55,212-2 (2.5 mg/kg i.p.) used in our study induced antinociception assessed using Von Frey filament testing (Pascual et al. 2005). Because psychotropic and motor effects can limit the therapeutic efficacy of CB₁-selective agonists and mixed CB₁/CB₂ agonists, it is noteworthy that the highest dose of WIN55,212-2 that eliminated vincristine-induced tactile allodynia in our study was insufficient to induce motor deficits in the bar test. Similar or higher doses of WIN55,212-2 (2.5–5 mg/kg i.p.) also attenuate tactile allodynia in animal models of traumatic nerve injury (Bridges et al. 2001; Fox et al. 2001; Herzberg et al. 1997; Ibrahim et al. 2003; LaBuda and Little 2005).

The CB₂-selective agonist AM1241 (2.5 mg/kg i.p.) also attenuated vincristine-induced mechanical hypersensitivity, albeit with lower potency than WIN55,212-2. CB₂ agonists may be a preferred analgesic relative to CB₁ or mixed (CB₁/CB₂) cannabinoid receptor agonists due to their reduced profile of CNS side-effects and failure to induce hypoactivity (Bridges et al. 2001; Malan et al. 2001). AM1241 is an effective analgesic in animal models of traumatic nerve injury (Ibrahim et al. 2003) and inflammation (Hohmann et al. 2004; Nackley et al. 2003; Nackley et al. 2004; Quartilho et al. 2003). AM1241 also suppresses C fiber-mediated afterdischarge responses and windup in spinal dorsal horn neurons (Nackley et al. 2004), providing a neurophysiological basis for a CB₂-mediated suppression of central nervous system sensitization. AM1241 may also reduce hyperalgesia and allodynia by releasing β-endorphin from keratinocytes at peripheral sites, which in turn act on the μ-opioid receptors located on primary afferent terminals (Ibrahim et al. 2005).
Opioids are commonly administered to cancer patients experiencing neuropathic pain due to their chemotherapeutic treatment (Cata et al. 2006b; Lynch et al. 2004). Our results, however, suggest that cannabinoids acting through either CB1 or CB2 specific mechanisms are more potent than morphine in suppressing vincristine-evoked painful peripheral neuropathy. The dose of morphine used in the present study suppressed neuropathic pain in the spinal nerve ligation model (LaBuda and Little 2005), but failed to suppress vincristine-induced tactile allodynia in our study. These observations are consistent with the enhanced antihyperalgesic efficacy of cannabinoids relative to opioids observed in animal models of traumatic nerve injury (Mao et al. 2000; Mao et al. 1995). Our data do not preclude the possibility that higher doses of the opiate analgesic would effectively suppress tactile allodynia induced by both vincristine and paclitaxel. A reversal of chemotherapy-induced tactile allodynia has been observed with higher doses of morphine (Nozaki-Taguchi et al. 2001), whereas doses of 8 mg/kg i.p. induced only a 50% reversal of paclitaxel-evoked mechanical allodynia/hyperalgesia (Flatters and Bennett 2004). The observation that morphine is less efficacious for reducing abnormal sensations related to myelinated as opposed to unmyelinated fiber activation (Taddese et al. 1995), is also consistent with the differential neuroanatomical distribution of µ-opioid and cannabinoid receptors observed at the level of the spinal cord and primary afferent (Hohmann et al. 1999; Hohmann and Herkenham 1998).

Vincristine induces a central sensitization in the spinal cord which may contribute to chemotherapeutic neuropathy. Vincristine induces abnormal spontaneous activity in wide dynamic range neurons as well as abnormal afterdischarges to mechanical stimulation (Weng et al. 2003). Abnormal windup responses and enhanced acute A-fiber
and C-fiber-mediated responses of WDR neurons are observed in vincristine-treated animals following transcutaneous electrical stimulation of the peripheral receptive field. An abnormal wind-down response of WDR neurons is also observed after repeated stimulation at 1.0 Hz (Weng et al. 2003). Abnormal windup responses are similarly observed in the spinal dorsal horn of paclitaxel-treated rats (Cata et al. 2006a). Thus, it is noteworthy that cannabinoids suppress C-fiber mediated responses and windup through activation of either CB$_1$ (Drew et al. 2000; Strangman and Walker 1999) or CB$_2$ (Nackley et al. 2004) receptors. More work is necessary to determine the neurophysiological basis for cannabinoid suppression of vincristine-evoked painful neuropathy and its sites of action. The observation of decreased protein levels for the glutamate-aspartate transporter (GLAST), the glial glutamate transporter-1 (GLT-1), and the excitatory amino acid carrier-1 (EAAC1) in paclitaxel-treated animals also suggests that enhanced glutamate levels may mediate both the abnormal behavioral phenotype and observed central nervous system sensitization (Cata et al. 2006a). It is noteworthy, however, that glutamate and NMDA receptor antagonists reverse hyperalgesia in a nerve-injury model (Mao et al. 1995), but have no effect in models of chemotherapeutic neuropathy (Aley and Levine 2002; Flatters and Bennett 2004), suggesting that distinct mechanisms underly the development of neuropathy induced by traumatic nerve injury and chemotherapeutic treatment, respectively.

Abnormal primary afferent input may lead to excessive activation of excitatory amino acid receptors at the level of the spinal cord, thereby enhancing neuronal excitability by increasing levels of intracellular Ca$^{2+}$ (Kawamata and Omote 1996). Ethosuxomide, a T-type calcium antagonist, and other drugs which reduce intra-
extracellular levels of Ca$^{2+}$ reduce mechanical allodynia and hyperalgesia in a vincristine model of neuropathic pain (Flatters and Bennett 2004; Siau and Bennett 2006). Cannabinoids also inhibit N and P/Q-type Ca$^{2+}$ channels to suppress neuronal excitability and neurotransmitter release (Mackie and Hille 1992; Mackie et al. 1995; Rao et al. 2004). Further investigation is required to determine if cannabinoid modulation of chemotherapeutic neuropathy is related to cannabinoid suppression of Ca$^{2+}$ channels and central nervous system sensitization.
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