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Cannabinoid agonist WIN 55,212-2 prevents the development of paclitaxel-induced peripheral neuropathy in rats. Possible involvement of spinal glial cells

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ABSTRACT

Spinal glial activation contributes to the development and maintenance of chronic pain states, including neuropathic pain of diverse etiologies. Cannabinoid compounds have shown antinociceptive properties in a variety of neuropathic pain models and are emerging as a promising class of drugs to treat neuropathic pain. Thus, the effects of repeated treatment with WIN 55,212-2, a synthetic cannabinoid agonist, were examined throughout the development of paclitaxel-induced peripheral neuropathy. Painful neuropathy was induced in male Wistar rats by intraperitoneal (i.p.) administration of paclitaxel (1 mg/kg) on four alternate days. Paclitaxel-treated animals received WIN 55,212-2 (1 mg/kg, i.p.) or minocycline (15 mg/kg, i.p.), a microglial inhibitor, daily for 14 days, simultaneous with the antineoplastic. The development of hypersensitive behaviors was assessed on days 1, 7, 14, 21 and 28 following the initial administration of drugs. Both the activation of glial cells (microglia and astrocytes) at day 29 and the time course of proinflammatory cytokine release within the spinal cord were also determined. Similar to minocycline, repeated administration of WIN 55,212-2 prevented the development of thermal hyperalgesia and mechanical allodynia in paclitaxel-treated rats. WIN 55,212-2 treatment also prevented spinal microglial and astrocytic activation evoked by paclitaxel at day 29 and attenuated the early production of spinal proinflammatory cytokines (interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α). Our results confirm changes in the reactivity of glial cells during the development of peripheral neuropathy induced by paclitaxel and support a preventive effect of WIN 55,212-2, probably via glial cells reactivity inactivation, on the development of this neuropathy.

1. Introduction

Similar to other antineoplastic agents, such as vincristine or cisplatin, painful peripheral neuropathy is a dose-limiting side-effect observed following paclitaxel therapy (Dougherty et al., 2004; Quasthoff and Hartung, 2002) and can be the origin of a chronic pain situation (Postma et al., 1995; van den Bent et al., 1997). Several rodent models have been developed to study the pathophysiology of paclitaxel-evoked neuropathy (Authier et al., 2000; Cavaletti et al., 1995; Cliffer et al., 1998; Polomano et al., 2001); however, the underlying cellular mechanisms have not been completely elucidated.

There is mounting evidence that central glial cells (microglia and astrocytes) play a prominent role in pain modulation (Hutchinson et al., 2008; Ren and Dubner, 2010). Over the last fifteen years, activation of spinal glia, evaluated by increased expression of glial activation markers (CD11b and GFAP), has been observed in different animal models of pathological pain, including inflammatory and neuropathic pain (reviewed in Cao and Zhang, 2008). Activated glia produce a large variety of inflammatory mediators, such as nitric oxide, prostaglandins and proinflammatory cytokines, which contribute to the initiation and maintenance of behavioral hypersensitivity associated with persistent pain states (DeLeo and Yezierski, 2001; Watkins et al., 2001; Wieseler-Frank et al., 2004, 2005). A reliable method for analyzing glial involvement in pain is the use of minocycline, a microglial inhibitor. Pre-emptive systemic administration of minocycline blocks the development of hypersensitive behaviors and the resultant proinflammatory cytokine release in diverse neuropathic pain models (Ledeboer et al., 2005; Padi and Kulkarni, 2008; Raghavendra et al., 2003; Zanjani et al., 2006). Therefore, spinal cord glial cells appear to be potential non-neuronal targets for neuropathic pain treatment.

Considerable evidence supports the effectiveness of cannabinoids in reducing the experimental neuropathic pain induced by peripheral nerve injury (reviewed in Walker and Hohmann, 2005). Regarding glial cells, since cannabinoid CB_1 and CB_2 receptors are predominantly expressed in astrocytes (Salio et al., 2002) and activated microglial cells (Romero-Sandoval et al., 2008; Zhang et al., 2003), respectively, it has been suggested that both receptors play an immune, modulatory

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function in the central nervous system (Cabral et al., 2008; Klein et al., 2003; Stella, 2004). Nevertheless, the mechanisms underlying these potential effects are not fully defined.

The ability of cannabinoids to suppress paclitaxel-induced neuropathic nociception has also been demonstrated (Naguib et al., 2008; Pascual et al., 2005; Rahn et al., 2008; Xu et al., 2010), but these investigations have shown only the effectiveness of cannabinoids acutely alleviating the typical signs of neuropathy. Recently, it has been reported that glial activation within the spinal cord also occurs after intravenous administration of high doses of paclitaxel (Peters et al., 2007a,b). On this basis, the goal of our study was to determine whether repeated administration of WIN 55,212-2, a mixed CB_1/CB_2 agonist, can modulate the development of peripheral neuropathy evoked by low doses of paclitaxel in rats and whether this effect is related with the participation of spinal glial cells and the release of inflammatory mediators in the spinal cord.

2. Material and methods

2.1. Animals

Adult male Wistar rats (200–250 g, Harlan Ibérica, Spain) were housed in clear plastic cages with soft bedding (four/cage) and with free access to food and water. They were maintained in a temperature-controlled room (23 ± 1 °C) on an automatic 12 h light–dark cycle for at least five days prior to the experiments. The number of animals per separate experimental group was at least eight. All the behavioral and immunohistochemical assays were conducted by researchers blinded to the drug treatment.

Experimental protocols used in this investigation were approved by the Ethical Committee of Rey Juan Carlos University and were conducted in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

2.2. Drugs and treatments

Paclitaxel (kindly supplied by Bristol–Myers Squibb, USA) was dissolved in a vehicle consisting of a mixture of saline and 10% Cremophor EL, a derivative of castor oil and ethylene oxide. This vehicle is clinically used for paclitaxel injections. Minocycline hydrochloride (Sigma-Aldrich, Spain) was dissolved in 0.9% saline. WIN 55,212-2 mesilate ((R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone) (Tocris, Spain) was dissolved in ethanol 1 mg/1 ml and subsequently in an ethanol and Tween 80 (1:2) solution, after which the ethanol was evaporated and saline was added to reach the desired final concentration (Pertwee et al., 1992). All drugs were administered intraperitoneally (i.p.) in a volume of 1 ml/kg.

After habituation to the test environments and baseline measurements of pain sensitivity (see below), animals were i.p. injected on four alternate days (days 1, 3, 5 and 7) with paclitaxel at a dose of 1 mg/kg (Polomano et al., 2001). Thus, the final cumulative paclitaxel dose administered was 4 mg/kg.

The aim of the first set of experiments was to examine the effects of repeated administration of minocycline, a selective inhibitor of microglial cell activation, on paclitaxel-induced painful neuropathy. Animals treated with paclitaxel were given minocycline (15 mg/kg i.p.) once a day for 14 days, starting together with the first dose of paclitaxel. A separate group of rats received minocycline (15 mg/kg i.p.) once a day for 14 days, in the absence of antineoplastic drug. The dose of minocycline, as well as the dose regimen, was in the range previously reported to prevent the development of pain hypersensitive behaviors in animal models of neuropathic pain (Padi and Kulkarni, 2008; Raghavendra et al., 2003; Zanjani et al., 2006).

A second set of experiments was performed to investigate the effects of repeated administration of WIN 55,212-2, a non-selective cannabinoid agonist, on peripheral neuropathy induced by paclitaxel. Paclitaxel-treated rats were given WIN 55,212-2 (1 mg/kg i.p.) or its vehicle once a day for 14 days, starting with the first dose of paclitaxel. A separate group of animals was also administered with WIN 55,212-2 (1 mg/kg i.p.) or its vehicle, daily over 14 days, in the absence of paclitaxel treatment. The dose of WIN 55,212-2 used in this study had previously shown antinociceptive effects without changes to motor performance in animals (Burgos et al., 2010; Liang et al., 2007), and the selected dose schedule had also been shown to prevent the development of hypersensitive behaviors after peripheral nerve injury (Costa et al., 2004; Guindon et al., 2007).

In order to minimize the impact of environment and manipulation and their possible effect on the observed behaviors, minocycline and WIN 55,212-2-treated groups each had their own control groups, which received only paclitaxel. These control groups were also included to confirm, in each set of experiments, the development of peripheral neuropathy due to antineoplastic treatment.

2.3. Behavioral assays

Test sessions were carried out between 09:00 and 17:00 h in a quiet room maintained at 23 °C, and the rats did not have access to food or water during the test. The plantar surface of hindpaws was tested for hypersensitive behaviors (heat-hyperalgesia and tactile-allodynia) to evaluate the effect of different pharmacological treatments. The paw withdrawal responses to thermal and mechanical stimulation as well as spontaneous locomotor activity were measured before beginning drug(s) administration (Day 1 or Basal (B)) and on days 7, 14, 21 and 28 after starting the administration of drug(s). When an injection of drug (paclitaxel and/or minocycline/WIN 55,212-2) had to be given on the same day as behavioral testing, rats were injected after the measurements had been taken.

2.3.1. Hind paw heat hyperalgesia

Heat hypersensitivity was tested according to the method described by Bennett and Hargreaves (1990). The latency (withdrawal time in seconds (s)) of the hindpaws from a focused beam of radiant heat applied to the plantar surface using a plantar test apparatus (Ugo Basile, Italy) was measured. Briefly, animals were placed within a plastic compartment on a glass floor and allowed to acclimatize for 5 min. A light source beneath the floor was aimed at the midplantar surface of the hindpaw. The withdrawal reflex interrupts the light and automatically turns the light and a timer off. The intensity of the light was adjusted at the start of the experiment such that the control average baseline latencies were about 10 s, and a cut-off latency of 30 s was imposed. The withdrawal latency of each paw was measured over three trials at 2 min intervals, and the mean of the three readings was used for data analysis.

2.3.2. Hind paw mechano allodynia

Tactile allodynia was assessed using the electronic model (EVF3) of von Frey filaments (Panlab S.A., Spain). On the day of the experiment, animals were placed in a Perspex chamber with a mesh metal floor and allowed to acclimatize for 15 min. The von Frey filament was applied perpendicular to the mid plantar surface of both hindpaws with sufficient force to cause slight bending against the paw and held until a response was achieved. This mechanical stimulation was maintained for 2 s (maximum). The tactile threshold was considered to be the force at which the paw was sharply withdrawn or when flinching occurred upon removal of the filament. The corresponding force (in grams (g)) applied was recorded by the system and displayed on the von Frey unit with a resolution of 0.1 g. The tactile threshold of each paw was measured over three trials at 2 min intervals, and the mean of the three readings was used for data analysis.

2.3.3. Locomotor activity

Spontaneous locomotor activity was evaluated using individual photocell activity chambers (CIBERTEC, Spain). Rats were placed in the recording chambers (55×40 cm, 3 cm spacing between beams) for 5 min before starting the experiment, and the number of interruptions of the photocell beams was recorded over a 30 min period. The mean number of crossings was compared with that obtained from normal naïve animals.

2.4. Immunohistochemistry

The correlation between behavioral hypersensitivity and glial activation within the spinal cord was assessed by testing the immunoreactivity of spinal cord sections (L4–L5) for integrin alpha M (CD11b) and glial fibrillary acidic protein (GFAP) obtained in all experimental groups. We examined lumbar spinal cord sections immunostained with antibodies against CD11b and GFAP as markers of microglia and astrocyte activation, respectively. While immunofluorescence staining of CD11b and GFAP was used for qualitative analysis, quantitative data were obtained using diaminobenzidine (DAB) immunohistochemistry.

Animals were deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and euthanized at day 29 following initial administration of drug(s) by transcardiac perfusion. Lumbar spinal cord sections were harvested and processed as previously described (Gómez-Nicola et al., 2008) to perform the immunohistochemical labeling on free-floating sections. These sections were blocked and then incubated overnight at 4 °C with mouse anti-rat CD11b (1:200; BD PharMingen, Palo Alto, CA) or mouse anti-GFAP (1:2000; Chemicon, Temecula, CA). Following primary antibody incubation, the sections were incubated with biotinylated goat anti-mouse antibody (1:200, Jackson ImmunoResearch, West Grove, PA) or with Alexa 488 or 594-conjugated secondary antibody (1:1000; 1 h 4 °C; Molecular Probes, Leiden, The Netherlands). For light microscopy, the sections were incubated with Vectastain ABC complex (Vector Labs, Burlingame, CA) and developed with DAB. Cell nuclei were labeled with Hoechst-33342 for immunofluorescent techniques. Sections were visualized in an Olympus Provis AX70 microscope coupled to an Olympus DP50 image acquisition system.

CD11b and GFAP expression in the spinal cord, detected by DAB immunohistochemistry, was measured with the help of an image analysis system (AIS, Imaging Research Inc., Linton, England) using a 4× lens. The proportional area stained for CD11b or GFAP (epitope-positive area/scan area) was determined in each visible spinal cord in normal naïve and drug-treated animals. Immunohistochemical data are expressed as the percentage of area stained for CD11b or GFAP relative to whole spinal cord section area (CD11b and GFAP-positive area). Measurements were carried out on 6 animals per group and 5–6 sections/animal. ImageJ software was used for image processing (ImageJ 1.38×; (Rasband, 2008)).

2.5. Western blotting

To confirm immunohistochemical results, we examined protein expression of microglial (CD11b, major histocompatibility complex class II (MHC-II)) and astrocytic (GFAP, vimentin) activation markers, as well as the inflammatory mediator inducible nitric-oxide synthase (iNOS), by western blot analysis.

CD11b, GFAP, MHC-II, vimentin and iNOS protein expression within the spinal cord were quantified using a separate group of rats. On the 29th day after initial drug(s) administration, the animals were euthanized by CO_2 asphyxiation followed by immediate decapitation. An 18-gauge needle was inserted into the caudal end of the vertebral column and the spinal cord was flushed out with ice-cold PBS. The spinal cord was immediately frozen in liquid nitrogen and stored at −80 °C until homogenization. Tissue processing and protein expression analysis were performed as described previously (Gómez-Nicola et al., 2008). Lumbar spinal cord (L4–L6 segment) samples were electrophoresed (20 μg protein/lane) and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The blots were blocked for non-specific binding and then incubated with mouse anti-rat CD11b (BD PharMingen, Palo Alto, CA), mouse anti-rat MHC-II (1:500;Serotec, Oxford, UK), mouse anti-GFAP (1:2000; Chemicon, Temecula, CA), mouse anti-vimentin (Dako, Glostrup, Denmark) or rabbit anti NOS2 (1:500; iNOS; Santa Cruz Biotechnologies, Santa Cruz, CA), using mouse anti-glyceraldehyde phosphate dehydrogenase (1:2000; GAPDH; Chemicon, Temecula, CA) as a loading control. Blots were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:5000; Jackson Immunoresearch, Cambridgeshire, UK), and protein bands were then detected using Supersignal west pico or femto chemiluminiscent substrate (Pierce, Rockford, IL). Image densitometry was performed with Quantity One 4.2 software (BIO-RAD Labs, Richmond, CA) referencing sample intensity to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (each $n \geq 4$).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Quantitative determination of IL-1β, IL-6 and TNF-α protein was performed on lumbar spinal cord (L4–L6 segment) from a separate group of rats. Enzyme-linked immunosorbent assays (ELISAs) were performed on lumbar spinal cord from animals treated with paclitaxel (each $n \geq 6$), paclitaxel and minocycline or WIN 55,212-2 (each $n \geq 5$), and minocycline or WIN 55,212-2 (each $n \geq 4$). Protein levels in the treated animals were also compared to protein levels of the normal naïve animals ($n \geq 6$). Spinal cord homogenization was adapted from the method of Sweitzer et al. (2001a). After CO_2 asphyxiation and decapitation, the spinal cord was isolated from the whole animal at 4, 8 and 29 days after initial administration of drug(s). Spinal cord isolation was achieved by inserting an 18-gauge needle into the caudal end of the vertebral column and flushing the spinal cord out with ice-cold PBS. The recovered spinal cord was flash frozen in liquid nitrogen and stored at −80 °C until homogenization. At the time of homogenization, a 0.5 cm section of lumbar spinal cord, which included the L4–L6 segment, was removed from the intact frozen cord and weighed. One complete protease inhibitor tablet (Roche Diagnostics, Barcelona, Spain) was added to 50 ml of homogenization buffer (50 mM NaCl, 10 mM Tris, 2.5 mM $MgCl_2$, pH 7.4). The tissue was minced and placed in 0.250 ml of ice-cold homogenization buffer containing protease inhibitors. Tissue was then homogenized using a B. Braun Labsonic U ultra-sonic homogenizer (B. Braun Biotech International, Spain) on high speed for 20 s. Samples were then spun at 13,200 g for 30 min at 4 °C, and the supernatant was aliquoted and stored at −80 °C for future protein quantification.

The concentration of IL-1β, IL-6 and TNF-α was measured with a quantitative sandwich enzyme immunoassay from Diaclone (bioNova científica, Spain), according to the procedure recommended by the manufacturer. The intensity of the color product, recorded at 450 nm with a spectrophotometer SPECTRAFluor Plus (Tecan Ibérica Instrumentación, Spain), is directly proportional to the concentration of the cytokine. IL-1β, IL-6 and TNF-α protein quantifications were determined by comparing the samples to the standard curves generated using the respective kits.

2.7. Statistical analyses

Behavioral data are expressed as means ± Standard Errors of the Mean (S.E.M.). In order to facilitate the comparison between the effects observed using different tests, data are expressed as percentages of the control values before beginning drug(s) administration (day B). One-way analysis of variance (ANOVA), followed by the Newman–Keuls test was used to compare values obtained from the same experimental group. Statistical analysis to identify significant differences

between multiple groups was performed by two-way ANOVA followed by the Bonferroni test. Graphpad Prism 4.0 software was used. $P<0.05$ was considered as statistically significant. Comparisons were established, using as reference values those data recorded before the start of drug(s) administration (day B) or obtained from paclitaxel-treated animals (paclitaxel control groups).

For the rest of experiments, data are expressed as means ± S.E.M. and were analyzed by one-way ANOVA followed by the Newman–Keuls test. Results were considered statistically significant at $P<0.05$.

3. Results

In the present study, we used a reliable model of peripheral neuropathy induced by intraperitoneal (i.p.) injection of paclitaxel at a cumulative dose of 4 mg/kg (1 mg/kg, on four alternate days) (Polomano et al., 2001). Normal weight gain was observed in all groups of treated and untreated animals during 28 days of testing (data not shown), and no animals died during paclitaxel therapy. As previously demonstrated (Pascual et al., 2005; Polomano et al., 2001), animals administered the paclitaxel vehicle, Cremophor, did not exhibit altered behavioral or morphological parameters throughout the experimental period (data not shown). None of the drug-treated animals showed any obvious change in spontaneous behavior when observed in their home cages.

3.1. Repeated administration of WIN 55,212-2 prevents the development of thermal hyperalgesia and mechanical allodynia evoked by paclitaxel

Cannabinoid vehicle did not modify thermal hyperalgesia or mechanical allodynia during 28 days of testing, either in naïve or in paclitaxel-treated rats (Table 1). Moreover, no significant differences in the spontaneous locomotor activity exhibited by cannabinoid vehicle-treated rats or naïve animals were found (Table 2).

As expected, paclitaxel treatment produced a significant decrease in the latency of paw withdrawal after noxious thermal stimulus (Fig. 1A, C) and innocuous mechanical stimulus (Fig. 1B, D), indicating the development of thermal hyperalgesia and mechanical allodynia, respectively. Baseline (day B) threshold of animals to the noxious heat was 9.64 ± 0.34 s for minocycline group (Fig. 1A) and 9.93 ± 0.34 s for WIN 55,212-2 group (Fig. 1C). Baseline threshold for mechanical stimulus was 19.48 ± 0.58 g for minocycline group (Fig. 1B) and 18.94 ± 0.51 g for WIN 55,212-2 group (Fig. 1D). These baseline values were considered as the 100% of the control response. The reduction of the threshold was statistically significant after day 14 for the thermal stimulus and after day 7 for mechanical stimulus (Fig. 1). Both the heat-hyperalgesia and the mechano-allodynia lasted until at least day 28. As in previous studies (Pascual et al., 2005), no differences were found between data recorded from the left or right paw, so data were calculated using the mean value obtained from both hindpaws. Paclitaxel treatment did not modify spontaneous locomotor activity of animals during testing days when compared to day B or to normal naïve animals (Table 2).

Repeated i.p. treatment with minocycline 15 mg/kg, once a day for 14 days, prevented the development of both thermal hyperalgesia (Fig. 1A) and mechanical allodynia (Fig. 1B) in paclitaxel-treated rats, when compared to the paclitaxel control group (heat hyperalgesia: $P<0.001$; tactile allodynia: $P<0.01$). With regard to spontaneous activity, animals receiving minocycline alone showed a transiently increased locomotor activity on day 28 ($P<0.05$ by Newman–Keuls *post hoc* analysis) (Table 2).

Similar to the minocycline group, paclitaxel-treated animals, which received 1 mg/kg of WIN 55,212-2 daily for 14 days, did not develop either thermal hyperalgesia (Fig. 1C) or mechanical allodynia (Fig. 1D) when compared to the paclitaxel control group (heat hyperalgesia: $P<0.001$, tactile allodynia: $P<0.001$). Animals receiving paclitaxel and WIN 55,212-2 showed paw withdrawal thresholds to heat and mechanical stimuli similar to those obtained in the pre-treatment period (day B) (Fig. 1). Furthermore, the prevention of hyperalgesia and allodynia by repeated administration of WIN 55,212-2 was sustained for up to fourteen days following the termination of cannabinoid treatment. The same prolonged treatment with WIN 55,212-2 (1 mg/kg, once daily for 14 days) was evaluated in the absence of paclitaxel, and the repeated administration of cannabinoid agonist on its own did not affect the response to thermal (Fig. 1C) or mechanical (Fig. 1D) stimuli. Moreover, repeated administrations of WIN 55,212-2 to paclitaxel-treated or naïve animals did not alter their spontaneous motility throughout the experiment, and no differences were observed when compared to normal naïve animals (Table 2).

3.2. WIN 55,212-2 prevents spinal cord glial cell activation induced by paclitaxel

Naïve rats exhibited no overt signs of microglial (Fig. 2A) or astrocytic (Fig. 2E) activation in the L4–L5 spinal cord (10×). As illustrated at a higher magnification (40×) (insets, Fig. 2A and E), the microglia and astrocytes from normal naïve animals showed characteristic morphologies of resting glia, with thin and highly ramified processes. By contrast, a strong microglial (Fig. 2B) and astroglial (Fig. 2F) activation was observed in the lumbar (L4–L5) spinal cord 29 days after the initial administration of paclitaxel. A cumulative dose of paclitaxel (4 mg/kg) increased the labeling intensity and number of CD11b- (microglia) and GFAP- (astrocytes) positive cells. These cells exhibited characteristic morphologies of activated glial cells: hypertrophied cell bodies and fibrous processes (insets, Fig. 2B and F). Moreover, CD11b and GFAP immunoreactivity was significantly (3- and 2-fold) increased in the lumbar spinal cord of paclitaxel-treated animals when compared with normal naïve rats (Fig. 2I and J). Confirming the immunohistochemical results, a constitutive expression of glial markers was observed in the lumbar (L5) spinal cord of

Table 1
Effect of repeated administration of cannabinoid vehicle, once a day for 14 days, on the development of paclitaxel-induced thermal hyperalgesia and mechanical allodynia in rats.

	Days									
	B (pre-treatment)		7		14		21		28	
Latency time (s)										
Paclitaxel	13.1	1.1	12.96	0.57	10.28	0.27	7.61	0.34[c]	7.15	0.27[c]
Paclitaxel + vehicle	10.24	0.46	10.71	0.43	9.77	0.47	7.65	0.46[b]	7.62	0.31[b]
Vehicle	11.51	0.67	11.33	0.85	10.32	0.75	9.94	0.33[f]	9.84	0.52[f]
Mechanical threshold (g)										
Paclitaxel	17.21	0.57	15.38	0.65	14.37	0.69[a]	12.37	0.69[c]	12.05	0.26[c]
Paclitaxel + vehicle	19.78	1.1	16.98	0.67	16.58	0.73	13.14	0.37[c]	12.31	0.44[c]
Vehicle	20.66	0.92	20.64	1.11	21.67	1[d]	19.6	0.68[e]	19.91	0.57[f]

Data are expressed as means ± S.E.M. Statistically significant differences *vs* day B (a $P<0.05$, b $P<0.01$, c $P<0.001$; one-way ANOVA plus *post-hoc* Newman–Keuls). + Statistically significant differences *vs* paclitaxel control group (d $P<0.05$, e $P<0.01$, f $P<0.001$; two-way ANOVA plus *post-hoc* Bonferroni test). ($n \geq 6$ per group). Vehicle: cannabinoid vehicle.

Table 2
Effect of repeated administration of minocycline, WIN 55,212-2 and cannabinoid vehicle on spontaneous locomotor activity of paclitaxel-treated animals.

Treatment	Days							
	7		14		21		28	
Naïve	507.6	50.41	480.50	33.65	576.33	91.16	400.17	63.67
Paclitaxel	615.33	116.65	478.42	90.56	505.75	71.06	457.75	77.9
Paclitaxel + minocycline 15 mg/kg	600.67	87.78	595	103.59	650	59.51	460.67	125.35
Minocycline 15 mg/kg	364.75	25.25	491.75	75.64	592.5	129.97	607	78.65[a]
Paclitaxel + WIN 55,212-2 1 mg/kg	537.11	70.68	492.89	87.72	530.67	70.24	405.33	46.32
WIN 55,212-2 1 mg/kg	627.29	108.49	674.86	113.55	596.13	101.22	595	108.81
Paclitaxel + vehicle	535.63	92.87	493.5	146.27	565.86	94.28	581	136.74
Vehicle	480.88	38.35	534.5	75.27	499.88	77.64	595.50	147.40

Data represent the means (±S.E.M.) number of beam crosses by treated and normal naïve animals, over the time course of the experiment. Chronic treatments consisted of daily i.p. injection of minocycline, WIN 55,212-2 or vehicle, for 14 days, to paclitaxel-treated and naïve rats. Statistically significant differences *vs* day 7. (n≥6 per group). Vehicle: cannabinoid vehicle.

[a] $P<0.05$; one-way ANOVA plus *post-hoc* Newman–Keuls.

normal naïve rats at day 29, whereas paclitaxel treatment produced robust overexpression of spinal CD11b, MHC-II, GFAP and vimentin proteins (Fig. 3A and B). Similarly, the administration of paclitaxel significantly enhanced the expression of spinal iNOS compared to that in normal naïve animals (Fig. 3A and B).

Repeated treatment of paclitaxel-treated animals with minocycline (15 mg/kg i.p.) resulted in attenuated microglia and astrocyte activation in the lumbar (L4–L5) spinal cord at day 29 (Fig. 2). As shown in Fig. 2, insets C and G, the appearance of microglia and astrocytes in paclitaxel-minocycline treated rats was similar to that of resting glial cells. The quantification of immunoreactivity levels revealed a significant decrease in immunostaining intensity for CD11b (microglia) and GFAP (astrocytes) when compared to paclitaxel-treated animals (Fig. 2I and J). However, while CD11b immunoreactivity was reduced almost to baseline levels (Fig. 2I), GFAP immunoreactivity remained significantly elevated when compared to normal naïve

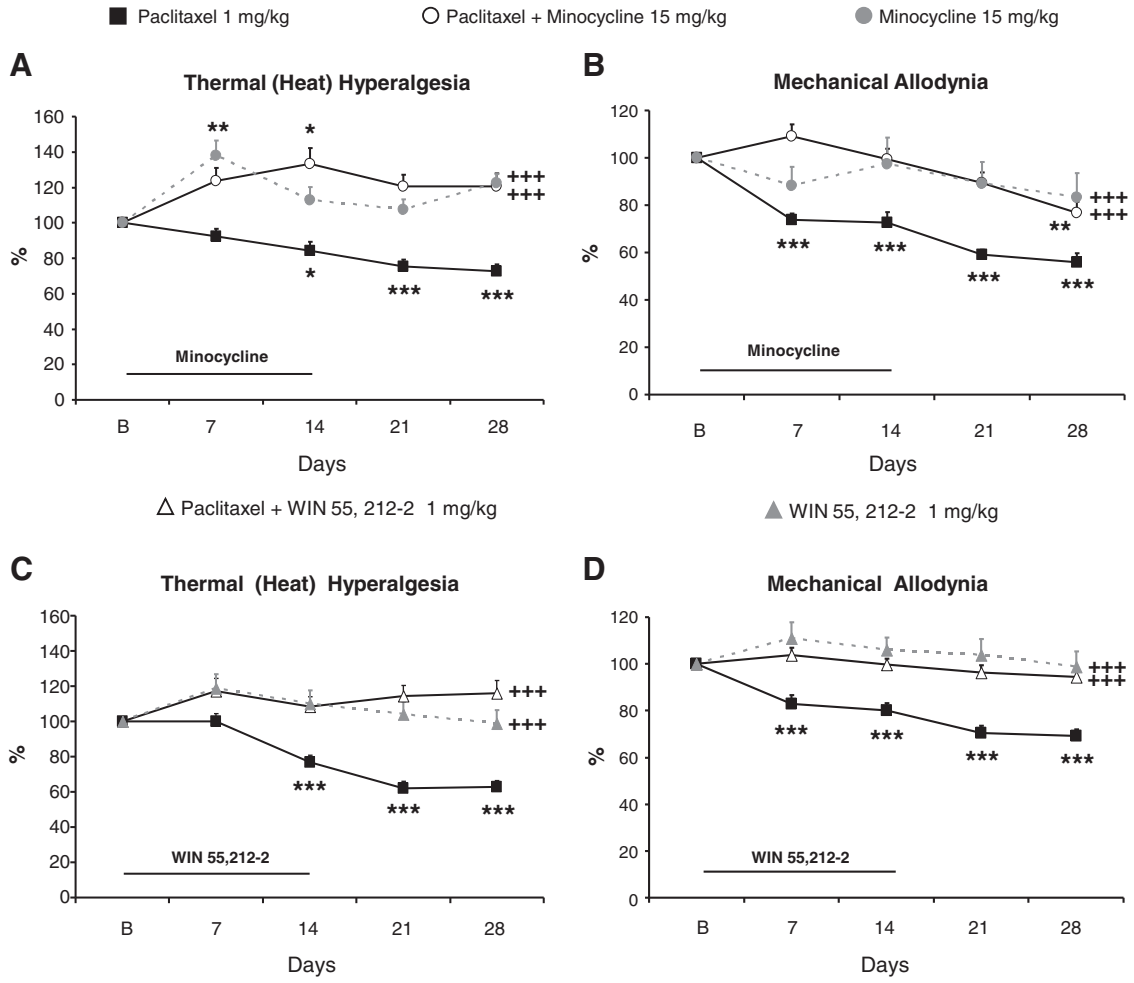

Fig. 1. Effect of repeated treatment with minocycline (A, B) and WIN 55,212-2 (C, D) on the development of paclitaxel-induced thermal hyperalgesia and mechanical allodynia. Data are expressed as the percentages of the means pre-drug control responses ± S.E.M. against the pre-treatment period (day B) and at successive treatment days (7, 14, 21, 28). * Statistically significant differences *vs* day B (*$P<0.05$, **$P<0.01$, ***$P<0.001$; one-way ANOVA plus *post hoc* Newman–Keuls test). + Statistically significant differences *vs* paclitaxel control group (++ $P<0.01$, +++ $P<0.001$; two-way ANOVA plus *post hoc* Bonferroni test). (n≥8 per group).

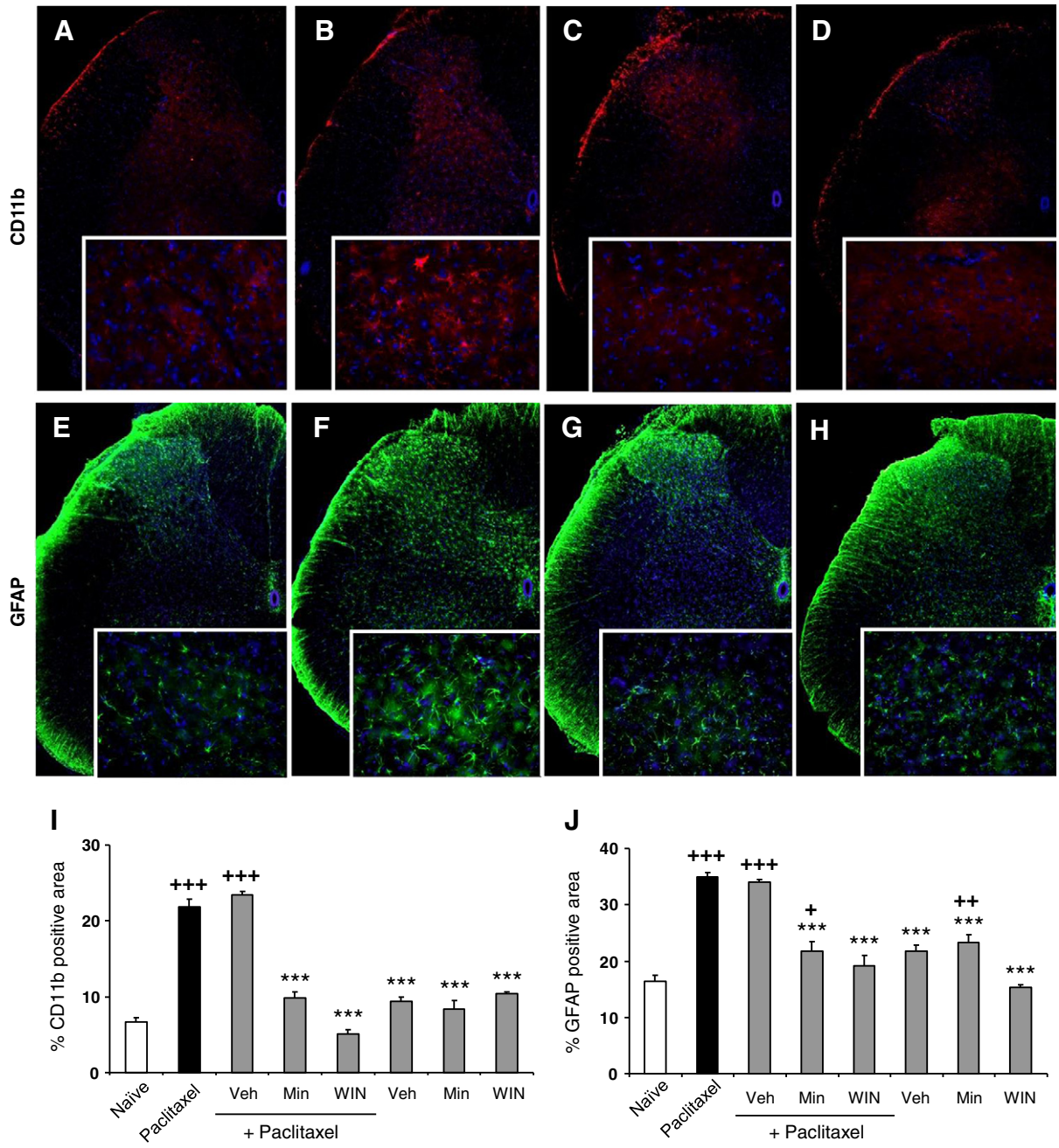

Fig. 2. Effect of repeated administration of minocycline (Min, 15 mg/kg i.p.), WIN 55,212-2 (WIN 1 mg/kg i.p.) and cannabinoid vehicle (Veh) on paclitaxel-induced microglial and astrocytic activation in the lumbar spinal cord at day 29 following initial administration of drug(s). Representative images of microglial (A–D) and astroglial (E–H) immunofluorescent staining in sections of L4–L5 spinal cord obtained from normal naïve animals (A and E), paclitaxel-treated animals (B and F) and paclitaxel-treated rats administered with minocycline (C and G) or WIN 55,212-2 (D and H), daily over 14 days (10×). Tissue harvested was immunohistochemically examined with antibodies against CD11b (red fluorescence) and GFAP (green fluorescence). Nuclei, labeled with Hoechst-33342, show blue fluorescence. Insets are examples of CD11b and GFAP immunostaining at 40× magnification. Histograms (I–J) show the effects of repeated treatment with minocycline, WIN 55,212-2 or cannabinoid vehicle in L4–L5 spinal cord CD11b-immunoreactivity (I) and GFAP-immunoreactivity (J). Relative labeling of CD11b and GFAP was quantified by AIS software and expressed as means ± S.E.M. of percent positive area. + Statistically significant differences *vs* naïve value (+ $P<0.05$, ++ $P<0.01$, +++ $P<0.01$; one-way ANOVA plus *post hoc* Newman–Keuls test). * Statistically significant differences *vs* paclitaxel-treated animals (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; one-way ANOVA plus *post hoc* Newman–Keuls test). ($n \geq 6$ per group).

animals (Fig. 2J). Minocycline treatment (15 mg/kg i.p.) alone did not induce any change in the immunoreactivity levels of CD11b (microglia) compared to naïve animals (Fig. 2I), but the immunoreactivity levels of GFAP (astrocytes) were significantly greater than naïve levels at day 29 (Fig. 2J). In agreement with these results, paclitaxel-induced overexpression of CD11b, MHC-II, GFAP and iNOS proteins in the L5 spinal cord was significantly attenuated by repeated treatment with minocycline (15 mg/kg i.p.) (Fig. 3A and B), but MHC-II expression levels remained significantly elevated when compared with naïve animals. A reduction of spinal vimentin expression was also observed in minocycline-treated animals, but the difference was not statistically significant (Fig. 3B).

Similar to the effect of minocycline, daily administration of WIN 55,212-2 at a dose of 1 mg/kg (i.p.) to paclitaxel-treated animals prevented both microglial (Fig. 2D) and astroglial (Fig. 2H) activation. Twenty-nine days after the initial administration of drugs, CD11b

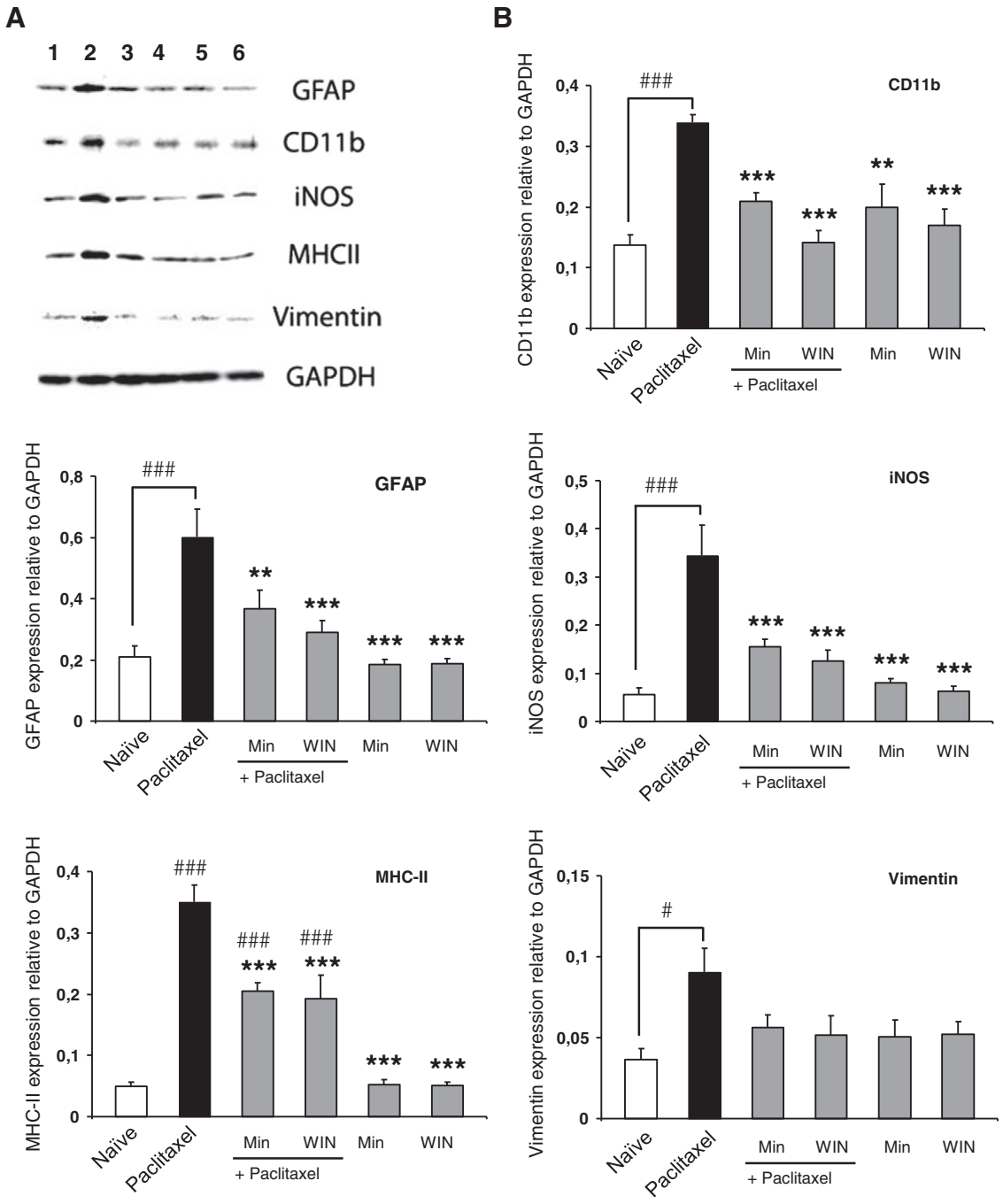

Fig. 3. Effect of repeated administration of minocycline (Min, 15 mg/kg i.p.) and WIN 55,212-2 (WIN, 1 mg/kg i.p.) on paclitaxel-evoked CD11b, GFAP, iNOS, MCH-II and vimentin protein expression in the lumbar spinal cord at day 29 following initial administration of drug(s). Chronic treatments consisted of daily injection of minocycline or WIN 55,212-2, for 14 days, to paclitaxel-treated and normal naïve rats. (A) Representative western blots of CD11b, GFAP, iNOS, MCH-II and vimentin proteins in spinal cord (L4-L6 segment) homogenate. Each lane was loaded with 20 μg of proteins. Lane 1: normal naïve animals; lane 2: paclitaxel; lane 3: paclitaxel + WIN 55,212-2; lane 4: paclitaxel + minocycline; lane 5: WIN 55,212-2; lane 6: minocycline. GAPDH is the protein loading control. (B) Quantification of CD11b, GFAP, iNOS, MCH-II and vimentin expression levels (rel. to GAPDH) in spinal cord (L4-L6 segment) homogenate obtained from normal naïve animals, paclitaxel-treated animals and paclitaxel-treated and untreated rats which received minocycline or WIN 55,212-2 once a day for 14 days. # Statistically significant differences *vs* naïve value (# $P<0.05$, ### $P<0.001$; one-way ANOVA plus *post hoc* Newman–Keuls test). * Statistically significant differences *vs* paclitaxel-treated animals (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; one-way ANOVA plus *post hoc* Newman–Keuls test). ($n \geq 4$ per group).

and GFAP immunostaining in the lumbar (L4–L5) spinal cord was unchanged from naïve animals, revealing microglia and astrocytes with the characteristic morphology of resting cells (insets, Fig. 2D and H). In addition, immunoreactivity of either CD11b (microglia) or GFAP (astrocytes) in paclitaxel-WIN 55,212-2-injected animals was comparable to that in normal naïve animals, whereas a significant reduction was observed when compared to paclitaxel-treated animals (Fig. 2I and J). Repeated administration of WIN 55,212-2 alone did not alter the expression of markers of microglia (CD11b, Fig. 2I) or astrocytes (GFAP, Fig. 2J) reactivity. In contrast to this effect of WIN 55,212-2, daily treatment with cannabinoid vehicle had no significant effect on glial activation since there was no difference in CD11b/GFAP immunoreactivity levels between paclitaxel and vehicle-paclitaxel treated animals (Fig. 2I and J). Quantification of glial activation proteins by western blot analysis revealed that repeated administration of WIN 55,212-2 strongly blocked the

overexpression of CD11b, GFAP and iNOS proteins in the L5 spinal cord of paclitaxel-treated animals (Fig. 3A and B). Like for the minocycline group, chronic treatment with WIN 55,212-2 significantly decreased spinal MHC-II expression induced by paclitaxel (Fig. 3B).

As expected, the constitutive expression of CD11b, MHC-II, GFAP, vimentin and iNOS proteins was not altered in the L5 segment from the spinal cord of animals that received minocycline or WIN 55,212-2 alone (Fig. 3).

3.3. Spinal proinflammatory cytokines are involved in the preventive effect of WIN 55,212-2 treatment

To test for a correlation between the effect of WIN 55,212-2 on paclitaxel-induced behavioral hypersensitivity and glial activation as well as proinflammatory cytokine expression, we analyzed the time course of IL-1β, IL-6 and TNF-α protein levels within the spinal cord. The results of this study revealed a statistically significant increase of IL-1β (Fig. 4A), IL-6 (Fig. 4B) and TNF-α (Fig. 4C) levels in the lumbar (L4-L6 segment) spinal cord of paclitaxel-treated rats on days 4 (after two injections of paclitaxel) and 8 (after four alternate injections of paclitaxel) when compared to normal naïve animals. In contrast, no differences in the proinflammatory cytokines content were detected between paclitaxel-treated rats and naïve rats at day 29 (Fig. 4). Daily administration of minocycline (15 mg/kg i.p.) to paclitaxel-treated animals resulted in the restoration of normal IL-1β (Fig. 4A) and IL-6 (Fig. 4B) levels at days 4 and 8, whereas spinal TNF-α protein levels were significantly reduced only at day 4 (Fig. 4C). On day 29, the spinal proinflammatory cytokine content in paclitaxel-minocycline treated animals was similar to that detected in normal naïve animals (Fig. 4).

Repeated treatment with 1 mg/kg of WIN 55,212-2 (i.p.) significantly prevented paclitaxel-induced upregulation of IL-1β levels at day 4 but not at day 8 (Fig. 4A). Spinal IL-6 (Fig. 4B) protein levels were also significantly reduced by WIN 55,212-2 treatment either at day 4 or 8 when compared to paclitaxel-treated animals. On days 4 and 8, paclitaxel-WIN 55,212-2 treated animals exhibited less spinal TNF-α than paclitaxel-treated animals (Fig. 4C), although a statistically significant reduction was only observed at day 4. As observed with the minocycline treatment, there were no statistically significant differences in the contents of spinal proinflammatory cytokines in paclitaxel-WIN 55,212-2 treated animals compared to paclitaxel-treated or naïve animals at day 29 (Fig. 4). Additionally, repeated i.p. administration of minocycline (15 mg/kg) or WIN 55,212-2 (1 mg/kg) alone had no effect on the lumbar spinal cord levels of IL-1β, IL-6 and TNF-α throughout the experimental period (Fig. 4).

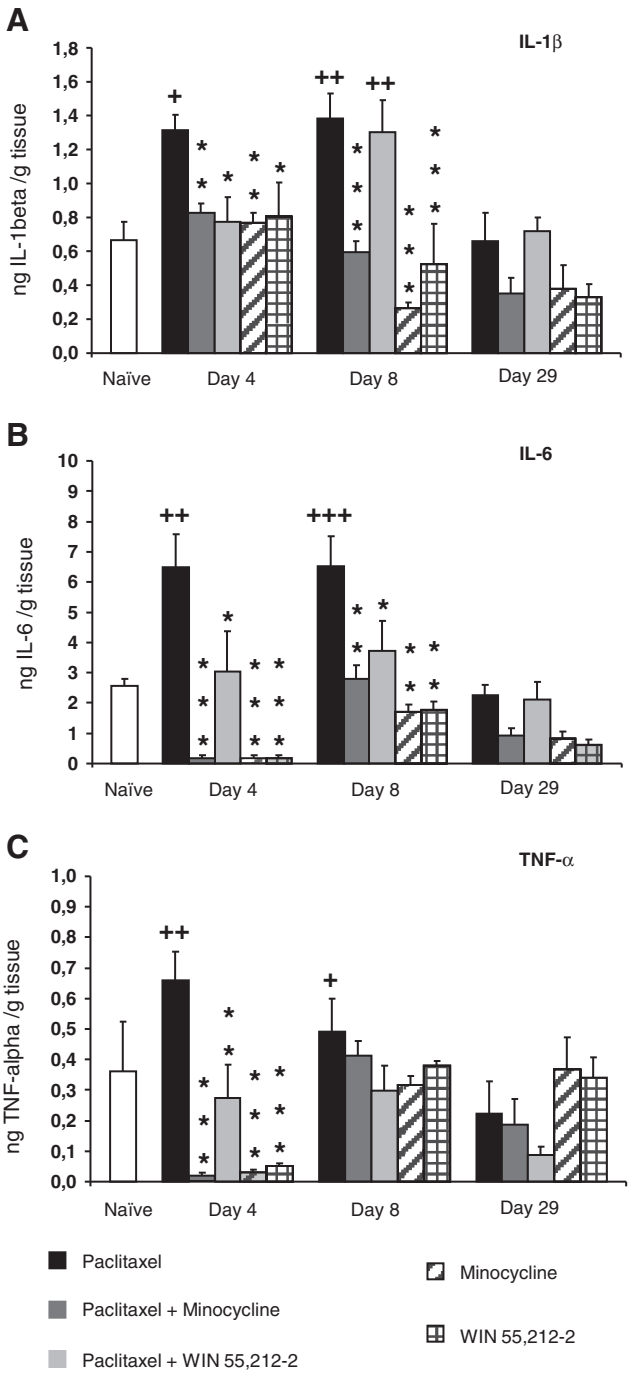

Fig. 4. Effect of repeated administration of minocycline (15 mg/kg i.p.) and WIN 55,212-2 (1 mg/kg i.p.) on the time course of proinflammatory cytokine levels in the lumbar spinal cord of paclitaxel-treated animals. Lumbar (L4–L6 segment) spinal cords of different group of rats were harvested on days 4, 8 and 29 after starting the administration of drug(s) to detect protein levels of IL-1β (A), IL-6 (B) and TNF-α (C). Data are in ng of protein per gram of L4–L6 spinal cord tissue homogenized (mean ± S.E.M.). + Statistically significant differences *vs* naïve value (+ $P<0.05$, ++ $P<0.01$, +++ $P<0.001$; one-way ANOVA plus *post hoc* Newman–Keuls test). * Statistically significant differences *vs* paclitaxel-treated animals (*$P<0.05$, ** $P<0.01$, *** $P<0.001$; one-way ANOVA plus *post hoc* Newman–Keuls test). ($n \geq 4$ per group).

4. Discussion

Painful peripheral neuropathy is a common side-effect observed following exposure of patients to paclitaxel, one of the most effective antineoplastics against solid tumors (Rowinsky and Donehower, 1995). Clinically, paclitaxel evokes neuropathic pain symptoms and sensory abnormalities, which can become chronic, persisting for months or years following the termination of paclitaxel therapy (Dougherty et al., 2004; van den Bent et al., 1997). From these clinical observations, the first animal model using paclitaxel was validated by Polomano in 2001, and behavioral modifications as well as biochemical evidences showing neuropathic symptoms were found in rats treated with paclitaxel. In animals, paclitaxel causes a dose-limiting distal, symmetrical, sensory peripheral neuropathy that is often accompanied by a neuropathic pain syndrome (Zheng et al., 2011). Thus, there is a clinical need to identify novel therapeutic agents for the treatment and prevention of this neuropathic pain.

The administration of paclitaxel induces several modifications in the somatosensory system and the threshold for nociceptive stimulus is reduced inducing allodynia and hyperalgesia. Moreover, paclitaxel shows to induce modifications in voltage-gated sodium channels present in nociceptors (Nieto et al., 2008), and changes in glutamate transporters in the lumbar spinal dorsal horn (Cata et al., 2006).

Analgesia is a major therapeutic indication for cannabinoids (Azad and Rammes, 2005; Guindon and Hohmann, 2009; Pacher et al., 2006). The antinociceptive activity of cannabinoid agonists has been

widely demonstrated, suggesting a regulatory role in nociceptive pathways (Cravatt and Lichtman, 2004; Martin and Lichtman, 1998; Pertwee, 2001; Rice et al., 2002; Seyrek et al., 2010; Walker and Hohmann, 2005). In neuropathic pain animal models, however, the efficacy of cannabinoid compounds has been mostly reported by evaluating the effect of a single injection (Bridges et al., 2001; Fox et al., 2001; Herzberg et al., 1997; Scott et al., 2004; Ulugol et al., 2004), including the paclitaxel-evoked neuropathy model (Pascual et al., 2005; Rahn et al., 2008; Xu et al., 2010) investigated herein.

In the present study, we demonstrated that repeated systemic administrations of the cannabinoid agonist WIN 55,212-2 could prevent the development of hypersensitive behaviors, such as thermal hyperalgesia and mechanical allodynia, induced by paclitaxel in rats. A few previous studies (Costa et al., 2004; Guindon et al., 2007; Hama and Sagen, 2009) have demonstrated the ability of this agonist, when administered daily, to attenuate behavioral hypersensitivity in neuropathic pain models during the cannabinoid administration period (Costa et al., 2004; Hama and Sagen, 2009) or up to six days after cannabinoid administration (Guindon et al., 2007). Interestingly, our results showed that the antihyperalgesic and antiallodynic effects of WIN 55,212-2 were time-independent because such effects lasted up to fourteen days after the termination of cannabinoid treatment. These results contrast with the findings of Guindon et al. (2007), who reported a loss of the WIN 55,212-2 antiallodynic effect concurrent with drug discontinuation. This discrepancy might be due to the lower dose of cannabinoid agonist used by Guindon's group (0.1 mg/kg *vs* 1 mg/kg) or to etiological differences between these neuropathic pain models. Moreover, in agreement with the results obtained in the chronic constriction injury model (Costa et al., 2004) and in the partial sciatic nerve ligation model (Guindon et al., 2007), we did not observe the development of tolerance to WIN 55,212-2-induced antinociception. Furthermore, we did not detect any altered spontaneous motility of WIN 55,212-2-treated animals over time, and therefore the effect of WIN 55,212-2 on allodynia and hyperalgesia should not be attributed to the ability of the agonist to depress motor activity.

Recent reports for rats that received high cumulative dose of intravenous paclitaxel (36 mg/kg) described cellular and neurochemical changes of the peripheral nerves as well as increased microglial and astrocyte activation within the spinal cord (Peters et al., 2007a,b). Our data, in agreement with the Polomano study (Polomano et al., 2001), clearly demonstrated that low cumulative dose of systemic paclitaxel (4 mg/kg) produces central neuroimmune activation characterized by glial activation and immune mediator production associated with the development of behavioral hypersensitivity.

Since there is solid evidence of glial cell contribution to the development and maintenance of central sensitization in chronic pain states (Cao and Zhang, 2008; DeLeo and Yezierski, 2001; Nakagawa and Kaneko, 2010), we tested the hypothesis that repeated administration of WIN 55,212-2 could modulate spinal glial activation using minocycline, a standard microglial inhibitor, as a comparative drug. Our findings confirmed that repeated systemic administration of minocycline prevents the development of paclitaxel-induced thermal hyperalgesia and mechanical allodynia in rats. This result is consistent with an earlier report that repeated administration of minocycline for 7, but not 14, days prevents the development of paclitaxel-induced pain behaviors (Cata et al., 2008). Similar results to ours have been observed in other animal models of neuropathic pain due to peripheral nerve injury (Leblanc et al., 2011; Ledeboer et al., 2005; Padi and Kulkarni, 2008; Raghavendra et al., 2003; Zanjani et al., 2006). Because minocycline inhibits microglial activation without direct effects on astrocytes or neurons (Yrjänheikki et al., 1998), our data support the idea that early microglial activation also plays a crucial role in the initiation and development of pain behaviors in paclitaxel-induced peripheral neuropathy.

Confirming behavioral observations, we showed that minocycline treatment suppressed the activation of microglia and, to a lesser extent, astrocytes. Minocycline consistently exhibited an inhibitory effect on the expression of glial activation markers (CD11b, MHC-II GFAP and vimentin) in chemotherapy-treated animals. Both microglial and astroglial activation occur in the spinal cord in some persistent pain states (Cao and Zhang, 2008; O'Callaghan and Miller, 2010; Tsuda et al., 2005; Wieseler-Frank et al., 2004). Glial activation is characterized morphologically by hypertrophy and overexpressed cell surface markers (CD11b) in microglial cells and the increased production of intermediate filaments (GFAP, vimentin) in astrocytes (Cao and Zhang, 2008; Hutchinson et al., 2008). Consistent with our results, other animal models have demonstrated that pain behaviors produced by peripheral nerve injury are correlated with spinal cord glial activation (Colburn et al., 1997; Garrison et al., 1991; Hidaka et al., 2011; Ledeboer et al., 2005; Sweitzer et al., 2001b, 2002, 2006; Tanga et al., 2004; Winkelstein et al., 2001). Moreover, accumulating data from neuropathic pain models indicate that microglial cells are activated before astrocytes and are responsible for the initial pain enhancement. On the other hand, several reports show that astrocytes, which are activated, at least in part, by activated microglia, become the primary glial cell responsible for maintenance of the persistent pain states (Cao and Zhang, 2008; Milligan and Watkins, 2009; Wieseler-Frank et al., 2004). Our findings are in agreement with the view that astrocyte activation is indirectly blocked following minocycline-evoked inhibition of microglia.

In our study, and consistently in other neuropathic pain models (Ledeboer et al., 2005; Padi and Kulkarni, 2008; Raghavendra et al., 2003; Zanjani et al., 2006), repeated administration of minocycline suppresses both the levels of spinal cord proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and the expression of inducible nitric-oxide synthase (iNOS). Microglia and astrocytes respond to these cytokines by upregulating enzymes such as iNOS and cyclooxygenase-2 to produce NO and prostaglandins that potentiate pain transmission by neurons (DeLeo and Yezierski, 2001; Watkins et al., 2001; Wieseler-Frank et al., 2004). In our study, the increased IL-1β, IL-6 and TNF-α levels confirmed the role of these cytokines as initiators for the cascade phenomena that accompany the development of neuropathic pain. Changes in the expression of many inflammatory mediators usually precede most of the physiological and behavioral changes happening in many models of nervous system injury. Similarly, our data also showed that the spinal cytokine content returned to basal levels at the end of the experiment while both neuropathic pain and glial cell activation were maintained. This result could indicate that proinflammatory cytokine expression is uniquely needed for the beginning of the process and, once neuropathic pain is established, other factors, which cannot be determined by this research, would fill in for the role of these cytokines (Cao and Zhang, 2008; Hutchinson et al., 2008; Wieseler-Frank et al., 2005). Daily treatment with WIN 55,212-2, similar to minocycline, prevents paclitaxel-induced microglial and astroglial reactivity in the lumbar spinal cord. WIN 55,212-2 inhibits both morphological changes and upregulated expression of microglial (CD11b, MHC-II) and astrocytic (GFAP, vimentin) activation markers observed in the lumbar spinal cord after paclitaxel treatment. We further demonstrated that chronic administration of WIN 55,212-2 also inhibited the enhanced expression of iNOS and attenuated the early production of proinflammatory cytokines induced by paclitaxel. Collectively, these data suggested that WIN 55,212-2 could prevent the development of hypersensitive behaviors by inhibiting glial activation. Nevertheless, since we used a non-selective cannabinoid agonist for this study, we could not elucidate the cannabinoid receptor (CB_1 or CB_2) implicated.

An additional feature of cannabinoids is their ability to modulate a variety of immune system functions (Klein et al., 2003; Pandey et al., 2009). Cannabinoid receptor activation by endogenous or synthetic cannabinoids has anti-inflammatory and neuroprotective effects, as demonstrated by a reduction in the release of IL-1β, IL-6 and TNF-α from microglia (Facchinetti et al., 2003; Puffenbarger et al., 2000), astrocytes (Ortega-Gutiérrez et al., 2005) or COX-2, and iNOS expression in

forebrain slices (Castillo et al., 2010). Moreover, an inhibition of iNOS production, which resulted in a suppression of NO release, was observed through the CB_1 receptor (Cabral et al., 2001; Waksman et al., 1999) and CB_2 (Castillo et al., 2010).

Our findings support, for the first time, the effectiveness of WIN 55,212-2 in preventing the development of paclitaxel-induced peripheral neuropathy in rats. Because the effects of WIN 55,212-2 resulted in a parallel inhibition of both glial reactivity and proinflammatory cytokine production, we suggest that the suppression of central neuroimmune activation by WIN 55,212-2 leads to the subsequent prevention of paclitaxel-induced neuropathic pain. Although additional work is required, endogenous cannabinoid system is emerging as a potential therapeutic target for the treatment of this neuropathic pain syndrome.

5. Conclusions

The activation of cannabinoid receptors can prevent the development of thermal hyperalgesia and mechanical allodynia induced by paclitaxel in rats. This effect could be related to with a blockade of spinal glial activity, since the morphological changes of microglia and astrocites, as well as the release of spinal proinflammatory substances (IL-1β, IL-6 and TNF-α) was abolished.

Acknowledgments

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