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A role for cannabinoid receptors, but not endogenous opioids, in the antinociceptive activity of the CB_2-selective agonist, GW405833

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Abstract

Several recent reports have demonstrated a role for selective cannabinoid CB_2 receptor agonists in pain modulation, showing both analgesic and antihyperalgesic activities. While the mechanism of action is poorly understood, it has been postulated that these effects may be indirect, involving release of endogenous opioids. We have previously reported that administration of the selective cannabinoid CB_2 receptor agonist GW405833 (2,3-dichloro-phenyl)-[5-methoxy-2-methyl-3-(2-morpholin-4-yl-ethyl)-indol-1-yl]-methanone) to rats elicits potent and efficacious antihyperalgesic effects against neuropathic and inflammatory pain and, at high dose (100 mg/kg), is analgesic and ataxic [Valenzano, K.J., Tafesse, L., Lee, G., Harrison, J.E., Boulet, J., Gottshall, S.L., Mark, L., Pearson, M.S., Miller, W., Shan, S., Rabadi, L., Rotstheyn, Y., Chaffer, S. M., Turchin, P.I., Elsemore, D.A., Toth, M., Koetzner, L., Whiteside, G.T., 2005. Pharmacological and pharmacokinetic characterization of the cannabinoid receptor 2 agonist, GW405833, utilizing rodent models of acute and chronic pain, anxiety, ataxia and catalepsy. Neuropharmacology 48, 658–672]. In the current study, we confirm these properties using mouse models and investigate the role of cannabinoid CB_2 receptors using knockout animals. Furthermore, we provide evidence that the antinociceptive properties of GW405833 are opioid independent. GW405833 elicited robust antihyperalgesic effects in mouse models of inflammatory (Freund's complete adjuvant) and neuropathic (Seltzer) pain. In contrast, GW405833 showed no antihyperalgesic activity against Freund's complete adjuvant-mediated inflammatory pain in cannabinoid CB_2 receptor knockout mice. As in rats, high-dose GW405833 (100 mg/kg) showed both analgesic and sedative activities in wild-type mice, activities that were also apparent in cannabinoid CB_2 receptor knockout mice. In rats, neither the antihyperalgesic effect in the Freund's complete adjuvant model nor the analgesic effects in tail flick and hot plate assays were inhibited by pre-treatment with the non-selective opioid receptor antagonist, naltrexone. These data demonstrate that the antihyperalgesic effects of GW405833 are mediated via the cannabinoid CB_2 receptor, whereas the analgesic and sedative effects are not. Furthermore, these data suggest that the mechanism of action for GW405833 does not depend on the release of endogenous opioids.

Keywords: Cannabinoid CB_2 receptor; GW405833; Inflammatory pain; Neuropathic pain; Knockout; Opioid

1. Introduction

Cannabinoid receptors belong to the superfamily of G protein-coupled receptors, and, to date, two have been cloned and characterized, cannabinoid CB_1 and CB_2 receptors, which share 48% identity at the amino acid level (Munro et al., 1993). In addition to Δ^9-tetrahydrocannabinol (THC), the psychotropic agent found in marijuana, numerous other natural products, endogenous ligands and synthetic small molecules can modulate the activity of the cannabinoid receptors.

Cannabinoids and cannabinoid receptors have been implicated in pain transduction and perception (for review, see Chapman and Finn, 2003) as well as neuroinflammation (for review, see Walter and Stella, 2004). The expression patterns for these receptors support a role in these processes. The expression of the cannabinoid CB_1 receptor is primarily restricted to neurons of the central and peripheral nervous

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systems (Galiegue et al., 1995). In contrast, the expression of the cannabinoid CB_2 receptor is primarily localized to cells of the immune system, including B and T cells, natural killer cells, monocytes and macrophages (Galiegue et al., 1995).

A number of publications have demonstrated the analgesic, antihyperalgesic and anti-inflammatory actions of selective cannabinoid CB_1 receptor agonists (for review, see Chapman and Finn, 2003). However, activation of cannabinoid CB_1 receptors within the central nervous system results in catalepsy, sedation and undesirable psychotropic effects. Interestingly, recent data suggest that selective agonism of cannabinoid CB_2 receptors may constitute a novel strategy for treating chronic pain, and that this approach may be devoid of the undesirable effects associated with central cannabinoid CB_1 receptor activation (for review, see Malan et al., 2003). While the mechanism of cannabinoid CB_2 receptor-mediated antinociception is poorly understood, recent work with the cannabinoid CB_2 receptor agonist, AM1241, suggests that cannabinoid CB_2 receptor activation stimulates the release of endogenous opioids from keratinocytes, allowing subsequent interaction with opioid receptors on peripheral nociceptors, and ultimately leading to analgesic and antihyperalgesic effects (Ibrahim et al., 2005).

GW405833 is a cannabinoid CB_2 receptor-selective agonist that has been previously shown to both reduce edema formation and inhibit the hypersensitivity associated with intraplantar injection of carrageenan (Clayton et al., 2002). These effects were inhibited by the cannabinoid CB_2 receptor-selective antagonist SR144528, providing evidence that the effect of GW405833 is mediated by cannabinoid CB_2 receptors (Clayton et al., 2002). Previous work from our laboratory extended the characterization and therapeutic potential of GW405833 (Valenzano et al., 2005). We demonstrated that GW405833 (up to 30 mg/kg) elicits potent and efficacious antihyperalgesic effects in rodent models of neuropathic (Seltzer), incisional and inflammatory (Freund's complete adjuvant) pain. In contrast, analgesia, sedation and catalepsy were not seen in this dose range, but were apparent at 100 mg/kg. In the current study, we confirm these properties using mouse models and investigate the role of cannabinoid CB_2 receptors in antihyperalgesia, analgesia and ataxia using knockout animals. Furthermore, using the non-selective opioid receptor antagonist, naltrexone, we have investigated the involvement of opioid-related mechanisms in GW405833-mediated antinociception.

2. Materials and methods

2.1. Compounds and reagents

The HCl salt form of GW405833 was synthesized as described previously (Valenzano et al., 2005). The free base form of the peripheral opioid receptor agonist, DiPOA ([8-(3,3-diphenyl-propyl)-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl]-acetic acid) (Valenzano et al., 2004), was synthesized as described in patent application WO 2003101953 (Victory and Chen, 2003). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

2.2. Administration procedures

For all in vivo pharmacological studies, GW405833 and DiPOA were administered intraperitoneally (i.p.) in 25% hydroxypropyl-β-cyclodextrin dissolved in distilled water. The mu opioid receptor agonist morphine was administered subcutaneously (s.c.) in 0.9% saline. The cycloxogenase-2 inhibitor celecoxib (Toronto Research Chemicals, Toronto, Canada) and the non-steroidal anti-inflammatory drug indomethacin were administered orally (p.o.) in 0.5% methylcellulose dissolved in distilled water. The anticonvulsant gabapentin (Kemprotec; Middlesborough, UK), the potent mu agonist, fentanyl, and the non-selective opioid receptor antagonist, naltrexone, were administered i.p. in 0.9% saline. All dosage formulations were sonicated for 15 min prior to administration. Dosing volumes for all compounds were 10 ml/kg in mice. The dosing volume for GW405833 in rats was 4 ml/kg. The dosing volume for all other compounds in rats was 2 ml/kg, with the exception of celecoxib and indomethacin, which were dosed orally at 10 ml/kg.

2.3. Animals

The Purdue Institutional Animal Care and Use Committee approved all animal procedures according to the guidelines of the Office of Laboratory Animal Welfare. Male Sprague–Dawley rats (Taconic; Germantown, NY) weighing 180–200 g were used. Male C57BL/6 mice (Jackson Labs; Bar Harbor, ME) and cannabinoid CB_2 receptor knockout mice in a C57BL/6 background (A. Zimmer, University of Bonn, Germany) (Buckley et al., 2000) weighing 15–30 g were used. Animals were group-housed and had free access to food and water at all times, except prior to oral administration of drugs when food was removed 12 h before dosing. For comparison with compound-treated groups, animals treated with appropriate drug vehicle were included in each experiment. The volume of administration and all other experimental procedures and conditions for vehicle and compound-treated mice or rats were identical.

2.4. Neuropathic hyperalgesia

The partial sciatic nerve ligation model (Seltzer et al., 1990) was used as a model of nerve injury-related pain in mice. Hind paw withdrawal thresholds to a non-noxious tactile stimulus (von Frey withdrawal threshold) were determined using automated electronic von Frey apparatus (IITC; Woodland Hills, CA). The electronic von Frey apparatus employs a single non-flexible filament to which the experimenter applies an increasing force. A force above 10 g was considered to be a noxious stimulus as this elicited a response in naïve animals. The stimulus was applied to site 2 of the plantar surface of the mouse hind paw as previously

described (Brennan et al., 1996). The endpoint was taken as nocifensive paw withdrawal and three thresholds were taken per test and averaged.

Baseline von Frey withdrawal thresholds were determined and partial ligation of the left sciatic nerve was performed under isofluorane (2% in oxygen) inhalation anesthesia. After induction of anesthesia, the left thigh was shaved and prepared in a sterile manner. The sciatic nerve was exposed at high thigh level through a small incision and was carefully cleared of surrounding connective tissue just distal to the bony prominence of the femur. A 7-0 silk suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle and tightly ligated so that the dorsal one-third to one-half of the nerve thickness was held within the ligature and the wound was closed. Sham-operated control mice underwent an identical procedure on the left hind limb; however, the sciatic was not manipulated or ligated. After surgery, animals ($n=9$–10/group) were weighed and returned to their home cages. Three weeks following nerve ligation, pre-drug von Frey withdrawal thresholds were measured and the mice received a single dose of either 3, 10 or 30 mg/kg GW405833, 100 mg/kg gabapentin (the positive control) or vehicle. Von Frey withdrawal threshold was again determined 1, 3, 5 and 24 h post-drug administration. Percent reversal of hyperalgesia for each mouse was calculated according to Eq. (1).

$$\%\ \text{reversal} = \frac{\text{Postdose threshold} - \text{predose threshold}}{\text{Baseline threshold} - \text{predose threshold}} \times 100 \quad (1)$$

2.5. Inflammatory hyperalgesia

The efficacy of GW405833 against hyperalgesia associated with inflammation was investigated using the Freund's complete adjuvant model in rats and mice. For mice, hind paw von Frey withdrawal thresholds were determined as described above. Baseline measurements were taken, the mice ($n=6$–10/group) were anesthetized with isofluorane (2% in oxygen) and received an intraplantar injection of 50% Freund's complete adjuvant (20 μl diluted in 0.9% saline) to the left hind paw. Twenty-four hours following Freund's complete adjuvant injection, pre-drug paw withdrawal thresholds were measured. Immediately following the pre-drug test, mice received a single dose of either 3, 10 or 30 mg/kg GW405833, 100 mg/kg indomethacin (the positive control) or vehicle. Von Frey withdrawal threshold was again determined 1, 3, 5 and 24 h post-drug administration. Percent reversal of hyperalgesia for each mouse was calculated as described by Eq. (1).

For rats, hind paw withdrawal thresholds to a noxious mechanical stimulus were determined using a model 7200 analgesymeter (Ugo Basile; Varese, Italy). Cut-off was set at 250 g and the endpoint was taken as complete paw withdrawal. Paw withdrawal threshold was determined once for each rat at each time point. Baseline paw withdrawal threshold was determined, the rats ($n=11$–12/group) were anesthetized with isofluorane (2% in oxygen) and received an intraplantar injection of 50% Freund's complete adjuvant (50 μl diluted in 0.9% saline) to the left hind paw. Twenty-four hours following Freund's complete adjuvant injection, pre-drug paw withdrawal thresholds were measured and the rats received a single dose of 30 mg/kg GW405833, 30 mg/kg celecoxib (the positive control) or vehicle. For naltrexone inhibition, rats received a single dose of 10 mg/kg naltrexone 10 min prior to 30 mg/kg GW405833. Paw withdrawal threshold was again determined 1 h post-drug administration.

2.6. Acute analgesia

The effect of GW405833 on acute analgesia was investigated using the tail flick and hot plate assays. For the tail flick, rats or mice ($n=10$–12/group) were placed on the apparatus (Ugo Basile; Varese, Italy) and an infrared beam was focused onto the tail (5 cm from the tip for rats and 2.5 cm for mice). The latency to tail flick was assessed. Cut-off was set at 20 s and the intensity was set to 35%. For the hot plate, mice ($n=10$–12/group) were placed on a metal plate maintained at 52 °C (Ugo Basile; Varese, Italy). The latency to nocifensive response, defined as hind paw lift, flutter, licking or escape behavior, was measured. Cut-off was set at 30 s. Baseline latency was determined for each animal. Approximately 1 h later, the animals received a single dose of either 10, 30 or 100 mg/kg GW405833, 10 mg/kg morphine or 0.125 mg/kg fentanyl (positive controls), or vehicle. For naltrexone inhibition, rats received a single dose of 10 mg/kg naltrexone 10 min prior to 30 mg/kg GW405833 or positive control. Tail flick and hot plate latencies were again determined 1, 3 and/or 5 h post-drug administration. Latency was determined once for each animal at each time point.

2.7. Ataxia/motor coordination

To examine the potential effects of GW405833 on motor performance, mice were tested using the accelerating rotarod (Accuscan; Columbus, OH) using established protocols. The rotarod was set to accelerate from 0 to 40 rpm over 300 s, with the maximum time spent on the rotarod set at 300 s. Wild-type and knockout mice ($n=8$–10/group) received two training trials on the first day and then received a single dose of 100 mg/kg GW405833, 30 mg/kg morphine (positive control) or vehicle. Animals were tested on the rotarod 1 h following drug administration. Animals unable to stay on the rotarod were assigned a latency of 0 s; animals completing a trial were assigned a latency of 300 s.

2.8. Statistical analysis

Data are presented as the mean ± S.E.M. Untransformed data (thresholds/latencies) were analysed using a one-way analysis of variance (ANOVA). In instances where a main effect was detected, planned comparisons were made using Fisher's PLSD post hoc analysis with $P \leq 0.05$ considered statistically significant from vehicle-treated controls.

3. Results

3.1. GW405833 reduces tactile allodynia associated with nerve injury and inflammation

Partial ligation of the sciatic nerve in mice resulted in the development of tactile allodynia within 2 weeks of surgery as indicated by a decreased paw withdrawal threshold (von Frey withdrawal threshold) to a non-noxious tactile stimulus measured with an automated electronic von Frey apparatus. Intraperitoneal administration of 30 mg/kg GW405833 to mice 3 weeks after surgery produced a reduction in mechanical hyperalgesia 1 h ($F_{(5,53)}$=9.990, P=0.0001) and 3 h ($F_{(5,53)}$=6.770, P<0.002) post-administration (Fig. 1A). The maximum percent reversal (59.4±11.0%) was achieved 1 h following the 30 mg/kg dose. Contrary to its effects on mechanical hyperalgesia in rats (Valenzano et al., 2005), doses of GW405833 below 30 mg/kg (10 and 3 mg/kg) had no significant effect on tactile allodynia in mice (Fig. 1A). Intraperitoneal administration of gabapentin (100 mg/kg) also produced a statistically significant reversal of hyperalgesia 1 and 3 h post-administration (Fig. 1A). These data indicate that GW405833 can partially reverse the tactile allodynia associated with nerve damage in mice, as shown previously with rats.

Intraplantar injection of 20 µl Freund's complete adjuvant into the hind paw of mice resulted in the development of tactile allodynia as indicated by a decreased von Frey withdrawal threshold to a non-noxious tactile stimulus measured with an automated electronic von Frey apparatus (Fig. 1B). Intraperitoneal administration of GW405833 produced a dose-dependent reduction in tactile allodynia 1 h ($F_{(5,52)}$=12.728, P<0.0001) and 3 h ($F_{(5,52)}$=10.076, P<0.0001) post-administration. At the 1-h time point, statistically significant antiallodynia by GW405833 was seen with the 10- and 30-mg/kg doses. At the 3-h time point, statistically significant antiallodynia was seen only with the 30-mg/kg dose. The maximum percent reversal (63.4±10.1%) was achieved 1 h following the 30 mg/kg dose. Oral administration of indomethacin (100 mg/kg) also produced a statistically significant reversal of tactile allodynia 1 h post-administration (Fig. 1B). Intraplantar injection of 20 µl Freund's complete adjuvant into the hind paw of cannabinoid CB_2 receptor knockout mice also resulted in the development of tactile allodynia as indicated by a decreased von Frey withdrawal threshold to a non-noxious tactile stimulus measured with an automated electronic von Frey apparatus (Fig. 1C). Unlike in wild-type mice (P=0.01), intraperitoneal administration of 30 mg/kg GW405833 to knockout animals did not produce a statistically significant reduction in tactile allodynia 1 h post-administration, as compared to knockout animals treated with vehicle (P=0.98) (Fig. 1C).

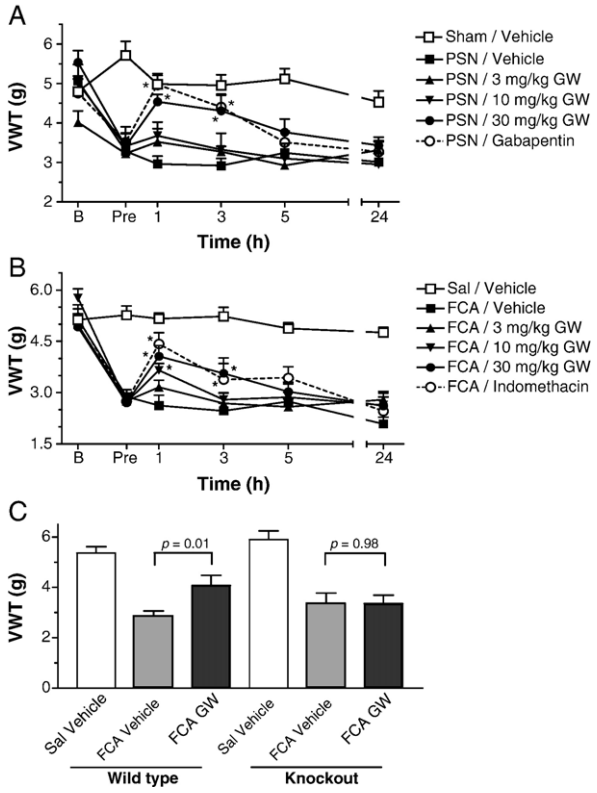

Fig. 1. GW405833 (GW) dose-dependently reverses tactile allodynia associated with nerve injury and inflammation in mice. Panel A: mice underwent surgery involving partial ligation of the sciatic nerve. Three weeks post-surgery, mice were administered increasing doses of GW405833 (i.p.), 100 mg/kg gabapentin (i.p.) or vehicle as indicated. von Frey withdrawal thresholds (VWT) in grams (g) were determined and are plotted vs. time. Asterisks denote significance ($P \leq 0.05$) from the partial sciatic nerve ligation (PSN)/vehicle-treated control group according to Fisher's PLSD post hoc test. Data shown are the mean±S.E.M. (n=9–10 mice/group). B, baseline; Pre, pre-drug reading. Panel B: wild-type mice received an intraplantar injection of 20 µl saline (open squares) or 50% Freund's complete adjuvant (FCA) (all other groups) into the hind paw, followed 24 h later by administration of increasing doses of GW405833 (GW) (i.p.), 100 mg/kg indomethacin (p.o.) or vehicle as indicated. Von Frey withdrawal thresholds (VWT) were determined and are plotted vs. time. Asterisks denote significance ($P \leq 0.05$) from Freund's complete adjuvant/vehicle-treated control group according to Fisher's PLSD post hoc test. Data shown are the mean±S.E.M. (n=10 mice/group). B, baseline; Pre, pre-drug reading. Panel C: wild-type or cannabinoid CB2 receptor knockout mice received intraplantar injections of Freund's complete adjuvant (FCA) or saline (sal) as described in Panel B, followed 24 h later by administration of 30 mg/kg GW405833 (i.p.) or vehicle as indicated. Paw withdrawal thresholds were measured 1 h after compound administration. Significance ($P \leq 0.05$) is shown according to Fisher's PLSD post hoc test. Data shown are the mean±S.E.M. (n=6–9 mice/group). Panels B and C are reprinted from Valenzano et al. (2005) with permission from Elsevier.

3.2. The central effects of high-dose GW405833 are not mediated by CB_2

We previously tested the effects of GW405833 on acute nociception in rats and showed that high dose (100 mg/kg, i.p.) produces a significant increase in both hot plate and tail flick latencies, with no effect at doses of 30 mg/kg or less (Valenzano et al., 2005). To investigate the contribution of the cannabinoid CB_2 receptor to these analgesic properties, we utilized cannabinoid CB_2 receptor knockout mice (Fig. 2, Panels A and B). In line with our previous findings in rats, 100 mg/kg GW405833 produced a significant increase in both hot plate ($F_{(4,45)}$=23.683, P=0.0003) and tail flick ($F_{(4,45)}$=9.639, P=0.031) latencies 1 h post-administration in wild-type mice. Similarly, morphine (10 mg/kg, s.c.) produced a significant increase in hot plate ($F_{(4,45)}$=23.683, P<0.0001) and tail flick ($F_{(4,45)}$=9.639,

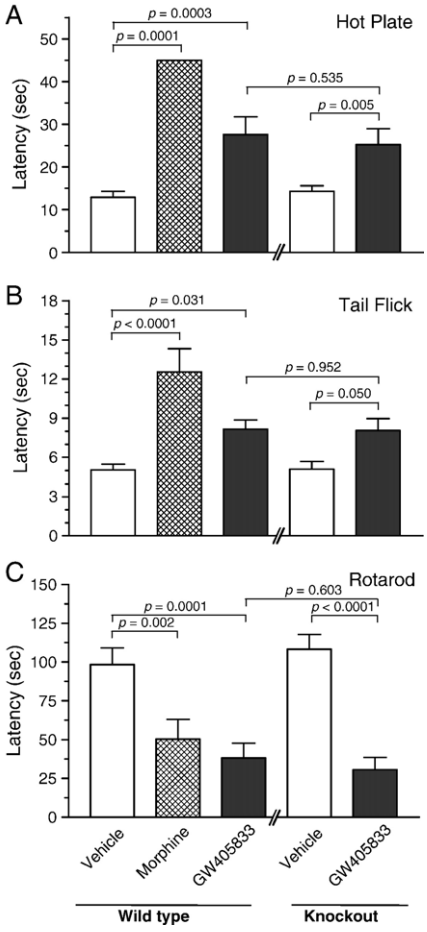

Fig. 2. The central effects of high-dose GW405833 are not mediated by CB2 receptors. The effects of GW405833 in assays of acute nociception were determined. Hot plate (Panel A) and tail flick (Panel B) latencies in wild-type and cannabinoid CB2 receptor knockout mice were determined 1 h after administration of vehicle, 100 mg/kg GW405833 (i.p.) or 10 mg/kg morphine (s.c.). The effects of GW405833 on motor performance were measured using the rotarod assay (Panel C). Wild-type and cannabinoid CB2 receptor knockout mice were placed on a rotarod set to accelerate from 0 to 40 rpm over 300 s and the latency to fall off the rotarod in seconds (sec) was recorded 1 h post-administration of vehicle, 100 mg/kg GW405833 (i.p.) or 30 mg/kg morphine (s.c.). Significance ($P \leq 0.05$) is shown according to Fisher's PLSD post hoc test. Data presented are mean latency values±S.E.M. (n=8–12 mice/group).

$P<0.0001$) latencies 1 h post-administration in wild-type mice. Importantly, 100 mg/kg GW405833 also produced a significant increase in hot plate ($F_{(4,45)}=23.683$, $P=0.005$) and tail flick ($F_{(4,45)}=9.639$, $P=0.05$) latencies 1 h post-administration in cannabinoid CB_2 receptor knockout mice. These data suggest that the analgesic properties of high-dose GW405833 are not mediated by the cannabinoid CB_2 receptor.

A common side effect of compounds used to treat inflammatory and neuropathic pain is ataxia, which can confound the interpretation of behavioral assays. We previously tested the effects of GW405833 on motor function using the rotarod assay in rats and mice and showed that high dose (100 mg/kg, i.p.) produces significant deficits in performance, with no effect at doses of 30 mg/kg or less (Valenzano et al., 2005). To investigate the contribution of the cannabinoid CB_2 receptor to these ataxic properties, we utilized cannabinoid CB_2 receptor knockout mice (Fig. 2C). In line with our previous findings, 100 mg/kg GW405833 produced a significant decrease ($F_{(4,45)}=12.276$, $P=0.0001$) in rotarod performance 1 h post-administration in wild-type mice. Similarly, morphine (10 mg/kg, s.c.) produced a significant decrease in performance ($F_{(4,45)}=12.276$, $P=0.002$) 1 h post-administration in wild-type mice. Importantly, 100 mg/kg GW405833 also produced a significant decrease in rotarod performance ($F_{(4,45)}=12.276$, $P<0.0001$) 1 h post-administration in cannabinoid CB_2 receptor knockout mice. These data suggest that the ataxic properties of high-dose GW405833 are not mediated by the cannabinoid CB_2 receptor.

3.3. The antihyperalgesic and analgesic effects of GW405833 are not dependent on release of endogenous opioids

Intraplantar injection of 50 μl Freund's complete adjuvant into the hind paw of rats resulted in the development of mechanical hyperalgesia as indicated by a decreased paw withdrawal threshold to a noxious mechanical stimulus (Fig. 3, Panels A and B). We previously showed that intraperitoneal administration of GW405833 dose-dependently reverses the mechanical hyperalgesia associated with Freund's complete adjuvant-induced inflammation (Valenzano et al., 2005). In line with these previous findings, intraperitoneal administration of 30 mg/kg GW405833 in this study produced a reduction in mechanical hyperalgesia 1 h ($F_{(6,53)}=15.954$, $P<0.0001$) post-administration (Fig. 3A). To test the hypothesis that the antihyperalgesic properties of GW405833 result from the stimulation of endogenous opioid release, rats were pretreated with the non-selective opioid receptor antagonist, naltrexone (10 mg/kg, i.p.), prior to administration of GW405833 (30 mg/kg, i.p.). Naltrexone pretreatment had no effect on the antihyperalgesic activity of GW405833 (Fig. 3A). As a positive control, the peripherally acting opioid receptor agonist, DiPOA (Valenzano et al., 2004; Whiteside et al., 2004), was tested in this model with and without naltrexone pretreatment (Fig. 3B). Pretreatment of rats with naltrexone (10 mg/kg, i.p.) significantly ($P=0.0002$) inhibited the antihyperalgesic activity of DiPOA (10 mg/kg, i.p.), indicating that naltrexone is available to antagonize peripheral opioid receptors by the route and at the dose utilized. Oral administration of celecoxib (30 mg/kg) also produced a statistically significant reversal of hyperalgesia 1 h post-administration (Fig. 3, Panels A and B). These data suggest that the antihyperalgesic activity of GW405833 is not dependent on the release of endogenous opioids.

As described above, GW405833 produced significant effects in rat and mouse assays of acute nociception (Valenzano et al., 2005 and Fig. 2). In this study, modest, but significant, analgesic effects were seen in the rat tail flick assay at the 1, 3 and 5 h time points after high-dose GW405833 administration (100 mg/kg, i.p.) (Fig. 3C). Similarly, morphine (10 mg/kg, s.c.) produced a significant increase in tail flick latency in this assay (Fig. 3C). To test the hypothesis that the analgesic properties of GW405833 result from the stimulation of endogenous opioid release, rats were pretreated with the non-selective

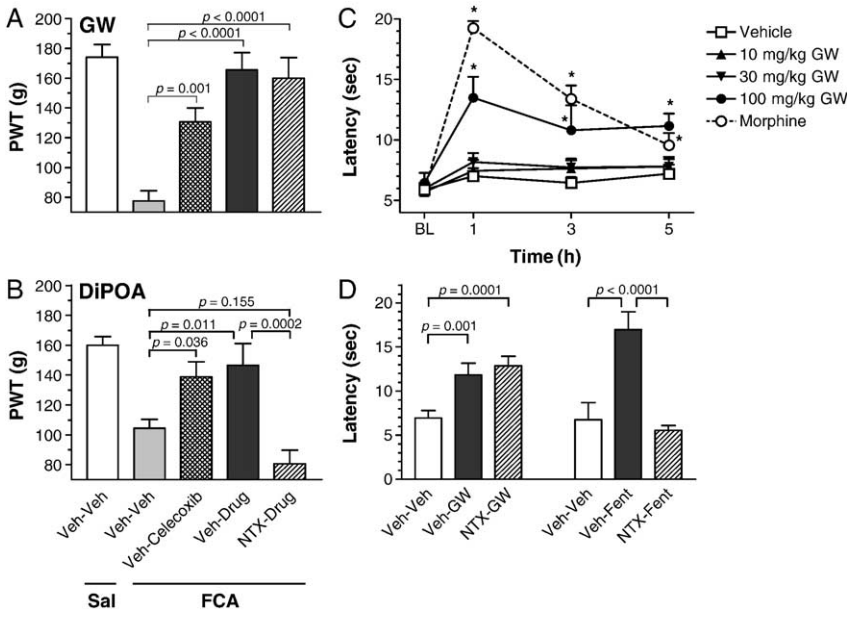

Fig. 3. The antihyperalgesic and analgesic effects of GW405833 are not dependent on release of endogenous opioids. Panels A and B: rats received an intraplantar injection of 50 μl saline (Sal group) or 50% Freund's complete adjuvant (FCA groups) into the hind paw, followed 24 h later by administration of drug. Rats were dosed with vehicle, 30 mg/kg celecoxib (p.o.) or 30 mg/kg GW405833 (GW) (i.p.) ±10 min pretreatment with 10 mg/kg naltrexone (i.p.) (NTX) as indicated (Panel A). Paw withdrawal thresholds were determined 1 h later. Similarly, rats were dosed with vehicle, 30 mg/kg celecoxib (p.o.) or 10 mg/kg DiPOA (i.p.) ±10 min pretreatment with 10 mg/kg naltrexone (i.p.) as indicated (Panel B). Paw withdrawal thresholds were determined 1 h later. Significance ($P \leq 0.05$) is shown according to Fisher's PLSD post hoc test. Data shown are the mean±S.E.M. ($n = 8$–10 rats/group). Panel C: Tail flick latency in seconds (sec) in rats was determined before and 1, 3 and 5 h after administration of 10, 30 or 100 mg/kg GW405833 (GW) (i.p.), 10 mg/kg morphine (s.c.) or vehicle as indicated. Asterisks denote significance ($P \leq 0.05$) from the vehicle-treated control group according to Fisher's PLSD post hoc test. Data shown are the mean±S.E.M. ($n = 11$–12 rats/group). BL, baseline latency. Panel D. Rats were dosed with vehicle alone or 30 mg/kg GW405833 (GW) (i.p.) ±10 min pretreatment with 10 mg/kg naltrexone (NTX) (i.p.) as indicated. Tail flick latencies in seconds (sec) were determined 1 h later. Similarly, rats were dosed with vehicle alone or 0.125 mg/kg fentanyl (Fent) (i.p.) ±10 min pretreatment with 3.125 mg/kg naltrexone (i.p.) as indicated, with tail flick latencies determined 0.5 h later. Significance ($P \leq 0.05$) is shown according to Fisher's PLSD post hoc test. Data shown are the mean±S.E.M. ($n = 10$ rats/group).

opioid receptor antagonist, naltrexone (10 mg/kg, i.p.), prior to administration of GW405833 (30 mg/kg, i.p.). Naltrexone pretreatment had no effect on the analgesic activity of GW405833 (Fig. 3D). As a positive control, the highly potent, centrally acting opioid receptor agonist, fentanyl, was tested in this assay with and without naltrexone pretreatment (Fig. 3D). Pretreatment of rats with naltrexone (3.125 mg/kg, i.p.) significantly ($P < 0.0001$) inhibited the analgesic activity of fentanyl (0.125 mg/kg, i.p.), indicating that naltrexone is available to antagonize opioid receptors by the route and at the dose utilized. These data suggest that the analgesic activity of GW405833 is not dependent on the release of endogenous opioids.

4. Discussion

While the antinociceptive mechanism of selective cannabinoid CB_2 receptor agonists is poorly understood, recent data suggest a role for endogenous opioids (Ibrahim et al., 2005). In this paper, we confirm and extend the antihyperalgesic, analgesic and ataxic activities of the cannabinoid CB_2 receptor-selective agonist, GW405833, using mouse models and demonstrate a role for cannabinoid CB_2 receptors in its antihyperalgesic, but not analgesic or ataxic, effects using knockout animals. Furthermore, we show that the antihyperalgesic and analgesic activities of GW405833 are not dependent on the release of endogenous opioids.

In the first set of experiments, GW405833 produced a significant and cannabinoid CB_2 receptor-dependent reversal of tactile allodynia in mouse models of chronic pain. Although presented in our previous report (Valenzano et al., 2005), here we include data from the mouse Freund's complete adjuvant model for comparison. These results are in line with our previous demonstration of GW405833-mediated antihyperalgesia in rat models of chronic pain (Valenzano et al., 2005). Similar to GW405833, reversal of neuropathic hyperalgesia has also been shown with the cannabinoid CB_2 receptor-selective and non-selective agonists, AM1241 and CP55,940, respectively (Ibrahim et al., 2003; Scott et al., 2004). Reversal of inflammatory pain has also been demonstrated using the cannabinoid CB_2 receptor-selective agonists, HU-308 (Hanuš et al., 1999), AM1241 (Nackley et al., 2003; Quartilho et al., 2003) and GW405833 (Clayton et al., 2002). In these studies, agonist efficacy was attenuated by co-administration of a selective cannabinoid CB_2 receptor antagonist. In our studies, we utilized cannabinoid CB_2 receptor knockout mice to provide evidence that the activity of GW405833 against Freund's complete adjuvant-induced tactile allodynia is indeed mediated by cannabinoid CB_2 receptors, as antiallodynia was not apparent in the knockout mice.

In the second set of experiments, we confirmed the analgesic and ataxic effects of high-dose GW405833 using mice. In line with our previous data from rats, 100 mg/kg

was both analgesic and ataxic. Interestingly, analgesia and ataxia were also evident in cannabinoid CB_2 receptor knockout mice demonstrating that, unlike in the Freund's complete adjuvant model, these effects are not mediated by cannabinoid CB_2 receptors. These data are indicative of a centrally mediated effect and would be consistent with activity at cannabinoid CB_1 receptors. Indeed, GW405833 shows both moderate affinity for rat cannabinoid CB_1 receptors and significant CNS penetration (Valenzano et al., 2005). However, studies investigating the effect of high-dose GW405833 in cannabinoid CB_1 receptor knockout mice would be required to unequivocally prove this hypothesis. Similar to GW405833, HU-308 was without effect in assays of acute nociception and ambulation/rearing (Hanuš et al., 1999). Likewise, AM1241 caused no deficits in the rotarod assay (Malan et al., 2001). However, AM1241 did show antinociceptive effects towards an acute thermal stimulus when administered either systemically or locally; this effect could be reversed with the cannabinoid CB_2 receptor-selective antagonist, AM630 (Malan et al., 2001). Similarly, CP55,940-induced thermal antinociception in the rat tail flick assay was partially inhibited in the presence of SR144528, suggesting a contribution of cannabinoid CB_2 receptors to acute thermal nociception (Scott et al., 2004). While the underlying cause of the differential activity in acute nociceptive assays is currently unknown for these cannabinoid CB_2 receptor agonists, differences in their interactions with cannabinoid receptors or in their downstream signaling properties may offer an explanation. Indeed, recent evidence suggests that AM1241 can stimulate the release of endogenous opioids from keratinocytes (Ibrahim et al., 2005), and that this release is responsible for at least some of its antinociceptive properties.

To this end, in the third set of experiments, we investigated the involvement of opioidergic mechanisms in the antinociceptive effects of GW405833 in rats. Pre-treatment with the non-selective opioid receptor antagonist, naltrexone, modulated neither the antihyperalgesic effect of GW405833 against inflammatory pain nor the analgesic effect of high-dose GW405833 in the tail flick assay. The dose and route of administration chosen for naltrexone were sufficient to inhibit both the antihyperalgesic effect of the peripheralized opioid receptor agonist, DiPOA (Valenzano et al., 2004; Whiteside et al., 2004) and the analgesic effect of fentanyl, indicating antagonism of both central and peripheral opioid receptors. These data strongly suggest that the antinociceptive properties of GW405833 are not dependent on opioid signaling. Thus, our data are inconsistent with the postulated mechanism of AM1241 against thermal hyperalgesia, which stems, at least partially, from stimulation of β-endorphin release from keratinocytes, which subsequently acts at local opioid receptors on surrounding peripheral neurons (Ibrahim et al., 2005). This mechanism is difficult to reconcile in light of previous studies demonstrating that local opioid agonist administration, or systemic administration of peripheralized opioid agonists, reverses the mechanical hyperalgesia associated with inflammation, but is without effect in the absence of an inflammatory insult (Stein et al., 1988, 1989; Whiteside et al., 2004). Similarly, stress-induced release and local administration of endogenous opioids, including β-endorphin, reverse mechanical hyperalgesia associated with inflammation, but are without effect in the non-inflamed paw (Stein et al., 1990). It is conceivable that thermal and mechanical nociception are modulated by different complements of endogenous mediators, which account, at least partially, for these discrepancies. However, this explanation is somewhat unsatisfactory, as both GW405833 and HU-308 (Hanuš et al., 1999) were without effect in naïve animals using the hot plate and tail flick assays. Differences in the compounds' interactions with cannabinoid CB_2 receptors (i.e., affinity, potency and efficacy) or differential modulation of cannabinoid CB_2 receptor-mediated intracellular signaling cascades may be involved. Indeed, recent data indicate that activation of cannabinoid CB_2 receptors on hematopoietic precursor cells results in cellular migration if activated by the endocannabinoid, 2-arachidonoylglycerol, and blockade of neutrophil differentiation if activated by CP55,940, indicating distinct effects depending on the agonist used (Alberich Jorda et al., 2004). Furthermore, off-target effects for one or more of these selective cannabinoid CB_2 receptor agonists could be at play. Hence, a better understanding of the molecular pharmacologies of these agents may shed light on their in vivo properties with respect to pain modulation.

Given that the antinociceptive activity of GW405833 is apparently independent of endogenous opioid release, alternative mechanisms may be at play. A more widely accepted hypothesis is that cannabinoid CB_2 receptor modulators affect pain indirectly via modulation of immune cell activity, possibly at the site of injury, resulting in decreased local levels of sensitizing agents, such as NGF, prostanoids, cytokines and histamine, that ultimately affect neuronal excitability. This hypothesis is less satisfactory in explaining the efficacy of GW405833 against neuropathic pain. It is possible that GW405833 acts at the site of nerve damage and reduces perineural inflammation, thus reducing sensitization of the peripheral nerve. Alternatively, GW405833 could act at central cannabinoid CB_2 receptors, the expression of which is increased in response to nerve damage (Zhang et al., 2003). The significant CNS penetration of GW405833 combined with significant reversal of neuropathic pain at doses that result in comparatively low plasma levels (Valenzano et al., 2005) may suggest that the latter is at least a contributing factor.

In conclusion these data demonstrate that the antihyperalgesic effects of GW405833 are mediated via the cannabinoid CB_2 receptor, whereas the analgesic and sedative effects are not. Furthermore, these data strongly suggest that the mechanism of action for GW405833 does not depend on release of endogenous opioids.

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