RESEARCH PAPER

Peripheral and central sites of action for the non-selective cannabinoid agonist WIN 55,212-2 in a rat model of post-operative pain

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Background and purpose: Activation of cannabinoid (CB) receptors decreases nociceptive transmission in inflammatory or neuropathic pain states. However, the effects of CB receptor agonists in post-operative pain remain to be investigated. Here, we characterized the anti-allodynic effects of WIN 55,212-2 (WIN) in a rat model of post-operative pain.

Experimental approach: WIN 55,212-2 was characterized in radioligand binding and *in vitro* functional assays at rat and human CB_1 and CB_2 receptors. Analgesic activity and site(s) of action of WIN were assessed in the skin incision-induced post-operative pain model in rats; receptor specificity was investigated using selective CB_1 and CB_2 receptor antagonists.

Key results: WIN 55,212-2 exhibited non-selective affinity and agonist efficacy at human and rat CB_1 versus CB_2 receptors. Systemic administration of WIN decreased injury-induced mechanical allodynia and these effects were reversed by pretreatment with a CB_1 receptor antagonist, but not with a CB_2 receptor antagonist, given by systemic, intrathecal and supraspinal routes. In addition, peripheral administration of both CB_1 and CB_2 antagonists blocked systemic WIN-induced analgesic activity.

Conclusions and implications: Both CB_1 and CB_2 receptors were involved in the peripheral anti-allodynic effect of systemic WIN in a pre-clinical model of post-operative pain. In contrast, the centrally mediated anti-allodynic activity of systemic WIN is mostly due to the activation of CB_1 but not CB_2 receptors at both the spinal cord and brain levels. However, the increased potency of WIN following i.c.v. administration suggests that its main site of action is at CB_1 receptors in the brain.

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Introduction

Cannabinoid (CB) receptors have emerged as an attractive therapeutic target for pain management in recent years (Walker and Hohmann, 2005; Pacher *et al.*, 2006). CB activity is mediated via the activation of inhibitory G-protein coupled CB receptors, namely CB_1 and CB_2 receptors (Pertwee, 2001; Walker and Hohmann, 2005). CB_1 receptors are expressed at

all levels of the pain-modulating pathways, including the primary afferent fibres, dorsal root ganglia (DRG), dorsal horn of the spinal cord and supraspinal sites, whereas CB_2 receptors are primarily expressed in immunocompetent cells and are not thought to be present on neuronal cells of the central nervous system (CNS) (Piomelli, 2005; Walker and Hohmann, 2005; Agarwal *et al.*, 2007). Correspondingly, endocannabinoid concentrations are increased along the nociception-relevant somatosensory neuraxis in various pain states (Walker and Hohmann, 2005; Mitrirattanakul *et al.*, 2006), and anandamide has been shown to inhibit carragee-nan-induced hyperalgesia, capsaicin-induced oedema and formalin-induced pain in rats (Calignano *et al.*, 1998;

Richardson et al., 1998a; Walker and Hohmann, 2005). Additionally, electrophysiological experiments have shown CB receptor activation following carrageenan injection and anandamide decreased carrageenan-evoked spinal neuronal response in rats (Sokal et al., 2003).

In animal studies, the anti-nociceptive efficacy of CBs and analogues has been demonstrated in several models of inflammatory and neuropathic pain (Walker and Hohmann, 2005). Synthetic CB agonists, such as WIN 55,212-2 (WIN), are potent activators of CB receptors and have demonstrable analgesic activity in rodents, reducing complete Freund's adjuvant (CFA)-induced inflammatory pain, formalin-induced persistent nociception (Tsou et al., 1996; Martin et al., 1999), and neuropathic pain caused by peripheral nerve injury (Fox et al., 2001; Costa et al., 2004), toxic nerve damage (Pascual et al., 2005) and diabetic neuropathy (Dogrul et al., 2004). Numerous studies have demonstrated that activation of CB receptors at several levels along the pain-modulating pathways can reduce nociceptive transmission, indicating that multiple sites of action are implicated in the anti-nociceptive effects of WIN. However, the relative contribution of each of these sites, or different subtypes of CB receptors, to the analgesic effects of systemic CB agonists remains unclear (Walker and Hohmann, 2005). Much less is known about the analgesic activity of CBs in post-operative pain (Valenzano et al., 2005).

Paw incision in rats induces a variety of nocifensive behaviours that parallel the time course of post-operative pain in humans (Brennan et al., 1996). This model has been widely used to determine the analgesic profile of novel and existing analgesic agents (Zahn and Brennan, 1998; Wang et al., 2000; Pogatzki et al., 2002; Brennan et al., 2005; LaBuda et al., 2005; Valenzano et al., 2005; Zhu et al., 2005). The present study was undertaken to characterize the anti-nociceptive effects of WIN, and to explore the relative contributions of supraspinal, spinal and peripheral CB receptors to mechanical hypersensitivity in a rat paw incision model of post-operative pain. Additionally, the potential CB receptor subtypes that mediate the analgesic activity of WIN in this model were also investigated.

Methods

Animals

All procedures were performed in an AAALAC-accredited facility and approved by the Institutional Animal Care and Use Committee of Abbott Laboratories. Adult male Sprague Dawley rats (300–350 g, Charles River Laboratories, Wilmington, MA, USA) were either housed five per cage or single-housed (animals implanted with i.t. catheters or i.c.v. cannulae). Animals were acclimated to the laboratory environment for 5–7 days before entering the study. All animals were kept in a temperature-regulated environment under a controlled 12 h light–dark cycle with lights on at 6:00 am. Food and water were provided ad libitum.

In vitro binding assays and measurement of cAMP in human embryonic kidney (HEK) cells

The selectivity of WIN for CB$_1$ or CB$_2$ receptors was assessed by performing competition-binding experiments in membranes prepared from HEK or the Chinese hamster ovary (CHO) cell lines, which stably express the human CB$_2$ (hCB$_2$) or CB$_1$ (hCB$_1$) receptors as previously described (Yao et al., 2006; 2008). For in vitro binding assays, membranes (CB$_1$ or CHO-K1) were incubated at 30°C for 90 min with 1 nmol·L^{-1} [^{3}H]-CP 55,940 in 250 µL of assay buffer (50 mmol·L^{-1} Tris, 2.5 mmol·L^{-1} EDTA, 5 mmol·L^{-1} MgCl$_2$ and 0.5 mg·mL^{-1} fatty acid free BSA, pH 7.4) in the presence of increasing concentration of unlabeled competitor compounds (Yao et al., 2008). Competitive binding at the hCB$_2$ receptor was conducted under the same conditions except the [^{3}H]-CP 55,940 concentration used was 0.5 nmol·L^{-1}. Data were analysed using GraphPad PRISM™, v. 3.0 (GraphPad, San Diego, CA, USA). K$_i$ values from competition binding assays were determined with one-site competition curve fitting using the Prism software (GraphPad, San Diego, CA, USA). Rat cortical membranes were prepared from rat cerebral cortex purchased from Pel-Freez Biologicals (Roger, AR, USA). The assay conditions for binding to rat cortical membranes were similar to those for rat CB$_1$ receptor binding using recombinant receptors, except that the protein concentration was 10-fold higher.

The cyclase functional assays were performed using the DiscoverX HitHunter cAMP assay kit according to the vendor's protocol as previously described (Yao et al., 2006; 2008). The CB receptor agonist CP 55,940 was used as the positive control for EC$_{50}$ determination, in which 10 µmol·L^{-1} CP 55,940 was measured as 100% efficacy. EC$_{50}$ values were calculated by curve fitting of sigmoidal dose response from Prism (GraphPad). All EC$_{50}$ values are presented as mean ± SEM.

Surgical preparation

The surgical procedure of paw incision was performed under isoflurane (2–3%) anesthesia as previously described (Brennan et al., 1996). Briefly, the plantar aspect of the right hind paw was placed through a hole in a sterile plastic drape. A longitudinal incision (1 cm) was made through the skin and fascia, starting 0.5 cm from the proximal edge of the heel and extending towards the toes, the plantar muscle was elevated and incised longitudinally leaving the muscle origin and insertion points intact. After haemostasis with gentle pressure, the skin was apposed with two mattress sutures (5–0 nylon).

Intrathecal (i.t.) catheterization was performed following the procedure as described (Yaksh and Rudy, 1976) in some animals under isoflurane (2–3%) anesthesia. In these animals, a PE-5 catheter (Marsil Enterprises, San Diego, CA, USA) was inserted through the atlanto-occipital membrane via a small hole in the cisterna magna. The catheter was then advanced 8.5 cm caudally such that the tip ended in the intrathecal space around the lumbar enlargement. The catheter was then secured to the musculature at the incision site. The catheter implantation was performed at least 7 days prior to paw incision surgery.

For intracerebroventricular (i.c.v.) cannulation, guide cannulae (22 gauge) were implanted into the left lateral ventricle of rats anesthetized with pentobarbital (50–60 mg·kg^{-1}, i.p.) in a separate group of animals. The cannula was located 1.6 mm lateral to midline and 1.0 mm caudal to Bregma, with a depth of 4.5 mm (Paxinos and Watson, 1982). Cannula placement

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was performed at least 7 days prior to paw incision. Cannula placement technique was verified by infusion of 0.5% fast-green dye in saline solution and subsequent dissection in a pilot study, with >95% accuracy of cannula implantation.

Behavioural assessment

A quantitative allodynia assessment technique was conducted to measure mechanical allodynia using calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) as previously described (Chaplan *et al.*, 1994) in all animals 120 min following surgery. Rats were placed into inverted individual plastic containers ($20 \times 12.5 \times 20$ cm) on top of a suspended wire mesh grid, and acclimated to the test chambers for 20 min. The von Frey filaments were presented perpendicularly to the plantar surface pointing towards the medial side of the incision (Brennan *et al.*, 1996), and then held in this position for approximately 8 s with enough force to cause a slight bend in the filament. Positive responses included an abrupt withdrawal of the hind paw from the stimulus, or flinching behaviour immediately following removal of the stimulus. A 50% paw withdrawal threshold (PWT) was determined using an up-down procedure (Dixon, 1980).

Horizontal exploratory behaviour was measured in an open field using photobeam activity monitors (AccuScan Instruments, Columbus, OH, USA). Rats were placed into $42 \times 42 \times 30$ cm activity chambers where photobeam breaks were recorded for 30 min. For rotarod performance, animal performance was measured using an accelerating rotarod apparatus (Omnitech Electronics, Inc. Columbus, OH). Rats were placed onto a 9 cm diameter rod, which increased in speed from 0 to 20 r.p.m. for a 60 s period. The time required for the rat to fall from the rod was automatically recorded, with a maximum cut-off of 60 s. Both Horizontal exploratory behaviour and rotarod performance were assessed 30 min following systemic WIN administration.

Experimental groups and compound administration

Systemic administration of WIN alone and pretreatment with a CB$_1$ or a CB$_2$ receptor antagonist. WIN (1, 3 and 10 µmol·kg^{-1}, i.e. 0.43, 1.3 and 4.3 mg·kg^{-1}) was administered i.p. 90 min following hind paw incision, and mechanical allodynia was determined 30 min post-treatment (2 h post-surgery). Because of the robust nociceptive behaviours observed in our previous time-course experiment (Zhu *et al.*, 2005), the 2 h time point was chosen to study the pharmacological characterization in acute post-operative pain and to be able to compare our data with the previously published pharmacological data using this model. To determine the CB receptor subtypes responsible for WIN-induced analgesic efficacy, animals were treated i.p. with either a CB$_1$ receptor antagonist SR141716A (SR1; 30 µmol·kg^{-1}, i.e. 14 mg·kg^{-1}), or a CB$_2$ receptor antagonist SR144528 (SR2; 10 µmol·kg^{-1}, i.e. 4.8 mg·kg^{-1}) 15 min prior to WIN (5 µmol·kg^{-1}, i.p.) treatment. Behavioural changes were evaluated 30 min following WIN administration. We have previously demonstrated that pretreatment with SR2 at 10 µmol·kg^{-1}, but not SR1 at 30 µmol·kg^{-1}, effectively blocked analgesic activity induced by A-796260 (a selec-

tive CB$_2$ agonist) in the same rat model of post-operative pain, whereas SR1 or SR2 given alone had no analgesic activity (Yao *et al.*, 2008).

To determine the potential confounding effects of WIN on behaviour, exploratory behaviour and motor coordination were examined by horizontal exploratory activity and rotarod performance in naïve rats 30 min following WIN (1, 3 and 10 µmol·kg^{-1}, i.p.).

Peripheral, injection of WIN in the paw, pretreatment with i.pl. SR1 or SR2 before systemic WIN. Systemically inactive doses of WIN (30, 100, 300 nmol per paw) were injected into the plantar region of the injured paw (i.pl.), 90 min following hind paw injury. To explore the possible systemic diffusion, WIN (100, 300 nmol per paw) was also injected into the contralateral, normal paw in a separate group of animals. To evaluate the peripheral contribution of the CB receptor subtypes to WIN-induced analgesic efficacy, SR1 or SR2 (150 nmol per paw) was subsequently injected i.pl. into the injured paw, 15 min prior to the systemic administration of WIN (5 µmol·kg^{-1}, i.p., 90 min following skin incision) in a separate experiment. Mechanical allodynia was assessed 30 min following i.pl. or i.p. injection of WIN.

Spinal application of WIN, pretreatment with i.t. SR1 or SR2 before systemic WIN. For intrathecal delivery, WIN (30, 100, 300 nmol per rat) was infused via an implanted catheter over 1 min, 90 min following surgery. The catheter was subsequently flushed with 5 µL of sterile water. To examine the spinal contribution of CB receptor subtypes to WIN-induced analgesic efficacy, SR1 or SR2 (150 nmol per rat) were delivered i.t. 15 min before the systemic administration of WIN (5 µmol·kg^{-1}, i.p., 90 min following skin incision) in a separate group of animals. Mechanical allodynia was assessed 30 min following i.t. or i.p. injection of WIN.

Supraspinal infusion of WIN, pretreatment with i.c.v. SR1 or SR2 before systemic WIN. For i.c.v. administration, WIN (3,10, 30, 100 nmol per rat) was infused via a 28-gauge injection cannula extending 1 mm beyond the guide cannula tip over 1 min. To investigate the supraspinal contribution of CB receptor subtypes to WIN-induced analgesic efficacy, SR1 or SR2 (150 nmol per rat) was i.c.v. infused 15 min before the systemic administration of WIN (5 µmol·kg^{-1}, i.p., 90 min following skin incision) in a separate group of animals. Mechanical allodynia was assessed 30 min following i.c.v. or i.p. injection of WIN.

Data analysis of behavioural experiments

In vivo data are presented as mean ± SEM. Statistical significance was evaluated using analysis of variance (ANOVA) followed by Bonferroni's multiple comparison (GraphPad Prism). $P < 0.05$ was considered to be significant. All behavioural experiments were performed by experimenters unaware of the treatment received by the animals.

Materials

SR141716A (a CB$_1$ receptor antagonist, SR1, molecular weight, 463.8), and SR144528 (a CB$_2$ receptor antagonist, SR2,

molecular weight, 476.1) were synthesized at Abbott Laboratories as previously described (Yao *et al*., 2006). [^{3}H]CP 55,940 (a non-selective CB receptor agonist), WIN 55212-2 (a non-selective CB receptor agonist, WIN, molecular weight, 426.5], dimethylsulphoxide (DMSO) and hydroxypropyl-β-cyclodextrin (HBC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SR1, SR2 and WIN were dissolved in a solution of DMSO/HBC at a volume of 2.0 mL·kg^{-1} for intraperitoneal (i.p.) administration (10:90, V/V), 25 µL for intraplantar (i.pl.) paw injection (20:80, V/V) and 10 µL for intrathecal (i.t.) delivery followed by a 5 µL sterile water flush, or intracerebroventricular (i.c.v.) infusion (40:60, V/V). All solutions were freshly prepared on the day of the experiments.

Results

Radioligand binding studies and measurement of cAMP

The affinity of WIN, SR1 and SR2 for human CB₁ (hCB₁) and CB₂ (hCB₂) receptors were measured using competition radioligand binding assays on CHO-K1 and HEK293 cell membranes expressing the recombinant receptors. WIN showed high affinity for hCB₂ (Ki = 1.32 ± 0.07 nmol·L^{-1}, *n* = 38) and a lower affinity for hCB₁ receptors (Ki = 15.34 ± 0.12 nmol·L^{-1}, *n* = 25). SR1 showed high hCB₁ receptor binding selectivity (Ki = 2.05 ± 0.13 nmol·L^{-1} for hCB₁, *n* = 24; Ki = 392.5 ± 0.12 nmol·L^{-1} for hCB₂, *n* = 10), whereas SR2 showed higher hCB₂ receptor binding affinity (Ki = 6.06 ± 0.09 nmol·L^{-1} for hCB₂, *n* = 14; Ki = 263.85 ± 0.08 nmol·L^{-1} for hCB₁, *n* = 12). Binding assays for rat CB₁ and rat CB₂ receptors were performed on HEK293 cell membranes expressing rat recombinant CB receptors. The affinity of WIN for rCB₂ (1.4 ± 0.12 nmol·L^{-1}, *n* = 18) was comparable to that of hCB₂ receptors but the affinity for rCB₁ (4.48 ± 0.08 nmol·L^{-1}, *n* = 11) was considerably higher than that of hCB₁ receptors. Similarly, SR1 showed high rCB₁ receptor binding selectivity (Ki = 0.7 ± 0.1 nmol·L^{-1} for rCB₁, *n* = 6; Ki = 126.55 ± 0.17 nmol·L^{-1} for rCB₂, *n* = 4), whereas SR2 showed higher rCB₂ receptor binding affinity (Ki = 1.65 ± 0.28 nmol·L^{-1} for rCB₂, *n* = 6; Ki = 428.26 ± 0.17 nmol·L^{-1} for hCB₁, *n* = 6). The affinity of WIN, SR1 and SR2 was also determined for native (rat) CB₁ receptor using cell membranes prepared from rat cerebral cortex. WIN and SR1 showed high binding affinity for rat cortex CB₁ receptors (Ki = 12.37 ± 0.057, *n* = 2; Ki = 2.77 ± 0.04 *n* = 4), whereas SR2 showed no binding affinity for rat cortex CB₁ receptors (Ki > 1 µmol·L^{-1}, *n* = 4). These data showed that the affinity of WIN, SR1 and SR2 in the native binding system is well correlated with its binding affinity in the recombinant system. Taken together, these binding data confirm that WIN is a non-selective ligand for both CB₁ and CB₂ receptors, SR1 is a selective ligand for CB₁ receptors and SR2 is a selective ligand for CB₂ receptors in our *in vitro* binding assays.

Cannabinoid receptors are seven trans-membrane receptors coupled to G proteins, specifically G$_{i/o}$, which negatively regulate adenylate cyclase. The ability of WIN to activate CB receptors and to functionally change the intracellular cAMP level was assessed in a cAMP accumulation assay using CHO K1 cells expressing human CB₁ and HEK293 cells expressing human CB₂ receptors. WIN inhibited forskolin-induced cAMP accumulation (EC₅₀: 31.87 ± 0.05 nmol·L^{-1}, *n* = 3 for hCB₁, and 0.77 ± 0.36 nmol·L^{-1}, *n* = 5 for hCB₂ receptors) in cells expressing recombinant hCB₁ and hCB₂ receptors respectively. Similarly, WIN was a potent agonist in rat adenylate cyclase assays, showing an EC₅₀ values of 21.82 ± 0.16 nmol·L^{-1}, *n* = 5 for rCB₁, and 1.28 ± 0.37 nmol·L^{-1}, *n* = 4 for rCB₂. However, in this assay, WIN showed partial agonist activity, with a maximum inhibition of 45%, *n* = 6 at 12.79 nmol·L^{-1} for rCB₂ receptors. SR2 showed inverse agonist activity at hCB₂ and rCB₂ receptors in these cyclase assays but SR1 demonstrated no activity in functional assays. Taken together, these data show that WIN is a non-selective, full agonist for rCB₁ and a partial agonist at rCB₂ receptors in functional assays *in vitro*.

Systemic WIN administration and systemic CB antagonists on systemic WIN-induced anti-allodynia

Hind paw injury resulted in the development of mechanical allodynia as indicated by a decreased PWT to a series of mechanical stimuli of calibrated von Frey filament 2 h post-incision (1.5 ± 0.1 g), compared with 15 g in non-injured paw (*P* < 0.01). WIN dose-relatedly reduced mechanical hypersensitivity, with 47 ± 6 and 63 ± 10% effect at 3 and 10 µmol·kg^{-1}, compared with vehicle-treated animals 2 h following surgery [Figure 1A, *F* (3,19) = 18, *P* < 0.001, *n* = 6 per group].

In the absence of CB antagonists, 5 µmol·kg^{-1} WIN produced 55 ± 7% anti-allodynic effects (*P* < 0.01). Pretreatment with selective CB₁ receptor antagonist SR1 (30 µmol·kg^{-1}, i.p.), but not with the CB₂ receptor antagonist SR2 (10 µmol·kg^{-1}, i.p.), fully blocked WIN (5 µmol·kg^{-1}, i.p.)-induced analgesic activity. Systemic administration of SR1 (30 µmol·kg^{-1}) or SR2 (10 µmol·kg^{-1}) alone produced no alteration of the PWT of the injured paw, compared with vehicle treated animals [Figure 1B, *F* (5,42) = 46, *P* < 0.001, *n* = 6–12 per group].

Peripheral effects of WIN and peripheral CB antagonists on systemic WIN-induced anti-allodynia

Peripheral injection of WIN (intraplantar; i.pl.) was designed to achieve localized application to the injured site, in doses ineffective at reducing post-operative pain when given systemically. Peripheral paw injection of WIN (30, 100, 300 nmol per paw, 90 min following surgery) reduced mechanical allodynia, by 46 ± 7% and 58 ± 6% at 100 and 300 nmol per paw,,respectively, compared with vehicle treated animals [Figure 2A, *F* (5,48) = 25, *P* < 0.001, *n* = 8–12 per group]. WIN applied to the contralateral side to the injury only produced anti-allodynic effects at 300 nmol per paw (38 ± 5%), which was less potent than the effects observed at the same dose when injected i.pl., at the site of injury (Figure 2A).

Systemic administration of 5 µmol·kg^{-1} WIN alone decreased allodynia, by 53 ± 17% effects. Pretreatment with i.pl. SR1 or SR2 (150 nmol per paw) reversed WIN (5 µmol·kg^{-1}, i.p.)-induced anti-allodynic effects. Intraplantar injection of SR1 or SR2 (150 nmol per paw) alone produced

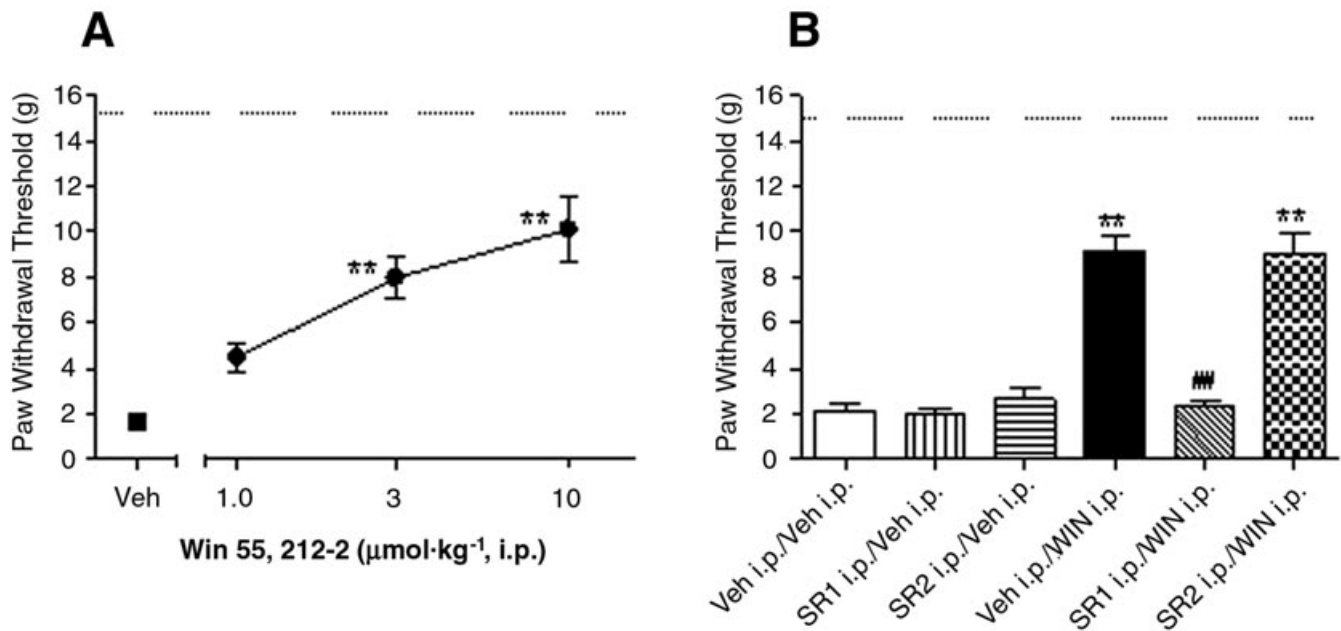Central CB$_1$ suppression of post-operative pain
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Figure 1 Effects of systemic WIN 55,212-2 (WIN) on skin incision-induced post-operative pain demonstrated as the increase of paw withdrawal threshold. (A) WIN (1, 3 and 10 µmol·kg^{-1}) was injected i.p. in rats 90 min after surgery. (B) Reversal of WIN-induced analgesic efficacy by pretreatment with CB receptor antagonists, selective CB$_1$ receptor antagonist SR141716A (SR1, 30 µmol·kg^{-1}, i.p.) or selective CB$_2$ receptor antagonist SR144528 (SR2, 10 µmol·kg^{-1}, i.p.) was injected 15 min before the administration of WIN (5 µmol·kg^{-1}, i.p.). Mechanical allodynia was assessed 30 min following WIN treatment. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus vehicle (Veh) treated group, ##$P < 0.01$, SR1 i.p./WIN i.p. versus Veh i.p./WIN i.p. group, $n = 6$ per group. Dashed line: paw withdrawal threshold of non-injured paw. CB, cannabinoid.
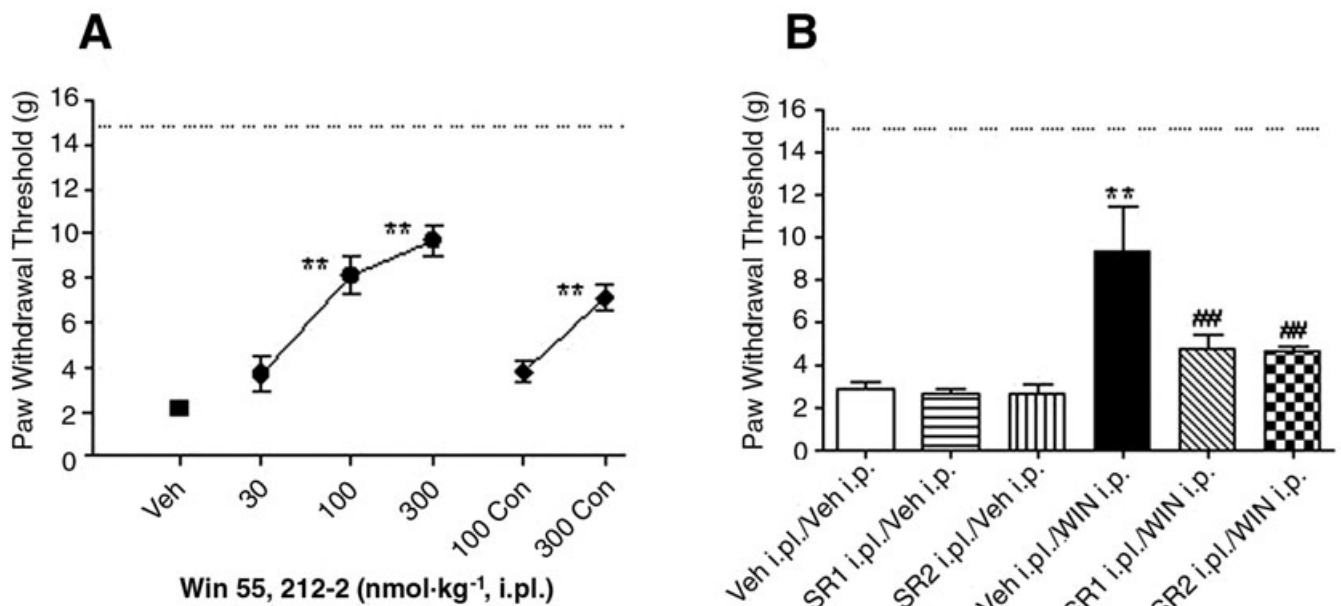

Figure 2 Effects of peripheral injection of WIN 55,212-2 (WIN) on skin incision-induced post-operative pain demonstrated as the increase of paw withdrawal threshold. (A) WIN (30, 100, 300 nmol per paw) was intraplantarly (i.pl.) injected in rats 90 min after surgery, WIN (100, 300 nmol per paw) was also injected i.pl. to the contralateral (Con) non-injured paw in a separate group of animals. (B) Reversal of WIN-induced anti-allodynic effects by peripheral pretreatment with CB antagonists, selective CB$_1$ receptor antagonist SR141716A (SR1, 150 nmol per paw) or selective CB$_2$ receptor antagonist SR144528 (SR2, 150 nmol per paw) was injected i.pl. 15 min before the administration of WIN (5 µmol·kg^{-1}, i.p.). Mechanical allodynia was assessed 30 min after WIN treatment. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus vehicle (Veh) treated group, ##$P < 0.01$, SR1 i.pl./WIN i.p. or SR2 i.pl./WIN i.p. versus Veh i.p./WIN i.p. group, $n = 6$ per group. Dashed line: paw withdrawal threshold of non-injured paw. CB, cannabinoid.
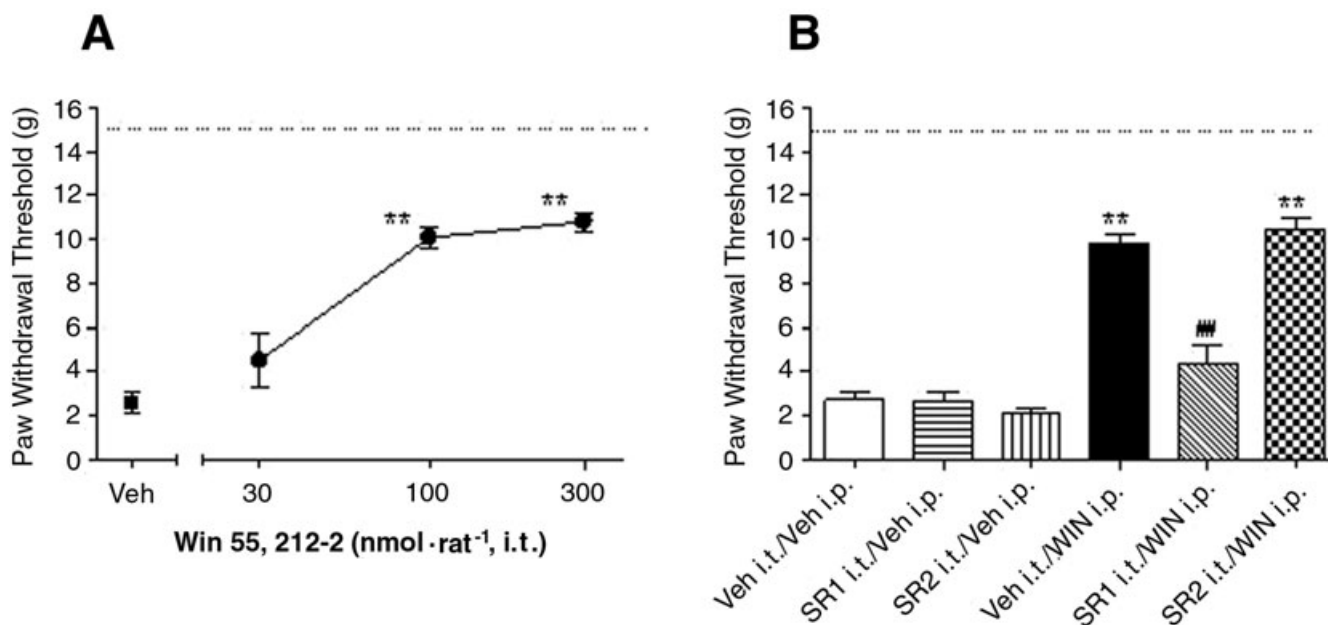
Figure 3 Effects of spinal application of WIN 55,212-2 (WIN) on skin incision-induced post-operative pain demonstrated as the increase of paw withdrawal threshold. (A) WIN (30, 100, 300 nmol per rat) was intrathecally (i.t.) applied in rats 90 min after surgery. (B) Reversal of WIN-induced analgesic efficacy by spinal pretreatment with CB antagonists, selective CB$_1$ receptor antagonist SR141716A (SR1, 150 nmol per rat) or selective CB$_2$ receptor antagonist SR144528 (SR2, 150 nmol per rat) was intrathecally delivered 15 min before the administration of WIN (5 µmol·kg^{-1}, i.p.). Mechanical allodynia was assessed 30 min after WIN treatment. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus vehicle (Veh) treated group, ##$P < 0.01$, SR1 i.t./WIN i.p. versus Veh i.t./WIN i.p. group, $n = 6$ per group. Dashed line: paw withdrawal threshold of non-injured paw. CB, cannabinoid.

no alteration of PWT of the injured paw, compared with vehicle treated animals [Figure 2B, $F_{(5,30)} = 7.3$, $P < 0.001$, $n = 6$ per group].

Spinal action of WIN and intrathecal CB antagonists on systemic WIN-induced anti-allodynia

Intrathecal (i.t.) application of WIN (30, 100, 300 nmol per rat, 90 min following surgery) reduced mechanical allodynia by 60 ± 4% and 66 ± 3%, at 100 and 300 nmol per rat respectively, compared with vehicle treated animals [Figure 3A, $F_{(3,24)} = 36$, $P < 0.001$, $n = 6$–10 per group].

Systemic administration of WIN (5 µmol·kg^{-1}, i.p.) decreased allodynia, by 58 ± 3% in the absence of CB antagonist(s). Pretreatment with i.t. SR1 (150 nmol per rat), but not SR2 (150 nmol per rat), completely reversed WIN (5 µmol·kg^{-1}, i.p.)-induced anti-allodynic effects . Intrathecal delivery of SR1 or SR2 (150 nmol per rat) alone produced no alteration of PWT of the injured paw, compared with vehicle treated animals [Figure 3B, $F_{(5,33)} = 56$, $P < 0.001$, $n = 6$–8 per group].

Supraspinal action of WIN and i.c.v. CB antagonists on systemic WIN-induced anti-allodynia

Intracerebroventricular (i.c.v.) infusion of WIN (3, 10, 30, 100 nmol per rat, 90 min following surgery) dose-dependently inhibited mechanical allodynia, by 48 ± 8, 61 ± 11 and 88 ± 6% at 10, 30 and 100 nmol per rat, respectively, compared with vehicle treated animals [Figure 4A, $F_{(4,25)} = 27$, $P < 0.001$, $n = 6$ per group].

Systemic administration of WIN (5 µmol·kg^{-1}) reduced allodynia, by 66 ± 8% in the absence of CB antagonist(s). Pretreatment with i.c.v. SR1 (150 nmol per rat) reversed WIN (5 µmol·kg^{-1}, i.p.)-induced anti-allodynic effects, whereas WIN-induced anti-allodynic effects (48 ± 4%) were not significantly affected by pre-administration of SR2 (150 nmol per rat). Intracerebroventricular infusion of SR1 or SR2 (150 nmol per rat) alone produced no alteration of PWT of the injured paw, compared with vehicle treated animals [Figure 4B, $F_{(5,39)} = 15.8$, $P < 0.001$, $n = 6$–10 per group].

CNS side effects of systemic WIN

Systemic administration of WIN (1, 3, 10 µmol·kg^{-1}) reduced horizontal exploratory activity in naïve rats, with a maximal efficacy of 71 ± 10% at 10 µmol·kg^{-1} [Figure 5A, $F_{(3,20)} = 37$, $P < 0.01$, $n = 8$ per group]. Similarly, WIN reduced fall latency in the rotarod assay by 23 ± 8% and 64 ± 5% at 3 and 10 µmol·kg^{-1} [Figure 5B, $F_{(3,20)} = 37$, $P < 0.01$, $n = 6$ per group].

Discussion

The current study is the first to demonstrate that the non-selective CB receptor agonist, WIN 55,212-2 (WIN), attenuates pain behaviour in a rat model of post-operative pain. WIN-induced anti-allodynic effects were mainly attributed to CB$_1$ receptor activation, as pretreatment with the CB$_1$ receptor antagonist SR1 by i.p., i.pl., i.t., and i.c.v. routes of administration prevented systemic WIN-induced analgesia, whereas

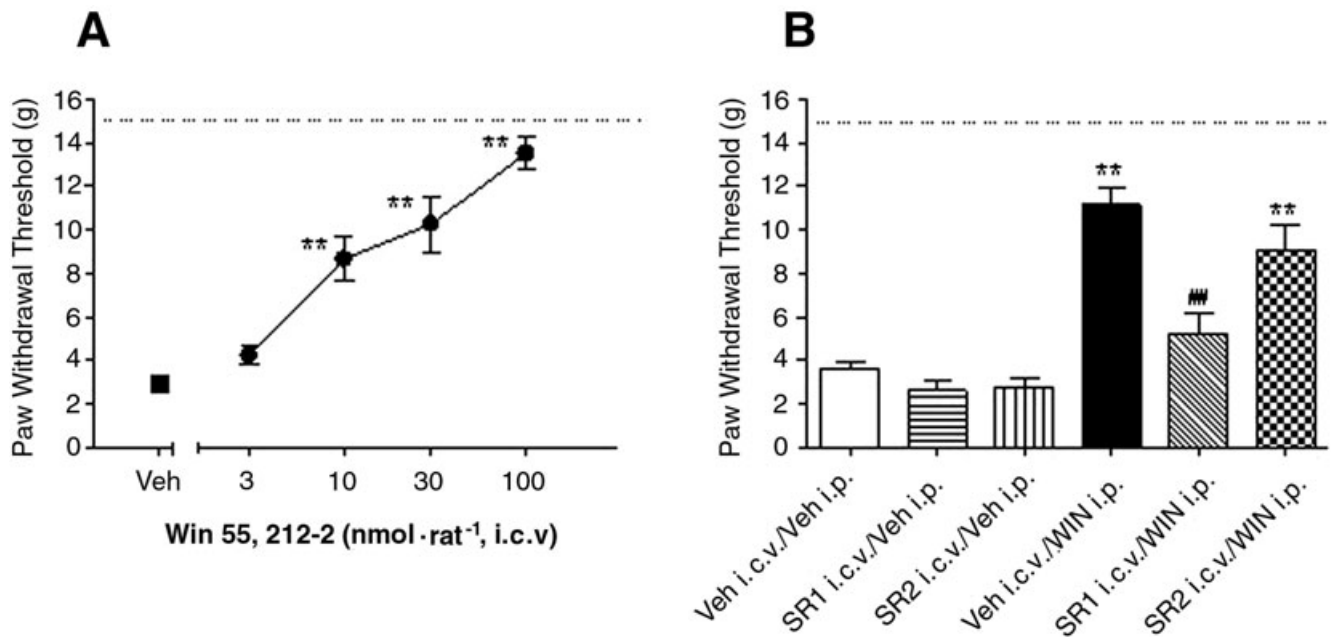Central CB$_1$ suppression of post-operative pain
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Figure 4 Effects of supraspinal infusion of WIN 55,212-2 (WIN) on skin incision-induced post-operative pain demonstrated as the increase of paw withdrawal threshold. (A) WIN (3,10, 30, 100 nmol per rat) was intracerebroventricularly (i.c.v.) infused in rats 90 min after surgery. (B) Reversal of WIN-induced analgesic efficacy by supraspinaly pretreatment with CB antagonists, selective CB$_1$ receptor antagonist SR141716A (SR1, 150 nmol per rat) or selective CB$_2$ receptor antagonist SR144528 (SR2, 150 nmol per rat) was i.c.v. infused 15 min before the administration of WIN (5 µmol·kg^{-1}, i.p.). Mechanical allodynia was assessed 30 min after WIN treatment. Data are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ versus vehicle (Veh) treated group, ## $P < 0.01$, SR1 i.c.v./WIN i.p. versus Veh i.c.v./WIN i.p. group, $n = 6$ per group. Dashed line: paw withdrawal threshold of non-injured paw. CB, cannabinoid.
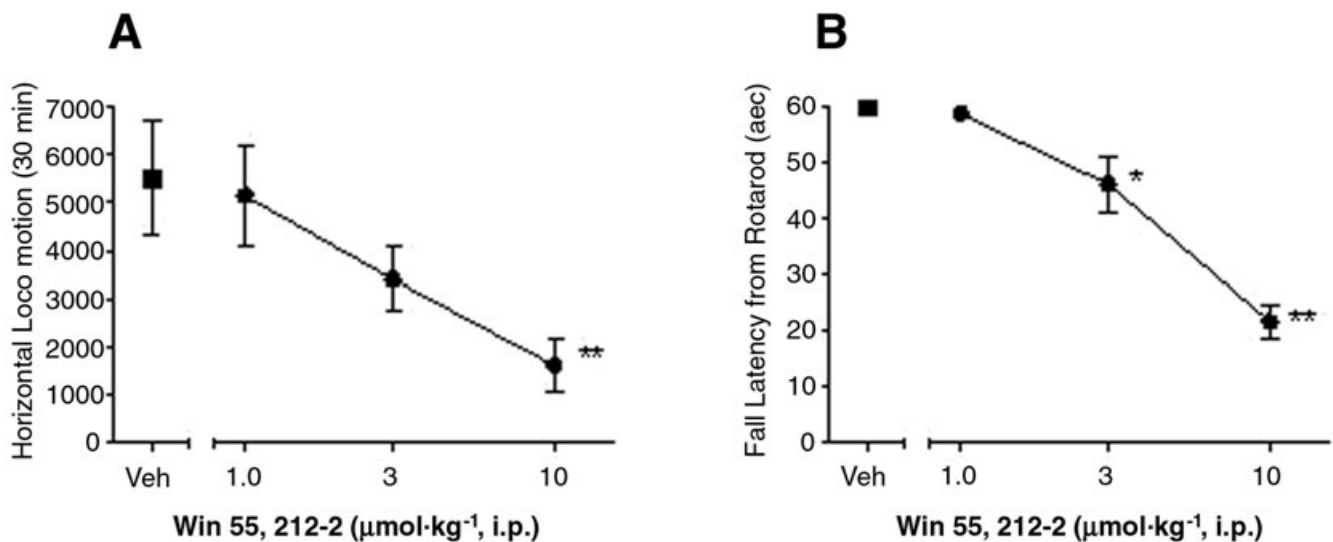

Figure 5 Effects of systemic WIN 55,212-2 (WIN) on spontaneous horizontal exploratory behaviours and rotarod performance. (A) WIN (1, 3 and 10 µmol·kg^{-1}) was i.p. administered in naïve rats 30 min before the assessment of horizontal exploratory activity. (B) WIN (1, 3 and 10 µmol·kg^{-1}) was i.p. administered in naïve rats 30 min before the recording of fall latency from rotarod performance. Data are mean $\pm$ SEM. *$P < 0.05$, **$P < 0.01$ versus vehicle (Veh) treated group, $n = 6–8$ per group.

the pretreatment with CB$_2$ receptor antagonist SR2 prevented systemic WIN-induced analgesia only following i.pl. injection. Finally, WIN-induced anti-allodynic effects were not clearly separable from CNS adverse effects, as analgesic doses of WIN also reduced horizontal exploratory activity and rotarod performance.

The non-selectivity and affinity of WIN for the CB receptor subtypes, CB$_1$ versus CB$_2$, reported from the present study are consistent with those reported in the literature (Howlett *et al.*, 2002; Yao *et al.*, 2008), indicating that the *in vivo* effects of WIN could be mediated non-selectively via activation of both CB$_1$ and CB$_2$ receptors. Following i.p. injection, WIN

produced dose-dependent anti-allodynic effects in the hind paw injury model of post-operative pain, with a maximal efficacy of 63% (10 µmol·kg⁻¹). In agreement with our observation, systemic WIN has been shown to reduce mechanical and thermal hyperalgesia in peripheral nerve injury-induced neuropathic pain (Fox *et al.*, 2001; Costa *et al.*, 2004; Walczak *et al.*, 2006), diabetic and/or paclitaxel-induced neuropathic pain (Dogrul *et al.*, 2004; Ulugol *et al.*, 2004; Pascual *et al.*, 2005), capsaicin-evoked hyperalgesia (Li *et al.*, 1999), formalin-induced persistent pain (Tsou *et al.*, 1996) and spinal cord injury-induced surgical pain (Hama and Sagen, 2007) in rodents. Although CBs have been shown to produce anti-nociceptive effects in several pre-clinical models of pain, a clinical trial of cannabis plant extract (Cannador) was terminated because of serious adverse events in patients receiving analgesic doses for post-operative pain (Holdcroft *et al.*, 2006). To further understand the potential for CB-induced post-operative pain relief, the present study evaluated the role of CB receptor subtypes in WIN-induced analgesia in a well-described rat model of post-operative pain.

To examine the site(s) of action at different levels of the nociceptive pathways, we subsequently investigated the effects of systemically inactive doses of WIN following central administration. Intrathecal delivery of WIN into the spinal enlargement reduced mechanical allodynia, with a maximal efficacy of 66% at 300 nmol per rat. Intracerebroventricular infusion of WIN potently reduced mechanical allodynia at even lower doses, with 61% efficacy at 30 nmol per rat. These data, in addition to the blockade by i.t. and i.c.v. CB₁ antagonist of the effects of systemic WIN, demonstrate that both the spinal cord and the brain play a role in the allodynic effects of W1N. The fact that local, i.c.v., infusion of WIN was 10-fold more potent than the local i.t. administration could suggest that the brain is a preferred site of action; it could also suggest that 30 min following i.c.v. administration, there is diffusion of the compound and activity at multiple sites that could synergize. Romero-Sandoval and Eisenach (2007) have reported that i.t. administration of another non-selective CB receptor agonist, CP 55,940, attenuated paw incision induced hypersensitivity, and this was prevented by co-application of both CB₁ and CB₂ receptor antagonists, supporting the possibility that both CB₁ and CB₂ receptors at the spinal cord level play an important role in nociceptive transmission following intrathecal administration of a non-selective agonist. These results contrast in part with the present findings that show that following systemic administration of a non-selective agonist, spinal CB₁ but not CB₂ receptors seemed to mediate the anti-allodynic effects observed *in vivo*. This difference could be due to the route of administration of the agonist and suggest that while CB₂ does play a role when the agonist is directly injected into the spinal cord, following systemic administration, CB₁ receptors are the major system responsible for the antiallodynic effects. In line with our result, i.t. application of WIN has been shown to suppress CFA-induced allodynia, carrageenan-induced thermal hyperalgesia (Richardson *et al.*, 1998a; Martin *et al.*, 1999), capsaicin-induced thermal and mechanical hyperalgesia (Johanek *et al.*, 2001), formalin-induced persistent nociceptive behaviours (Yoon and Choi, 2003; Kang *et al.*, 2007), peripheral nerve injury-induced neuropathic pain (Fox *et al.*, 2001; Lim *et al.*, 2003)

and penicillin-induced central pain in rodents (Kukushkin *et al.*, 2006). In addition, i.c.v. infusion of WIN also inhibited thermal hyperalgesia in the tail-flick assay (Welch *et al.*, 1995; Lichtman *et al.*, 1996; Raffa *et al.*, 1999).

A large body of evidence indicates that the anti-nociceptive effect of CBs is mediated via central sites of action. First, CB receptors are expressed abundantly in the spinal cord and brain, and activation of CB receptors can induce anti-nociceptive activity (Tsou *et al.*, 1996; 1998; Johanek and Simone, 2005), whereas knock-down of CB receptors could enhance the pain response (Dogrul *et al.*, 2002; Walker and Hohmann, 2005). Second, CB receptors are up-regulated in the spinal cord following sciatic nerve injury and CFA-induced inflammatory pain (Amaya *et al.*, 2006), and WIN-induced analgesic efficacy is reduced when such up-regulation is reversed (Dogrul *et al.*, 2002; Lim *et al.*, 2003). Third, electrophysiological studies have shown that WIN inhibits responses of thalamic and spinal dorsal horn neurons to acute peripheral noxious thermal or mechanical stimuli (Martin *et al.*, 1996; Hohmann *et al.*, 1999b; Fox *et al.*, 2001), as well as the wind-up response to prolonged noxious stimulation (Strangman and Walker, 1999). WIN also inhibits the formalin-induced c-fos expression in the spinal cord and thalamus (Martin *et al.*, 1996; 1999; Tsou *et al.*, 1996; Hohmann *et al.*, 1999a) and capsaicin-induced peptide release in the spinal cord (Richardson *et al.*, 1998a).

In addition to central modulation, recent anatomical and behavioural data have provided compelling evidence for a role of peripheral CB receptors in nociceptive transmission (Agarwal *et al.*, 2007). In the present study, i.pl. injection of WIN reduced mechanical allodynia, with a maximal efficacy of 58% at 300 nmol per paw, demonstrating that post-operative pain can also be attenuated by the activation of CB receptors at the peripheral site of injury. Previous studies have demonstrated that i.pl. anandamide inhibits carrageenan or capsaicin-induced hyperalgesia and paw oedema (Richardson *et al.*, 1998b; Amaya *et al.*, 2006) and formalin-induced persistent pain (Calignano *et al.*, 1998). Intra-plantar application of WIN also suppressed capsaicin and/or carrageenan-induced thermal and mechanical hyperalgesia (Li *et al.*, 1999; Johanek *et al.*, 2001; Nackley *et al.*, 2003), and heat-injury induced thermal hyperalgesia (Johanek and Simone, 2004). In addition, i.pl. injection of WIN or application to the site of a peripheral nerve injury reduced mechanical and cold allodynia (Fox *et al.*, 2001; Lever *et al.*, 2007), and topical application of WIN produced analgesic activity in tail flick assay (Dogrul *et al.*, 2003). Consistent with a peripheral site of action, CB receptors are localized to nerve fibres and cutaneous nociceptors (Agarwal *et al.*, 2007; Lever *et al.*, 2007), activation of these receptors inhibits excitatory calcium responses and reduces exocytosis of neuropeptides from these neurons (Ahluwalia *et al.*, 2003) as well as their peripheral terminals stimulated in the skin (Ellington *et al.*, 2002). Consequently, topical application of CBs to skin has a suppressive effect on the pro-inflammatory and pro-nociceptive efferent functions of C-fibre sensory neurons (Rukwied *et al.*, 2003; Yesilyurt *et al.*, 2003), whereas, peripheral knockout of CB receptors leads to a major reduction in the analgesia produced by systemically administered CBs (Agarwal *et al.*, 2007). Collectively, our results suggest that CB receptors expressed in

primary afferent neurons are also involved in nociceptive transmission of post-operative pain (Farquhar-Smith and Rice, 2003). Interestingly, contralateral paw application of WIN at 100 nmol per paw produced no anti-allodynic effects, whereas 300 nmol per paw revealed a weaker effect compared with ipsilateral injection, suggesting that diffusion after i.pl. injection may occur and produce some degree of systemic efficacy.

To explore the specific contribution of CB receptor subtypes at different levels of the nociceptive pathways in post-operative pain, the selective CB$_1$ receptor antagonist SR1 or CB$_2$ receptor antagonist SR2 were both systemically and locally applied prior to i.p. WIN administration. WIN-induced anti-allodynic effects were prevented by the pretreatment with i.p. SR1 but not i.p. SR2; pretreatment with i.t. or i.c.v. SR1, but not SR2, also prevented i.p. WIN-induced anti-allodynic effects, supporting the hypothesis that WIN-induced anti-allodynic effects are mediated via CB$_1$ receptor activation, and such mediation is predominantly centrally mediated. It was previously reported that systemic pretreatment with SR1 antagonized systemic WIN-induced analgesia in neuropathic pain caused by sciatic nerve injury or paclitaxel toxicity (Pascual et al., 2005). In addition, the topical analgesic effects of WIN were blocked by systemic pretreatment with a selective CB$_1$ receptor antagonist (AM 251) in the tail-flick test (Yesilyurt et al., 2003). Intrathecal delivery of SR1 reversed CP 55,940-induced attenuation of spinal neuronal sensitization (Johanek and Simone, 2005), blocked WIN-induced analgesia in capsaicin-hyperalgesia (Johanek et al., 2001) and abolished WIN-induced reversal of peripheral nerve injury-induced mechanical hyperalgesia (Fox et al., 2001). Taken together, these data show that the WIN-induced anti-allodynic effects are mediated via the activation of centrally located CB$_1$ receptors. The dose of 5 μmol·kg^{-1} (2.13 mg·kg^{-1}) of WIN was chosen for i.p. administration for all experiments of the action site(s), as this is an approximate ED$_{50}$ value of analgesic efficacy from our systemic dose-response study. The dose of 150 nmol per rat of SR1 or SR2 were chosen for site application, which is about 20- to 60-fold lower than that used for systemic blockade (10 μmol·kg^{-1} for SR2, 30 μmol·kg^{-1} for SR1), as l WIN was 10- to 100-fold more potent given locally than when given by systemic routes. Higher doses SR1 or SR2 for central application were attempted without success due to limited solubility of these two compounds.

Our study also provides evidence that systemic WIN-induced anti-allodynic effects are partially peripherally mediated by both CB$_1$ and CB$_2$ receptors, as both CB$_1$ and CB$_2$ antagonists applied at the site of injury partially prevented i.p. WIN-induced anti-allodynic effects. Previous reports demonstrated that peripheral SR1 or SR2 administration reversed WIN-induced effect on mechanical hypersensitivity in nerve injury (Lever et al., 2007), and i.pl. co-application SR1 and/or SR2 blocked WIN-induced suppression of carrageenan-evoked Fos protein expression and pain behaviour (Nackley et al., 2003). These data provide direct evidence that a peripheral CB mechanism suppresses inflammation-evoked neuronal activity at the level of the spinal dorsal horn, suggesting that the peripheral anti-allodynic effects of CBs may be exploited for treatment of inflammatory pain states (Nackley et al., 2003; Gutierrez et al., 2007).

It is well-established that the analgesic properties of non-selective CB agonists are usually associated with undesired CNS side effects (Walker and Hohmann, 2005). The current study demonstrated that systemic WIN reduced horizontal exploratory activity and rotarod performance at systemic analgesic doses, suggesting that some of the anti-allodynic effects could be due to locomotor deficit. However, local administration of WIN at the site of injury and/or the central sites, at doses devoid of motor effects, also reduced post-operative pain behaviour, demonstrating that the ability of central and/or peripheral WIN administration to attenuate allodynia was not solely due to motor deficits. In supporting this notion, microinfusion of WIN at dose of approximately 60 nmol·kg^{-1} into different brain regions produced no alteration of locomotor activity in rats (Wegener et al., 2008). Furthermore, electrophysiological and immunocytochemical studies have demonstrated that WIN had antinociceptive properties without the potential disadvantage of motor impairment (Martin et al., 1996; Tsou et al., 1996).

These results demonstrate that both CB$_1$ and CB$_2$ receptors are involved in the peripheral anti-allodynic effect produced by WIN in a pre-clinical model of post-operative pain. In contrast, the centrally mediated anti-allodynic activity of WIN is due to the activation of CB$_1$ but not CB$_2$ receptors. Furthermore, the increased potency of WIN following i.c.v. administration suggests that its main site of anti-allodynic effect is CB$_1$ receptors in the brain.

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Conflict of interest

The authors state no conflict of interest.

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