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RESEARCH PAPER

Opioid and cannabinoid synergy in a mouse neuropathic pain model

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BACKGROUND AND PURPOSE

Clinical studies have reported that pan-cannabinoid receptor agonists may have efficacy in neuropathic pain states and that this might be enhanced by co-administration with opioids. While cannabinoid–opioid analgesic synergy has been demonstrated in animal models of acute pain, it has not been examined in neuropathic pain models. We examined the effect of combination treatment with cannabinoid and opioid receptor agonists on allodynia and side effects in a nerve injury-induced neuropathic pain model.

EXPERIMENTAL APPROACH

C57BL/6 mice were subjected to chronic constriction injury (CCI) of the sciatic nerve. The effects of systemic administration of morphine and the pan-cannabinoid receptor agonist, WIN55212, on allodynia and side effects were examined at 7–10 days post-CCI surgery. Isobolographic analysis was used to determine whether the effects of the combination were synergistic.

KEY RESULTS

The opioid agonist morphine reduced CCI-induced mechanical and cold allodynia and produced motor incoordination, in a dose-dependent manner. WIN55212 reduced CCI-induced allodynia and produced motor incoordination, catalepsy and sedation, in a dose-dependent manner, as we have observed previously. When administered together, WIN55212 and morphine reduced allodynia in a synergistic manner but had only an additive effect on motor incoordination.

CONCLUSIONS AND IMPLICATIONS

These findings indicate that administration of a combination of a non-selective opioid and cannabinoid receptor agonist synergistically reduces nerve injury-induced allodynia, while producing side effects in an additive manner. This suggests that this combination treatment has an improved anti-allodynic potency and therapeutic index in a neuropathic pain model.

Abbreviations

CCI, chronic constriction injury; MPE, maximum possible effect; PWT, paw withdrawal threshold; THC, Δ^9-tetrahydrocannabinol; WIN55212, [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulphonate

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Tables of Links

TARGETS
GPCRs
μ receptor
CB_1 receptor
CB_2 receptor

LIGANDS	
AM251	Δ^9-tetrahydrocannabinol (THC)
AM630	WIN55212
Morphine	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Chronic neuropathic pain is a debilitating pain syndrome induced by trauma, or disease of the somatosensory system (Jensen *et al.*, 2011). The recommended pharmacological approaches for this condition have problematic effectiveness and side effects which are often intolerable (Dworkin *et al.*, 2010). New pharmacological targets that have improved effectiveness and a better therapeutic window are therefore needed.

Non-selective cannabinoid receptor agonists reduce the allodynia induced by neuropathic pain models in animals but also produce a range of side effects, such as the disruption of motor control, catalepsy, immobility and cognitive impairment (Herzberg *et al.*, 1997; Fox *et al.*, 2001; Lim *et al.*, 2003; Costa *et al.*, 2004; De Vry *et al.*, 2004; Scott *et al.*, 2004). Importantly, the therapeutic window between analgesia and side effects of pan-cannabinoid receptor agonists is poor in a rat neuropathic pain model (Fox *et al.*, 2001). In clinical studies, *Cannabis sativa* and combinations of its constituents, including the major psychoactive ingredient THC, have variable efficacy against neuropathic pain syndromes (Attal *et al.*, 2004; Berman *et al.*, 2004; Abrams *et al.*, 2007; Ware *et al.*, 2010; Bestard and Toth, 2011; Toth *et al.*, 2012; Langford *et al.*, 2013; Wilsey *et al.*, 2013).

In order to enhance analgesia and reduce side effects, neuropathic pain is often treated with combination therapies (Dworkin *et al.*, 2010). It has been suggested that this might also apply to cannabinoids (Bushlin *et al.*, 2010). A recent small-scale clinical trial found that combination treatment with an opioid and vaporized cannabis produced a greater relief of chronic pain than the opioid alone (Abrams *et al.*, 2011). It is unclear whether this was a synergistic, or additive effect. Preclinical animal studies using isobolographic analysis have shown that combination treatment with non-selective opioid and cannabinoid receptor agonists produces antinociception in a synergistic manner in assays of acute pain (Cichewicz and McCarthy, 2003; Tham *et al.*, 2005; Williams *et al.*, 2008) and reduces hyperalgesia in an arthritic chronic pain model (Cox *et al.*, 2007). The effect of combined cannabinoid and opioid agonist treatment, however, has not been examined in animal models of neuropathic pain. We therefore used isobolographic analysis to determine whether a combination treatment with a non-selective opioid and cannabinoid receptor agonist synergistically reduced neuropathic pain in a mouse model and whether this resulted in an improved therapeutic index.

Methods

Animal studies were carried out on 168 adult male C57BL/6 mice and are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015) and those of the 'NH&MRC Code of Practice for the Care and Use of Animals in Research in Australia' and with the approval of the Royal North Shore Hospital Animal Ethics Committee. Mice initially weighing 20–25 g were obtained from the Kolling Institute Animal Facility and housed individually in ventilated cages (23 ± 1°C, humidity 70%) with environmental enrichment (various toys and tunnels) and had free access to food and water. Each animal was tested with only one drug, or drug combination. The experimenter was blinded to the drug/procedure being tested. Animals were killed by carbon dioxide asphyxiation at the end of the final testing period.

Pain model

In this study, the chronic constriction injury (CCI) model of neuropathic pain was used (Bennett and Xie, 1988). The animals were anaesthetized with isoflurane (2–2.5% in saturated O_2, 1 mL·min^{-1}), the left common sciatic nerve and two 7-0 chromic gut sutures were tied loosely around the nerve. The incision was closed in layers and the animal recovered. Following CCI surgery, the animals were monitored before being returned to their home cages and monitored until the day of the experiment.

Behavioural and pain testing

Mechanical and cold allodynia, two common symptoms of neuropathic pain, were measured (Maier *et al.*, 2010). Mice were placed in elevated perspex cages with a wire mesh floor (15 × 10 × 10 cm) and were allowed to acclimatize for a period of 30 min prior to any testing. For mechanical allodynia, the paw withdrawal threshold (PWT) to mechanical stimulation of the left hind paw was measured using a series of von Frey hairs, which exerted forces ranging from 0.2 to 8.5 g (North Coast Medical, San Jose, CA, USA). The von Frey hairs were pressed perpendicularly onto the plantar surface of the hind paw for 2 s (four times for each hair), and a positive response

was noted if there was a sharp flinching of the hind paw. The mechanical PWT was calculated using the up-down method (Chaplan *et al.*, 1994). For cold allodynia, 20 μL of acetone was sprayed onto the plantar surface of the left hind paw to induce evaporative cooling. The number of acetone-induced left hindlimb lifts, shakes and/or licks was counted over a 2 min period.

A range of assays was used to measure the side effects of the cannabinoids. Motor disruption was determined by using a rotarod (Ugo Basile, Comerio, Italy), which slowly accelerated from 3–30 r.p.m. over a 300 s period. The rotarod latency was measured as the delay until the mouse fell off the rotarod or held onto the cylinder without running for two consecutive rotations. Catalepsy was measured with the bar test. For this assay, the animal's forepaws were placed on an elevated bar (4.5 cm height). The bar latency was the time it took for the animal to remove its forepaws from the bar, up to a cut-off time of 120 s. Spontaneous locomotor activity was measured in a dark open field. The animal was placed in an open topped, perspex enclosure (40 × 40 × 40 cm) and monitored for 2 min. The enclosure was divided into a 4 × 4 grid and the number of forepaw grid crossings counted. These tests were carried out in low-level white light, except for the open field test, which was in low-level red light (both <3 lx).

Drugs and administration

In this study, the opioid morphine, the pan-cannabinoid agonist WIN55212 and the cannabinoid CB_1 and CB_2 receptor antagonists AM251 and AM630 were used (Alexander *et al.*, 2015). Morphine sulphate was obtained from the National Measurement Institute (Lindfield, Australia). AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide), AM630 ([6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl] (4-methoxyphenyl)-methanone) and (+)-WIN55212 mesylate ([(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-meth anone, monomethanesulphonate) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Stock solutions of morphine were prepared in saline; AM251, AM630 and WIN55212 were prepared in a vehicle solution, which comprised 2% randomly methylated β-cyclodextrin, 15% DMSO and 5% Tween-80 in saline. The drugs were injected s.c. at a volume of 0.1 mL 10 g^{-1} body weight. Solutions of all drugs were made up immediately prior to administration.

Protocols

After arrival, each group of four animals was acclimatized to their holding cages and testing devices (except for the open field) over an initial 4–5 day period. We first examined the time course of action of a near maximal dose of morphine, but not WIN55212, as this has been characterized in our recent study (Adamson Barnes *et al.*, 2016). In these experiments, animals underwent pain and side effect testing (except open field), and then CCI surgery. At 8–10 days post-CCI surgery, von Frey, acetone, rotarod and bar testing was carried out twice over a 30 min period; the animal then received a single drug injection, and the testing was repeated at set time points.

For the single drug dose–response experiments and antagonist experiments, animals underwent pain and side effect testing (except open field), and then CCI surgery. At 8–10 days post-CCI surgery, animals underwent pain/side effect testing (except open field) twice over a 45 min period and then received a single injection of WIN55212 (alone or with AM251, or AM630) or morphine over a range of doses similar to those used in previous neuropathic pain model studies (Yu *et al.*, 1997; Adamson Barnes *et al.*, 2016). Pain and side effect testing (all tests, including open field) was carried out again at 30 or 60 min post-injection for morphine and WIN55212 respectively.

For the combination drug dose–response experiments, animals underwent pain and side effect testing (except open field), and then CCI surgery. At 8–10 days post-CCI surgery, animals underwent pain and side effect testing (all tests except open field) twice over a 45 min period and then received a WIN55212 injection and a morphine injection 30 min later. Pain and side effect testing (including open field) was carried out 30 min after the morphine injection (i.e. at 30 and 60 min post-morphine and -WIN55212 respectively). The post-drug testing time points in the dose–response and antagonist experiments were chosen to coincide with the peak drug effects determined in the initial time course experiments (see Results section).

Analysis

For the time course experiments, rotarod latency and acetone response data were compared using two-way repeated measures ANOVAs, with time and drug treatment as a within- and between-subjects factors respectively (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA; Prism, GraphPad Software, La Jolla, CA, USA). The raw data satisfied Levene's test for equality of error variances. *Post hoc* comparisons were made using the Bonferroni adjustment for multiple comparisons. For the dose–response experiments, all data except that for the open field were normalized as a percentage of the maximum possible effect (MPE), as we have done previously (Anderson *et al.*, 2013). For mechanical PWT and the bar latency, this was calculated as [100 (post-drug − pre-drug)/(cut-off)], with cut-off values of 8.5 g and 120 s respectively. For the acetone responses and rotarod latency, this was calculated as [100 (pre-drug − post-drug)/(pre-drug)]. Raw data were used for open-field crossings as this was only assessed post-injection. Data in all figures are presented as mean ± SEM. Data were considered significantly different when $P < 0.05$. There were six animals per treatment group in all experiments. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Dose–response curves were constructed for the individual WIN55212 and morphine experimental data by fitting sigmoidal curves with variable slope, of the form

$$E(a) = E_A \frac{a^q}{(a^q + C_a^q)} \quad (1)$$

$$E(b) = E_B \frac{b^p}{(b^p + C_b^p)} \quad (2)$$

for doses a and b of morphine (drug A) and WIN55212 (drug B), with E_A and E_B their maximal effects, C_a and C_b the

doses producing half-maximal effects (ED_{50}s) and q and p the Hill coefficients (Prism). The therapeutic index was calculated as the ratio of the ED_{50}s for mechanical and cold anti-allodynia divided by the ED_{50} for each of the side effects.

Isobolographic analysis

This was used to examine the effect of combined morphine and WIN55212 administration (Tallarida, 2006; Tallarida and Raffa, 2010). To do this, experimental dose–response data were obtained for a fixed-ratio combination of morphine and WIN55212. To reduce animal numbers, one fixed ratio of 1:1 for their respective ED_{50}s was examined (C_a : C_b by weight). Dose–response curves were constructed for the combination morphine–WIN55212 experimental data by fitting a sigmoidal curve with variable slope, as in equations (1) and (2). The dose–response curves for morphine, WIN55212 and their combination had different maximal effects, and their Hill slopes were greater than one. Therefore, the dose B_i of drug B to produce the effect level of interest, for fixed-ratio combinations of doses $a + b$ of drugs A and B, was calculated using

$$B_i = b + b_{eq(a)} = b + \frac{C_b}{\left[\gamma\left(1 + \frac{C_a^q}{a^q}\right) - 1\right]^{1/p}}, \text{ where } \gamma = {}^{E_b}/_{E_a} \quad (3)$$

and $b_{eq(a)}$ is the dose of drug B equi-effective with dose a of drug A. The isobolograms for different effect levels were plotted using equation (3) for comparison with the experimentally obtained data (Tallarida and Raffa, 2010).

To determine whether the combination had a synergistic effect, the predicted additive dose–response curves were calculated using

$$E(a,b) = E_B \frac{(b + b_{eq(a)})^p}{(b + b_{eq(a)})^p + C_b^p} \quad (4)$$

and were compared with the experimentally obtained data using a modified t-test (Tallarida, 2000; Tallarida and Raffa, 2010). For this comparison, the variance of the predicted additive effect at each dose of interest was calculated using the δ method, where

$$Var(E_{ab}) \cong \left(\frac{\delta E(a,b)}{\delta C_A}\right)^2 Var(C_A) + \left(\frac{\delta E(a,b)}{\delta C_B}\right)^2 Var(C_B) \quad (5)$$

The partial derivatives of $E(a,b)$ were calculated using two-point numerical estimation.

Results

Time course of action of morphine

We first examined the time course of action of near-maximal doses of morphine in order to establish the time of maximal effect for subsequent dose–response analysis. The effects of morphine (10 mg·kg^{-1}) and its matching vehicle on mechanical PWT, acetone responses and rotarod latency, but not bar latency, differed over time (Figure 1A–D, $F_{6,60}$ = 6.4, 5.2, 17.1, $P < 0.05$; $F_{6,60} = 0.6$, $P > 0.05$). The increase in mechanical PWT produced by morphine peaked at 30 min post-injection and was significantly greater than vehicle at 30–90 min (Figure 1A, $P < 0.05$). The decrease in acetone responses produced by morphine peaked at 30–60 min post-injection was significantly less than vehicle at 30–60 min

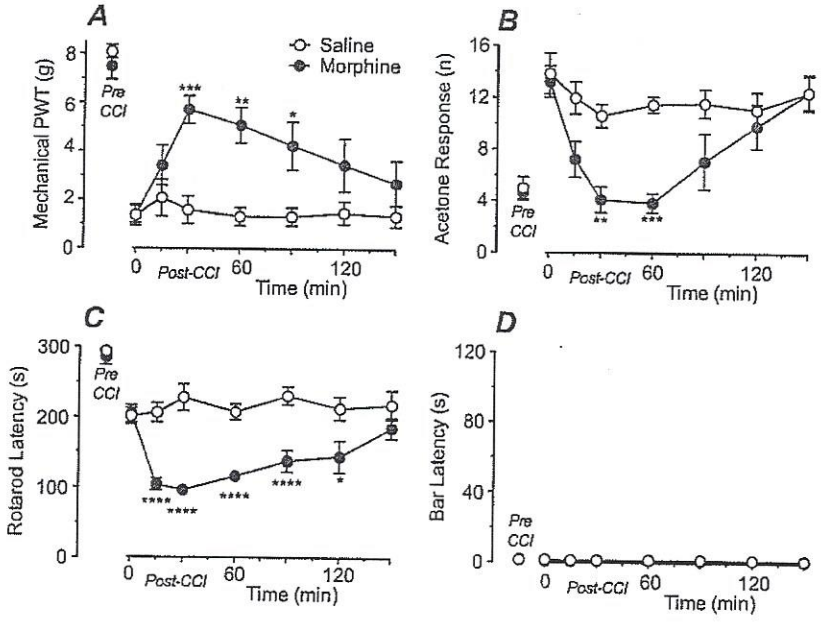

Figure 1

Time course of action of systemically delivered morphine. Time plots of the effect of morphine (10 mg·kg^{-1}) and vehicle on (A) mechanical PWT, (B) acetone responses, (C) rotarod latency and (D) bar latency. Animals received a s.c. injection of morphine or vehicle at time 0 h, 8–10 days after CCI surgery (post-CCI). The data for morphine and vehicle treatment groups are also shown prior to CCI surgery (pre-CCI). *, **, *** and **** denote $P < 0.05$, 0.01, 0.001 and 0.0001 for morphine versus vehicle at the corresponding time points.

($P < 0.05$). The decrease in rotarod latency produced by morphine peaked at 15–30 min post-injection was significantly less than vehicle at 15–120 min post-injection ($P < 0.05$).

Dose–response profile of morphine

We next obtained dose–response curves for morphine (1.78–30 mg·kg^{-1}) for all pain behaviour and side effect assays. The dose-dependent effects of morphine were assessed at 30 min post-injection, which corresponded to its peak effect time (see Figure 1A–D). Morphine reversed the CCI-induced reduction in mechanical PWT in a dose-dependent manner (Figure 2A). Similarly, morphine reversed the CCI-induced increase in acetone-induced responses in a dose-dependent manner (Figure 2A). The ED_{50} of morphine for mechanical PWT was greater than that for acetone responses (Table 1, 10.1 and 7.4 mg·kg^{-1} respectively, $P < 0.05$).

In these animals, morphine produced a decrease in rotarod latency with an ED_{50} of 6.1 mg·kg^{-1} (Figure 2B). The ED_{50} of morphine for rotarod latency was less than that for mechanical PWT ($P < 0.05$) but was not significantly different from that for acetone-induced responses (Table 1, $P > 0.05$). Morphine did not have a significant effect on bar latency and produced an increase in open field crossings at intermediate doses (Figure 2C, D).

Dose–response profile of WIN55212

We next obtained dose-response curves for WIN55212 (0.1–10 mg·kg^{-1}) for all pain behaviours and side effects, as we have done recently (Adamson Barnes *et al.*, 2016). The effect of WIN55212 was assessed at 1 h post-injection, which corresponded to its time of peak effect (Adamson Barnes *et al.*, 2016). WIN55212 reversed the CCI-induced reduction in mechanical PWT in a dose-dependent manner (Figure 3A). Similarly, WIN55212 reversed the CCI-induced increase in acetone-induced responses in a dose-dependent manner (Figure 3B). The ED_{50} of WIN55212 for mechanical PWT was greater than that for acetone responses (Table 1, 2.1 and 1.1 mg·kg^{-1}, $P < 0.05$). The maximal effects of morphine and WIN55212 on mechanical PWT and acetone responses were not significantly different ($P > 0.05$).

In these animals, WIN55212 produced a decrease in rotarod latency and an increase in bar latency, with ED_{50}s of 1.2 and 2.2 mg·kg^{-1} respectively (Figure 3C, D, Table 1). WIN55212 also produced a decrease in open field crossings, with an ED_{50} of 1.4 mg·kg^{-1} (Figure 3E, Table 1). The ED_{50} of WIN55212 for mechanical PWT was greater than that for rotarod latency and open field crossings ($P < 0.05$) but was not significantly different from that for bar latency ($P > 0.05$). By contrast, the ED_{50} of WIN55212 for acetone-induced responses was less than that for bar latency and open

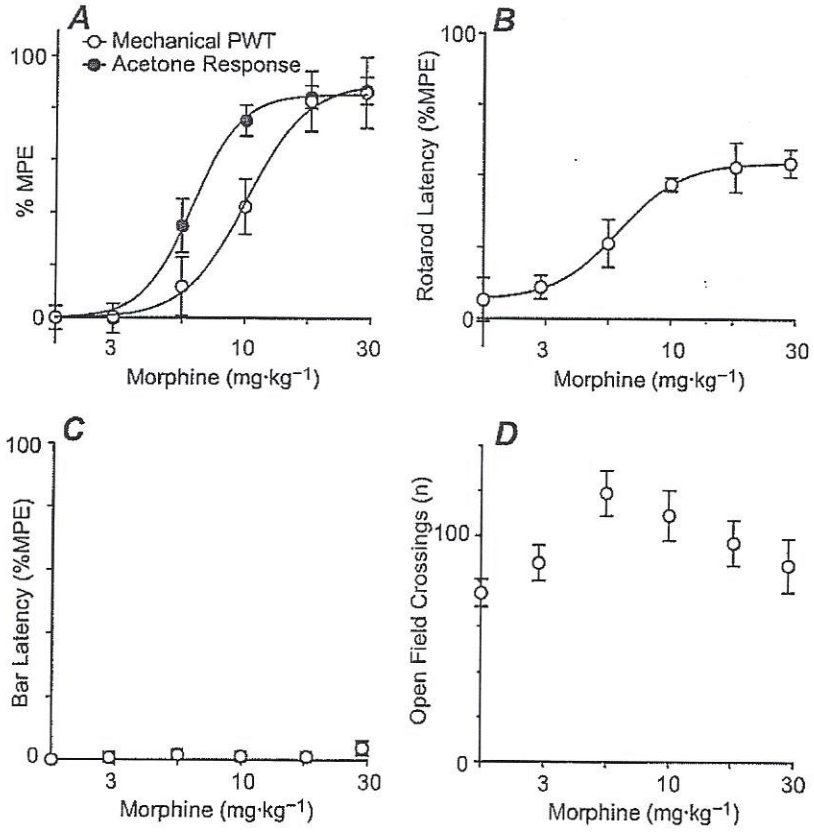

Figure 2

Dose–response profile of systemically delivered morphine. Dose–response curves for the effect of morphine on (A) mechanical PWT and acetone responses, (B) rotarod latency, (C) bar latency and (D) open field crossings. The lines in (A)–(B) represent sigmoidal curve fits to the data. Data shown are a percentage of the MPE (% MPE), except for the open field in which raw values are shown.

Table 1

Dose–response characteristics and therapeutic index of WIN55212, morphine and their combination

	Anti-allodynia		Side effects			
	Mechanical PWT	Acetone responses	Rotarod latency	Bar latency	Open field crossings	T.I.
WIN55212						
ED_{50}	2.1 (0.08)	1.1 (0.07)	1.2 (0.2)	2.2 (0.2)	1.4 (0.11)	1.1 (0.7–2.1)
E_{max}	92 (2)	95 (3)	86 (6)	100 (5)	–	–
Hill slope	4.3 (0.1)	3.2 (0.2)	3.7 (1.2)	3.4 (0.5)	4.5 (0.9)	–
Morphine						
ED_{50}	10.1 (0.5)	7.4 (0.7)	6.1 (0.5)	N.D.	N.D.	0.7 (0.6,0.8)
E_{max}	87 (1)	89 (5)	56 (2)	–	–	–
Hill slope	3.9 (0.1)	2.8 (0.5)	2.8 (0.4)	–	–	–
Morphine + WIN55212 (1:1) experimental						
ED_{50}	3.4 (0.1)	2.4 (0.1)	4.0 (0.1)	N.D.	4.7 (0.4)	1.6 (1.2–2.0)
E_{max}	100 (2)	100 (2)	91 (2)	–	–	–
Hill slope	3.5 (0.2)	2.9 (0.2)	2.5 (0.2)	–	3.6 (1.0)	–
Morphine + WIN55212 (1:1) predicted additive						
ED_{50}	6.3	3.8	4.2	N.D.	N.D.	0.9 (0.7,1.1)

Parameters, including ED_{50} ($mg \cdot kg^{-1}$), E_{max} (% MPE, and not determined for open field crossing), Hill slope and T.I. for sigmoidal dose–response curve fits for WIN55212 and morphine, individually. Values are shown as the mean (SEM or range).
T.I., therapeutic index; N.D., not determined.

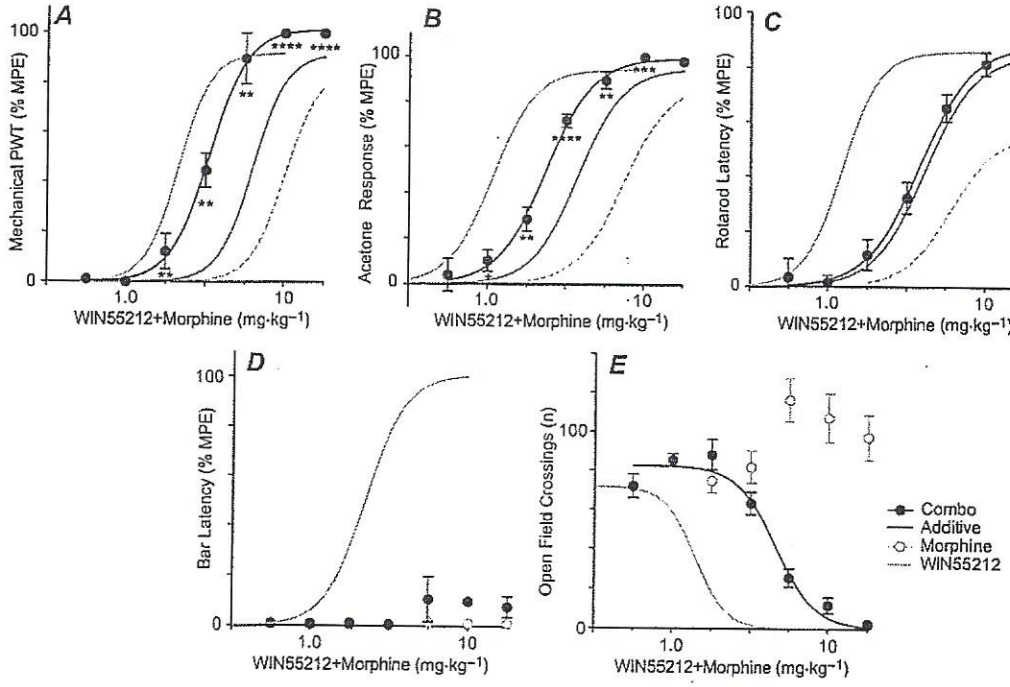

Figure 3

Dose–response profile of systemically delivered morphine plus WIN55212. Dose–response curves for the effect of co-administration of a fixed ratio of morphine plus WIN55212 (Combo, 5.6:1 fixed weight ratio, black filled circles) on (A) mechanical PWT, (B) acetone responses, (C) rotarod latency, (D) bar latency and (E) open field crossings. The dose–response curves for the predicted additive effects of morphine plus WIN55212 are shown in (A)–(C) (additive, blue line). The individual dose–response data or sigmoidal fits for morphine and WIN55212 are reproduced from Figures 3 and 4 for comparison (grey lines/symbols). The lines in (A)–(E) represent sigmoidal curve fits to the data where appropriate. *, **, *** and **** denote $P < 0.05$, 0.01, 0.001 and 0.0001 when comparing the experimentally obtained morphine/WIN55212 data points with the predicted additive effect at equivalent doses. Data shown are a percentage of the MPE (% MPE), except for the open field in which raw values are shown.

field crossing ($P < 0.05$) but was not significantly different from that for rotarod latency ($P > 0.05$). The maximal effect of WIN55212 on rotarod latency was significantly greater than that of morphine ($P < 0.05$).

We also examined the effect of the cannabinoid CB_1 and CB_2 receptor antagonists AM251 and AM630 (3 mg·kg^{-1}) on the actions of a near-maximal dose of WIN55212 (3 mg·kg^{-1}) (Figure 4). The effect of the treatment groups was different significantly for mechanical PWT, acetone responses, rotarod latency and bar latency ($F_{3,20}$ = 26.6, 16.7, 38.8, 12. 4, $P < 0.0001$) and for open field crossings ($F_{3,20}$ = 5.3, $P = 0.008$). WIN55212 had a significantly different effect from vehicle for all allodynia and side effect measures ($P < 0.05$–0.0001). Co-administration of WIN55212 with AM251 did not have a significantly different effect from vehicle ($P > 0.05$) and had a lesser effect than WIN55212 alone for all allodynia and side effect measures ($P < 0.05$–0.0001). Co-administration of WIN55212 with AM630 had a significantly different effect from vehicle for all allodynia and side effect measures ($P < 0.05$–0.0001). Compared with that with WIN55212 alone, co-administration with AM630 had a lesser effect on mechanical PWT ($P < 0.01$), but not acetone responses, rotarod latency, bar latency or open field crossings ($P > 0.05$).

Effect of a fixed-ratio WIN55212–morphine combination

We next examined the effect of a 1:1 fixed dose ratio combination of morphine : WIN55212 by ED_{50}. For the pain/behavioural assays in which both morphine and WIN55212 had sigmoidal dose-dependent effects (mechanical PWT, acetones responses and rotarod latency), the ratio of ED_{50}s by weight for morphine : WIN55212 varied from 4.8–6.9:1, with a mean of 5.6:1. Thus, morphine/WIN55212 co-administration was tested at a fixed ratio by weight of 5.6:1, over combined doses ranging from 0.56 to 18 mg·kg^{-1}.

Co-administration of the morphine/WIN55212 fixed-ratio combination reversed the CCI-induced reduction in mechanical PWT, with an ED_{50} of 3.4 mg·kg^{-1} (Figure 3A, Table 1). In addition, the morphine/WIN55212 combination reversed the CCI-induced increase in acetone-induced responses, with an ED_{50} of 2.4 mg·kg^{-1} (Figure 3B, Table 1). The morphine/WIN55212 combination also reduced rotarod latency and open field crossings, with ED_{50}s of 4.0 and 4.7 mg·kg^{-1} (Figure 3C, E, Table 1). By contrast, the morphine/WIN55212 combination had no significant effect on bar latency over the range of doses tested (Figure 3D).

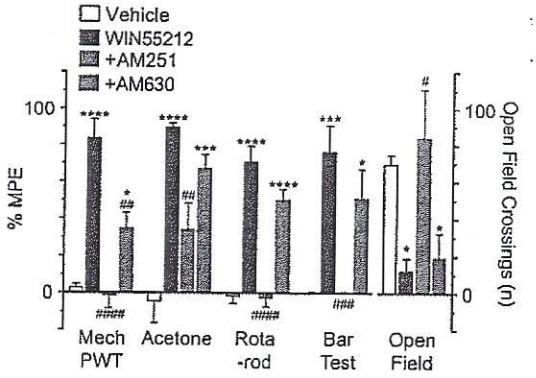

Figure 4

Effect of cannabinoid receptor antagonists on the actions of WIN55212. Bar chart of the effect of vehicle, WIN55212 (3 mg·kg^{-1}), WIN55212 plus AM251 (+AM251, 3 mg·kg^{-1}) and WIN55212 plus AM630 (+AM630, 3 mg·kg^{-1}) on mechanical paw withdrawal threshold (Mech PWT), acetone responses, rotarod latency, bar test latency and open field crossings. *, *** and **** denote $P < 0.05$, 0.001 and 0.0001 versus vehicle. #, ##, ### and #### denote $P < 0.05$, 0.01, 0.001 and 0.0001 versus WIN55212. Data shown are a percentage of the MPE (% MPE), except for the open field in which raw values are shown.

WIN55212–morphine isobolographic analysis

We finally examined whether this morphine/WIN55212 combination had synergistic effects. This was examined only for mechanical and cold allodynia, and rotarod performance, the assays in which both drugs and their combinations displayed sigmoidal dose-dependence (Figure 3). To do this, we used isobolographic analysis without the assumption of equal maximal effects, or Hill slopes of unity (equations 3 and 4). Using this approach, the predicted additive ED_{50}s for WIN55212 and morphine were 6.3, 3.8 and 4.2 mg·kg^{-1} for mechanical allodynia, cold allodynia and rotarod performance respectively (Figure 3A–C, Table 1).

The experimentally obtained dose–response curves for the effect of combined morphine/WIN55212 on both mechanical PWT and acetone responses were leftward shifted compared with their predicted additive dose–response curves (Figure 3A, B). Thus, the experimentally obtained effect of this combination on mechanical PWT and acetone responses was greater than that predicted by their additive effects over doses ranging from 1.0 to 18 mg·kg^{-1} ($P < 0.05$). By contrast, the experimentally obtained dose–response curve for the effect of the combination on rotarod latency overlapped its predicted additive dose–response curve (Figure 3C). Thus, the experimentally obtained and predicted additive dose–response curves for rotarod latency were not significantly different at any of the doses tested ($P > 0.05$, 0.3–18 mg·kg^{-1}).

To put the differences into context, we compared the isoboles of the experimentally obtained morphine/WIN55212 combination data with their theoretical additive effects over a range of effect levels. For the mechanical PWT isobologram, the experimentally obtained ED_{50} for morphine/WIN55212 was equivalent to a predicted additive ED_7 effect level (Figure 5A, i.e. 7% of MPE). For the acetone isobologram, the experimentally obtained ED_{50} for morphine/WIN55212 was equivalent to the predicted additive ED_{20} effect level (Figure 5B, i.e. 20% of MPE). By contrast, for the rotarod isobologram, the experimentally obtained ED_{50} for morphine/WIN55212 was equivalent to the predicted additive ED_{46} effect level (Figure 5C, i.e. 46% of MPE).

The presence and absence of morphine/WIN55212 synergy for anti-allodynia and motor performance, respectively, suggested that combination treatment might improve the therapeutic index of these agonists. The therapeutic index, measured as a ratio of the ED_{50}s of side effects versus anti-allodynia, was 1.6 for the morphine/WIN55212 combination

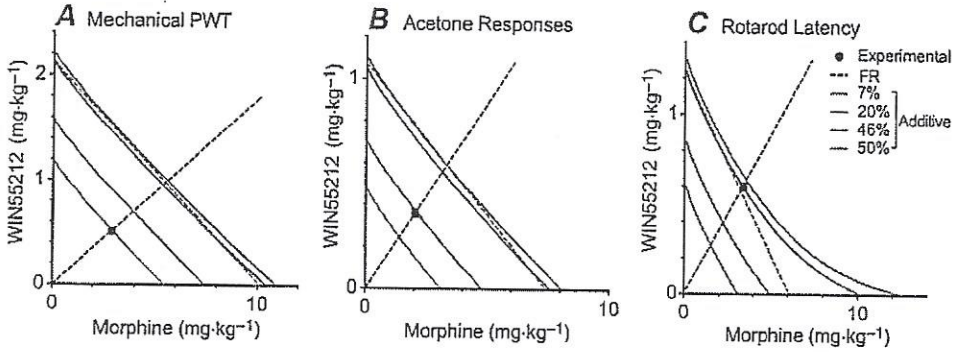

Figure 5

Isoboles for combined morphine plus WIN55212 at a range of effect levels. Isoboles for morphine/WIN55212 co-administration on (A) mechanical PWT, (B) acetone responses and (C) rotarod latency. The experimental data for the combination, at its ED_{50} (50% effect level, black symbol), is shown as part of the continuum of fixed-ratio effects (black dotted line). Theoretical isoboles of additivity for effect levels of 7, 20, 46 and 50% of MPE for combination morphine/WIN55212 are shown for comparison (solid lines, no constraint on Hill slope and maximal effect). The theoretical isobole for the 50% effect level is also shown with the assumption of similar maximal effects and unity Hill slopes (dotted blue line).

and 0.9 for the predicted additive effect of morphine and WIN55212 (Table 1).

Discussion

In the present study, it has been demonstrated that co-administration of the cannabinoid and opioid agonists, WIN55212 and morphine, synergistically reduces the mechanical and cold allodynia induced by a mouse model of neuropathic pain. By contrast, this combination had only an additive effect on motor side effects measured by the rotarod. Together, this led to an improvement in anti-allodynic potency and therapeutic window.

In the present study, WIN55212 produced dose-dependent reductions in the mechanical and cold allodynia induced by chronic constriction of the sciatic nerve. This was similar to that observed in our recent study in mice (Adamson Barnes *et al.*, 2016) and in previous studies on other pan-cannabinoid receptor agonists in rat and mouse neuropathic pain models (Herzberg *et al.*, 1997; Fox *et al.*, 2001; Lim *et al.*, 2003; Costa *et al.*, 2004; De Vry *et al.*, 2004; Scott *et al.*, 2004; Rahn *et al.*, 2007). The WIN55212-induced reduction in mechanical allodynia was mediated by both CB_1 and CB_2 cannabinoid receptors, as observed previously by others. By contrast, the reduction in cold allodynia was largely mediated by CB_1 receptors, although we cannot rule out CB_2 receptors, or other receptor systems (Rahn and Hohmann, 2009). WIN55212 also produced dose-dependent side effects, including reduced motor performance with the rotarod, catalepsy in the bar test and sedation in a dark open field as we have observed recently (Adamson Barnes *et al.*, 2016). These effects were largely mediated by cannabinoid CB_1 receptors. There was little dose separation between the anti-allodynic actions of WIN55212 and its side effects. Indeed, WIN55212 displayed a lower potency for reducing mechanical allodynia, compared with the induction of side effects. This is consistent with the poor separation between the relief of neuropathic pain symptoms and cannabinoid-like side effects observed in previous studies in rats (Fox *et al.*, 2001; Scott *et al.*, 2004). Thus, the good anti-allodynic efficacy of pan-cannabinoid receptor agonists in rodent neuropathic pain models is hampered by their poor therapeutic window.

Like WIN55212, morphine also produced a reduction in mechanical and cold allodynia with high efficacy. While opioids have reduced antinociceptive potency in rodent neuropathic pain models, the current observations are consistent with previous studies, which have shown that morphine reduces the allodynia induced by rodent neuropathic pain models (Lee *et al.*, 1994; Bian *et al.*, 1995). Morphine also produced a disruption of motor performance in the rotarod test, and like WIN55212, there was little dose separation between the anti-allodynic and rotarod side effects of morphine. It might be noted, however, that morphine had relatively lower efficacy in the rotarod test, indicating that ED_{50} alone provides an overestimate of its motor side effect profile. Morphine also produced an increase in spontaneous locomotion over intermediate doses, as observed by others, but had no consistent cataleptic effect in the bar test. Other side effects specifically associated with opioids were not examined because the aim was to examine the effects of opioid/cannabinoid combinations.

Previous isobolographic studies have shown that co-administration of non-selective opioid and cannabinoid receptor agonists has a synergistic analgesic effect in assays of acute nociception in naïve and diabetic rodents (Cichewicz and McCarthy, 2003; Tham *et al.*, 2005; Williams *et al.*, 2008) and more recently for mechanical hyperalgesia in an arthritic pain model (Cox *et al.*, 2007). To date, however, the combined effect of cannabinoid and opioid agonists on the abnormal pain symptoms associated with neuropathic pain models, such as allodynia, has not been explored (Bushlin *et al.*, 2010). In the present study, it was found that co-administration of morphine and WIN55212 synergistically reduced both mechanical and cold allodynia in a neuropathic pain model. This synergy approximately equated to a quarter decade reduction in ED_{50} compared with the predicted additive effect of morphine and WIN55212. Indeed, the combination had a 50% effect level at doses which, additively, were predicted to have 7 and 20% effect levels for mechanical and cold allodynia respectively. This

demonstration of synergy, using isobolographic analysis in an animal neuropathic pain model, supports the study of opioid/cannabinoid combination treatment in chronic pain patients (Abrams *et al.*, 2011). The present study, however, used only a single fixed 1:1 ED_{50} dose ratio combination. It remains to be determined whether this is the optimal dose ratio for anti-allodynic synergy, as has been done for a recent isobolographic study of endocannabinoid/COX combination treatment in a neuropathic pain model (Crowe *et al.*, 2015).

The potential benefit of a synergistic anti-allodynic action of morphine and WIN55212 could be offset by a synergistic induction of side effects, which might circumvent any improvement in the therapeutic index (Stone *et al.*, 2014). In the present study, the morphine/WIN55212 combination produced a dose-dependent disruption of motor performance in the rotarod test, but with an ED_{50} which was similar to its predicted additive effect. Thus, synergy in the neuropathic pain model was restricted to the anti-allodynic actions of the morphine/WIN55212 combination. In terms of the other side effect assays, the morphine/WIN55212 combination did not have a cataleptic effect in the bar test and produced sedation in the open field test which was similar to that predicted for WIN55212 alone. Thus, the morphine/WIN55212 combination had a therapeutic index which was greater than predicted if they acted in an additive manner (Table 1). It must be noted, however, that only cannabinoid-specific side effects were examined as the aim was to examine the effect potential of opioid–cannabinoid synergies. Other side effects, including abuse liability and tolerance, need further exploration. It is interesting to note, however, that combined administration of cannabinoid and opioid agonists produce less tolerance to the acute antinociceptive actions of morphine (Cichewicz and Welch, 2003; Smith *et al.*, 2007). In addition, it would be interesting to examine the therapeutic index of these side effects with respect to other measures of neuropathic pain, such as spontaneous pain and hyperalgesia (Maier *et al.*, 2010).

Unlike previous isobolographic studies, which have investigated analgesic synergies between cannabinoids and opioids, or COX inhibitors (Cichewicz and McCarthy, 2003; Tham *et al.*, 2005; Cox *et al.*, 2007; Crowe *et al.*, 2015), the current study did not assume that dose–response curves had similar maximal effects, or Hill slopes of unity. The dose–response curves for the anti-allodynic effects of morphine and WIN55212 had different Hill slopes, but similar maximal effects. This introduced a minor non-linearity in the isobolograms that did not have an impact on the statistical detection of synergy (Figure 5A, B). The dose–response curves for the disruption of motor performance produced by morphine and WIN55212 had different Hill slope and maximal effects. The difference in maximal effects introduced a substantial non-linearity which increased at higher effect levels (Grabovsky and Tallarida, 2004). Indeed, the predicted additive isobole at the 50% effect level was shifted towards the sub-additivity region with the assumption of unity Hill slope and similar maximal effectiveness (Figure 5C). Thus, assumptions of similar maximal effectiveness could underestimate the occurrence of synergy particularly when fixed dose ratios are used, which have a higher proportion of the agent with lower maximal effectiveness.

In summary, treatment with the combination of WIN55212 and morphine had a synergistic effect on allodynia, but only an additive effect on motor disruption. This suggests that combination opioid–cannabinoid agonist treatment has a greater relative impact upon allodynia compared with motor side effects and potentially represents an improved treatment option for neuropathic pain. This improvement in therapeutic window should, however, be viewed against a background of a poor therapeutic window for both morphine and WIN55212, when administered individually.

Author contributions

N.P.K., S.L.C., P.W.S., V.A.M. and C.W.V. performed the study, analysed the data and wrote the manuscript. C.W.V. conceived and designed the study.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Abrams DI, Couey P, Shade SB, Kelly ME, Benowitz NL (2011). Cannabinoid–opioid interaction in chronic pain. Clin Pharmacol Ther 90: 844–851.

Abrams DI, Jay CA, Shade SB, Vizoso H, Reda H, Press S *et al.* (2007). Cannabis in painful HIV-associated sensory neuropathy: a randomized placebo-controlled trial. Neurology 68: 515–521.

Adamson Barnes NS, Mitchell VA, Kazantzis NP, Vaughan CW (2016). Actions of the dual FAAH/MAGL inhibitor JZL195 in a murine neuropathic pain model. Br J Pharmacol 173: 77–87.

Alexander SP, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015). The Concise Guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. Br J Pharmacol 172: 5744–5869.

Anderson WB, Gould MJ, Torres RD, Mitchell VA, Vaughan CW (2013). Actions of the dual FAAH/MAGL inhibitor JZL195 in a murine inflammatory pain model. Neuropharmacology 81: 224–230.

Attal N, Brasseur L, Guirimand D, Clermond-Gnamien S, Atlami S, Bouhassira D (2004). Are oral cannabinoids safe and effective in refractory neuropathic pain? Eur J Pain 8: 173–177.

Bennett GJ, Xie YK (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33: 87–107.

Berman JS, Symonds C, Birch R (2004). Efficacy of two cannabis based medicinal extracts for relief of central neuropathic pain from brachial

plexus avulsion: results of a randomised controlled trial. Pain 112: 299–306.

Bestard JA, Toth CC (2011). An open-label comparison of nabilone and gabapentin as adjuvant therapy or monotherapy in the management of neuropathic pain in patients with peripheral neuropathy. Pain Pract 11: 353–368.

Bian D, Nichols ML, Ossipov MH, Lai J, Porreca F (1995). Characterization of the antiallodynic efficacy of morphine in a model of neuropathic pain in rats. Neuroreport 6: 1981–1984.

Bushlin I, Rozenfeld R, Devi LA (2010). Cannabinoid–opioid interactions during neuropathic pain and analgesia. Curr Opin Pharmacol 10: 80–86.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994). Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53: 55–63.

Cichewicz DL, Mccarthy EA (2003). Antinociceptive synergy between delta(9)-tetrahydrocannabinol and opioids after oral administration. J Pharmacol Exp Ther 304: 1010–1015.

Cichewicz DL, Welch SP (2003). Modulation of oral morphine antinociceptive tolerance and naloxone-precipitated withdrawal signs by oral delta 9-tetrahydrocannabinol. J Pharmacol Exp Ther 305: 812–817.

Costa B, Colleoni M, Conti S, Trovato AE, Bianchi M, Sotgiu ML *et al.* (2004). Repeated treatment with the synthetic cannabinoid WIN55,212-2 reduces both hyperalgesia and production of pronociceptive mediators in a rat model of neuropathic pain. Br J Pharmacol 141: 4–8.

Cox ML, Haller VL, Welch SP (2007). Synergy between delta9-tetrahydrocannabinol and morphine in the arthritic rat. Eur J Pharmacol 567: 125–130.

Crowe MS, Leishman E, Banks ML, Gujjar R, Mahadevan A, Bradshaw HB *et al.* (2015). Combined inhibition of monoacylglycerol lipase and cyclooxygenases synergistically reduces neuropathic pain in mice. Br J Pharmacol 172: 1700–1712.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461–3471.

De Vry J, Denzer D, Reissmueller E, Eijckenboom M, Heil M, Meier H *et al.* (2004). 3-[2-cyano-3-(trifluoromethyl)phenoxy]phenyl-4,4,4-trifluoro-1-butanesulfonate (BAY 59-3074): a novel cannabinoid CB1/CB2 receptor partial agonist with antihyperalgesic and antiallodynic effects. J Pharmacol Exp Ther 310: 620–632.

Dworkin RH, O'connor AB, Audette J, Baron R, Gourlay GK, Haanpaa ML *et al.* (2010). Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. Mayo Clin Proc 85: S3–14.

Fox A, Kesingland A, Gentry C, Mcnair K, Patel S, Urban L *et al.* (2001). The role of central and peripheral cannabinoid(1) receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. Pain 92: 91–100.

Grabovsky Y, Tallarida RJ (2004). Isobolographic analysis for combinations of a full and partial agonist: curved isoboles. J Pharmacol Exp Ther 310: 981–986.

Herzberg U, Eliav E, Bennett GJ, Kopin IJ (1997). The analgesic effects of R(+)-WIN 55,212-2 mesylate, a high affinity cannabinoid agonist, in a rat model of neuropathic pain. Neurosci Lett 221: 157–160.

Jensen TS, Baron R, Haanpaa M, Kalso E, Loeser JD, Rice AS *et al.* (2011). A new definition of neuropathic pain. Pain 152: 2204–2205.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Langford RM, Mares J, Novotna A, Vachova M, Novakova I, Notcutt W *et al.* (2013). A double-blind, randomized, placebo-controlled, parallel-group study of THC/CBD oromucosal spray in combination with the existing treatment regimen, in the relief of central neuropathic pain in patients with multiple sclerosis. J Neurol 260: 984–997.

Lee SH, Kayser V, Desmeules J, Guilbaud G (1994). Differential action of morphine and various opioid agonists on thermal allodynia and hyperalgesia in mononeuropathic rats. Pain 57: 233–240.

Lim G, Sung B, Ji RR, Mao J (2003). Upregulation of spinal cannabinoid-1-receptors following nerve injury enhances the effects of WIN 55,212-2 on neuropathic pain behaviors in rats. Pain 105: 275–283.

Maier C, Baron R, Tolle TR, Binder A, Birbaumer N, Birklein F *et al.* (2010). Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): somatosensory abnormalities in 1236 patients with different neuropathic pain syndromes. Pain 150: 439–450.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br J Pharmacol 172: 3189–3193.

Rahn EJ, Hohmann AG (2009). Cannabinoids as pharmacotherapies for neuropathic pain: from the bench to the bedside. Neurotherapeutics 6: 713–737.

Rahn EJ, Makriyannis A, Hohmann AG (2007). Activation of cannabinoid CB1 and CB2 receptors suppresses neuropathic nociception evoked by the chemotherapeutic agent vincristine in rats. Br J Pharmacol 152: 765–777.

Scott DA, Wright CE, Angus JA (2004). Evidence that CB-1 and CB-2 cannabinoid receptors mediate antinociception in neuropathic pain in the rat. Pain 109: 124–131.

Smith PA, Selley DE, Sim-Selley LJ, Welch SP (2007). Low dose combination of morphine and delta9-tetrahydrocannabinol circumvents antinociceptive tolerance and apparent desensitization of receptors. Eur J Pharmacol 571: 129–137.

Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SP *et al.* (2016). The IUPHAR/BPS Guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. Nucl. Acids Res 44: D1054–D1068.

Stone LS, German JP, Kitto KF, Fairbanks CA, Wilcox GL (2014). Morphine and clonidine combination therapy improves therapeutic window in mice: synergy in antinociceptive but not in sedative or cardiovascular effects. PLoS One 9: e109903.

Tallarida RJ (2000). Drug synergism and dose-effect data analysis. Boca Raton: Chapman and Hall/CRC: London.

Tallarida RJ (2006). An overview of drug combination analysis with isobolograms. J Pharmacol Exp Ther 319: 1–7.

Tallarida RJ, Raffa RB (2010). The application of drug dose equivalence in the quantitative analysis of receptor occupation and drug combinations. Pharmacol Ther 127: 165–174.

Tham SM, Angus JA, Tudor EM, Wright CE (2005). Synergistic and additive interactions of the cannabinoid agonist CP55,940 with mu opioid receptor and alpha2-adrenoceptor agonists in acute pain models in mice. Br J Pharmacol 144: 875–884.

Toth C, Mawani S, Brady S, Chan C, Liu C, Mehina E *et al.* (2012). An enriched-enrolment, randomized withdrawal, flexible-dose, double-blind, placebo-controlled, parallel assignment efficacy study of nabilone as adjuvant in the treatment of diabetic peripheral neuropathic pain. Pain 153: 2073–2082.

Ware MA, Wang T, Shapiro S, Robinson A, Ducruet T, Huynh T *et al.* (2010). Smoked cannabis for chronic neuropathic pain: a randomized controlled trial. CMAJ 182: E694–E701.

Williams J, Haller VL, Stevens DL, Welch SP (2008). Decreased basal endogenous opioid levels in diabetic rodents: effects on morphine and delta-9-tetrahydrocannabinoid-induced antinociception. Eur J Pharmacol 584: 78–86.

Wilsey B, Marcotte T, Deutsch R, Gouaux B, Sakai S, Donaghe H (2013). Low-dose vaporized cannabis significantly improves neuropathic pain. J Pain 14: 136–148.

Yu W, Hao JX, Xu XJ, Wiesenfeld-Hallin Z (1997). Comparison of the anti-allodynic and antinociceptive effects of systemic; intrathecal and intracerebroventricular morphine in a rat model of central neuropathic pain. Eur J Pain 1: 17–29.