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, Δ⁹-tetrahydrocannabinol; WIN55212, (3R)-2,3-dihydro-5-methyl-3-(4-morpholinylnethyl)pyrrolo[1,2,3-de]-1,4-benzoazin-6-yl]-1-naphthalenyl-methanone, monomethanesulphonate
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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 et 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 dosimetric 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 dosimetric 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 (2.2-2.5% in saturated O₂, 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 allosthesia, 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 x 10 x 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 (Chapman 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 cutoff 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 CB1 and CB2 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-1H-pyrazole-3-carboxamide), AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl] (4-methoxyphenyl)-methanone and (+)-WIN55212 mesylate ([3(3β,6α,9α)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2-d:1,4-benzoxazin-6-y1]-1-naphthalenyl-meth ane, monomethanesulfonate) 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-cycloextrin, 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 group 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

\[
E(a) = E_a \frac{a^p}{(a^p + C_a)}
\]

(1)

\[
E(b) = E_b \frac{b^p}{(b^p + C_b)}
\]

(2)

for doses a and b of morphine (drug A) and WIN55212 (drug B), with \(E_a\) and \(E_b\) their maximal effects, and \(C_a\) and \(C_b\) the
doses producing half-maximal effects (ED_{50}) and g and p the Hill coefficients (Prism). The therapeutic index was calculated as the ratio of the ED_{50} for mechanical and cold 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} 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_{1} 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

\[
B_{1} = \frac{b + b_{eq}(a)}{\left(1 + \frac{b}{b_{eq}(a)}\right)^{1/\gamma}}, \text{ where } \gamma = \frac{b}{E_{a}} \tag{3}
\]

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

\[
E(a, b) = E_{a} \frac{(b + b_{eq}(a))^{\gamma}}{(b + b_{eq}(a))^{\gamma} + C_{b}} \tag{4}
\]

and were compared with the experimentally obtained data using a modified t-test (Tallarida, 2006; 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

\[
Var(E_{ab}) = \left(\frac{\partial E(a,b)}{\partial C_{a}}\right)^{2} Var(C_{a}) + \left(\frac{\partial E(a,b)}{\partial C_{b}}\right)^{2} Var(C_{b}) \tag{5}
\]

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](image_url)

**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 ED₅₀ 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 ED₅₀ of 6.1 mg·kg⁻¹ (Figure 2B). The ED₅₀ 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 ED₅₀ 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 ED₅₀s 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 ED₅₀ of 1.4 mg·kg⁻¹ (Figure 3E, Table 1). The ED₅₀ 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 ED₅₀ 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

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<td>Acetone responses</td>
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<tr>
<td>WIN55212</td>
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<tr>
<td>$ED_{50}$</td>
<td>2.1 (0.08)</td>
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<tr>
<td>$E_{max}$</td>
<td>92 (2)</td>
<td>95 (3)</td>
</tr>
<tr>
<td>Hill slope</td>
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<td>3.2 (0.2)</td>
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<td>$ED_{50}$</td>
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<td>7.4 (0.7)</td>
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<td>$E_{max}$</td>
<td>87 (1)</td>
<td>89 (3)</td>
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<td>Hill slope</td>
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<td>Morphine + WIN55212 (1:1) experimental</td>
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<tr>
<td>$ED_{50}$</td>
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<td>2.4 (0.1)</td>
</tr>
<tr>
<td>$E_{max}$</td>
<td>100 (2)</td>
<td>100 (2)</td>
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<tr>
<td>Hill slope</td>
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<td>2.9 (0.2)</td>
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<td>Morphine + WIN55212 (1:1) predicted additive</td>
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<tr>
<td>$ED_{50}$</td>
<td>6.3</td>
<td>3.8</td>
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</table>

Parameters, including $ED_{50}$ (mg·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, 5:61 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 CB1 and CB2 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 ($F_{2,20} = 26.6, 16.7, 38.8, 12.4, P < 0.0001$) and for open field crossings ($F_{2,20} = 5.3, P = 0.008$). WIN55212 had a significantly different effect from vehicle for all alldynia 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 alldynia and side effect measures ($P < 0.05-0.0001$). Co-administration of WIN55212 with AM630 had a significantly different effect from vehicle for all alldynia 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 sigmoideal dose-dependent effects (mechanical PWT, acetone responses and rotarod latency), the ratio of ED$_{50}$ 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).

**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 sigmoideal 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 4.3 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$_{7}$ 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$_{14}$ 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.
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 antiallodynic 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 CB1 and CB2 cannabinoid receptors, as observed previously by others. By contrast, the reduction in cold allodynia was largely mediated by CB1 receptors, although we cannot rule out CB2 receptors, or other receptor systems (Rahn and Hofmann, 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 CB1 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 ED50 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 naive 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 ED50 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 SC). 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.

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