


RESEARCH PAPER

Mechanisms of imidazoline I₂ receptor agonist-induced antinociception in rats: involvement of monoaminergic neurotransmission

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BACKGROUND AND PURPOSE

Although the antinociceptive efficacies of imidazoline I₂ receptor agonists have been established, the exact post-receptor mechanisms remain unknown. This study tested the hypothesis that monoaminergic transmission is critical for I₂ receptor agonist-induced antinociception.

EXPERIMENTAL APPROACH

von Frey filaments were used to assess antinociceptive effects of two I₂ receptor agonists, 2-BFI and CR4056 on chronic constriction injury (CCI)-induced neuropathic pain or complete Freund's adjuvant (CFA)-induced inflammatory pain in rats. Rectal temperature was measured to assess hypothermic effects of 2-BFI. A two-lever drug discrimination paradigm in which rats were trained to discriminate 5.6 mg·kg⁻¹ 2-BFI (i.p.) from its vehicle was used to examine the discriminative stimulus effects of 2-BFI. In each experiment, pharmacological mechanisms were investigated by combining 2-BFI or CR4056 with various pharmacological manipulations of the monoaminergic system including selective reuptake inhibition, monoamine depletion and monoamine receptor antagonism.

KEY RESULTS

In the CCI model, selective reuptake inhibitors of 5-HT (fluoxetine) or noradrenaline (desipramine), but not dopamine (GBR12909), enhanced 2-BFI-induced antinociception. Selective depletion of 5-HT or noradrenaline almost abolished 2-BFI-induced antinociception. 5-HT_{1A}, 5-HT_{2A} and α₁-adrenoceptor antagonists, but not other monoaminergic antagonists, attenuated 2-BFI and CR4056-induced antinociception in CCI and/or CFA models. However, none of these monoamine receptor antagonists significantly altered 2-BFI-induced hypothermia or discriminative stimulus effects.

CONCLUSIONS AND IMPLICATIONS

Antinociception induced by I₂ receptor agonists was mediated by serotonergic and noradrenergic mechanisms with 5-HT_{1A}, 5-HT_{2A} and α₁-adrenoceptor being particularly important. In contrast, the hypothermic and discriminative stimulus effects of I₂ receptor agonists were mediated by distinct, independent mechanisms.

Abbreviations

2-BFI, 2-(2-benzofuranyl)-2-imidazoline; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CL, confidence limit; CR4056, 2-phenyl-6-(1H-imidazol-1yl) quinazoline; MPE, maximal possible effect; PWT, paw withdrawal threshold

Introduction

Despite efforts by researchers and funding agencies in recent years to address the issue of chronic pain, it remains one of the United States' largest healthcare problems, affecting almost one-third of Americans (NIH, 2013). While opioids have been relied upon as first-line pain therapies, they are ill-suited to extended pain management because of unwanted effects such as analgesic tolerance, dependence, constipation and addiction. When viewed in light of the current opioid epidemic, the lack of mechanistically novel painkillers entering the market in the past 50 years (Kissin, 2010) highlights the urgent need for the development of novel, safer and more effective analgesics.

Recent preclinical investigations have established the **imidazoline I₂ receptor** as a promising target for the development of such analgesics. Effective in several rodent chronic pain models when administered alone, I₂ receptor agonists also enhance opioid antinociception in an additive to synergistic manner while preventing some opioid use-related side effects such as tolerance and dependence (Thorn *et al.*, 2015; Siemian *et al.*, 2016; Thorn, Zhang, and Li, 2016b; Thorn *et al.*, 2017). However, despite many investigations on I₂ receptors since their discovery decades ago, they have not yet been cloned or had their structure elucidated. As such, their functional mechanisms are still poorly understood. The most widely accepted theory regarding I₂ receptor function stems from the localization of I₂ receptors to allosteric inhibitory binding sites on the **enzymes, MAO A** (Jones *et al.*, 2007) and **MAO B** (McDonald *et al.*, 2010), which implies the involvement of monoaminergic systems. Indeed, acute systemic administration of I₂ receptor agonists in rats increases **5-HT**, **noradrenaline**, and **dopamine** in several areas of the CNS, including the striatum, frontal cortex, hippocampus, dorsal raphe and spinal cord (Nutt *et al.*, 1995; Ugedo *et al.*, 1999; Ferrari *et al.*, 2011). Although I₂ receptors appear to have dense populations in several brain areas important for monoaminergic function such as dorsal raphe and locus coeruleus (MacKinnon *et al.*, 1995; Lione *et al.*, 1998), no study has systematically examined monoaminergic involvement in any I₂ receptor-mediated behaviours. MacInnes and Handley (2003) proposed the potential involvement of 5-HT and noradrenaline in the discriminative stimulus effects of one I₂ receptor agonist, 2-BFI, but more recent studies have failed to replicate these findings (Qiu *et al.*, 2014).

This study has systematically examined the antinociceptive effects of the selective I₂ receptor agonist 2-BFI alone and in combination with various pharmacological manipulations of the monoamine system in adult male rats with chronic constriction injury (CCI)-induced neuropathic pain. Similar tests were conducted which used either another selective I₂ receptor agonist, CR4056, or another pain model, complete Freund's adjuvant (CFA)-induced inflammatory pain. In a separate set of experiments, the hypothermic effects of 2-BFI alone and in combination with several selective monoaminergic receptor antagonists were examined. In a final experiment, rats were trained to discriminate 5.6 mg·kg⁻¹ 2-BFI from its vehicle. The discriminative stimulus effects of 2-BFI alone and in combination with either selective monoaminergic reuptake inhibitors or selective monoaminergic receptor antagonists were examined.

Methods

Animals

All animal care and experimental protocols complied with the 2011 *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and the guidelines of the International Association for the Study of Pain (Zimmermann, 1983), and were approved by the Institutional Animal Care and Use Committee, University at Buffalo, the State University of New York (Buffalo, NY, USA). Care was taken to minimize animal suffering and any animals showing clear signs of discomfort or illness were killed. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Male ($n = 192$ rats) Sprague–Dawley rats (Envigo, Indianapolis, IN, USA) approximately 12 weeks old and weighing approximately 250 g at experiment onset were housed individually in standard housing conditions on a 12/12 h light/dark cycle with behavioural experiments conducted during the light period. All rats had free access to water, except during test sessions. Rats used in nociception ($n = 5–6$ per group) and body temperature ($n = 6–7$ per group) tests had free access to standard rodent chow in their home cages and were randomly assigned to different study groups. Group size was determined by previous studies from our laboratory to ensure sufficient statistical power. Rats used in drug discrimination studies ($n = 7$) were provided with restricted access to food after their daily sessions, such that their bodyweights were maintained at 85% of their free-feeding counterparts. Specific group numbers are given in the figure legends.

Experimental procedures

Validation of models. The CCI and CFA-induced pain models are well-described and widely used animal models of chronic neuropathic and inflammatory pain respectively. In rodents, each manipulation induces persistent hypersensitivity to a range of stimuli (Bennett and Xie, 1988; Nagakura *et al.*, 2003; Li *et al.*, 2014), making them appropriate models with which to study potential analgesics. Hypothermia was studied as a well-characterized physiological endpoint of I₂ receptor activation (Thorn *et al.*, 2012). Drug discrimination is a well-validated model first described in 1951 (Conger, 1951) and has been used to characterize the discriminative properties of many drugs since; I₂ receptor agonists have been previously characterized to produce discriminative stimulus effects (Qiu *et al.*, 2014; Qiu *et al.*, 2015).

Induction of inflammatory pain and neuropathic pain. Inflammatory pain was induced by CFA, as previously described (Li *et al.*, 2014). Briefly, rats were anesthetized in an induction chamber with 2% isoflurane mixed with 100% oxygen. Adequate anaesthesia was determined by loss of righting and toe-pinch reflexes. The right hind paw was cleaned and disinfected with a gauze pad saturated with 70% isopropyl alcohol; 0.1 mL of CFA (Sigma-Aldrich, St. Louis, MO, USA) containing approximately 0.05 mg of *Mycobacterium butyricum* dissolved in paraffin oil was injected s.c. into the plantar

surface of the right hind paw. Rats were then returned to their home cages.

Neuropathic pain was induced by CCI, using the procedure described earlier by Bennett and Xie, (1988) and Li *et al.*, (2014). Rats were anesthetized with a mixture of ketamine (60 mg·kg⁻¹) and xylazine (15 mg·kg⁻¹) i.p. prior to surgery. Sufficient anaesthesia was determined by loss of righting and toe-pinch reflexes. Under aseptic technique, the common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatic nerve's trifurcation, approximately 7 mm of nerve was freed of adhering tissue, and four ligatures (4.0 chromic gut suture; Patterson Veterinary, Devens, MA, USA) were tied loosely around it with approximately 1 mm spacing. Care was taken such that the ligatures just barely constricted the nerve so that circulation through the superficial epineurial vasculature was uninterrupted. The incisions were closed with surgical clips. Rats were given post-operative saline and antibiotics and allowed to recover on heating pads until righting reflex was regained.

Mechanical nociception. A total of 147 rats were used for these studies. Mechanical hyperalgesia was measured by the von Frey filament test using equipment and procedures as described in detail previously (Siemian *et al.*, 2016). Briefly, rats ($n = 5-6$ per group) were placed in transparent test chambers on top of a wire mesh platform through which filaments were applied perpendicularly to the medial plantar surface of the hind paw from below the mesh floor to determine the paw withdrawal threshold (PWT), defined as the lowest strength filament that elicited a behavioural response in at least two out of three applications. Behavioural testing began 1 day after CFA treatment or 5 days after CCI surgery as described previously (Li *et al.*, 2014). By design, all groups contained six rats each; some rats failed to develop mechanical hypersensitivity following CCI or CFA treatment, leading to five rats in certain groups; specific group numbers are given in the figure legends. For all nociception tests, a cumulative dosing procedure was used to establish dose-effect curves with a 20 min (2-BFI) or 30 min (CR4056 and reuptake inhibitors) inter-injection interval as described previously (Siemian *et al.*, 2016b) with the cumulative dose increasing by 0.25 log unit per injection, except for the reuptake inhibitors for which the doses increased by 0.5 log unit, up to doses that could be safely studied. For the investigations of 2-BFI or CR4056 with monoamine receptor antagonists or reuptake inhibitors, each group of rats was tested on three or four occasions. The first test was always with an I₂ receptor agonist alone, and subsequent tests were with a study compound in combination with the I₂ receptor agonist. Monoamine receptor antagonists and reuptake inhibitors were administered 20 and 30 min prior to the first injection of the I₂ receptor agonist respectively. Each group of rats was assigned to one study compound, and the test order for study compounds was arranged in an ascending dose order. A final test of monoamine receptor antagonist or reuptake inhibitor alone was conducted following combination studies. All tests were separated by at least 3 days.

For the 5-HT depletion experiment, one group of rats was used. One 2-BFI dose-effect curve was established prior to a

3 day treatment consisting of an injection of 200 mg·kg⁻¹ i.p. **p-chlorophenylalanine (pCPA)** on each of the first and second days and an injection of 10 mg·kg⁻¹ i.p. **fenfluramine** on the third day, which depletes brain 5-HT levels by approximately 93% (Prinssen *et al.*, 2002; Li *et al.*, 2009a,b). On the subsequent day (day 4), a second 2-BFI dose-effect curve was established. For the noradrenaline depletion experiment, one group of rats was used. One 2-BFI dose-effect curve was established, and on the subsequent day, an injection of 50 mg·kg⁻¹ *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (DSP-4) was administered (Jaim-Etcheverry and Zieher, 1980), which depletes brain noradrenaline by approximately 60% after 10 days (Kudo *et al.*, 2011). On day 10 after the DSP-4 injection, a second 2-BFI dose-effect curve was established. In all experiments, experimenters were blind to the treatments, and they received extensive training with the von Frey procedure to ensure accurate judgement of paw withdrawal responses and minimize experimenter bias.

Body temperature. A total of 38 rats were used in body temperature tests which were performed as previously described (Li *et al.*, 2009a). Briefly, rats were habituated to a quiet procedure room for at least 30 min before each test. Body temperature was measured by gently inserting a lubricated probe approximately 5.0 cm into the rectum and recording the temperature from the digital thermometer (BAT7001H, Physitemp Instruments Inc., Clifton, NJ, USA). A cumulative dosing procedure was used with rats receiving vehicle prior to the first 30 min cycle followed by increasing doses of 2-BFI every 30 min with the cumulative dose increasing by 0.25 log unit per injection. Body temperature was measured in the last minute of each 30 min cycle. Rats were handled and habituated to the procedure for 3 days before the testing began. Each group of rats ($n = 6-7$ per group, specific group numbers are given in the figure legend) was tested on three or four occasions. The first test was always with 2-BFI alone, and subsequent tests were with a monoamine receptor antagonist in combination with 2-BFI. Each group of rats was assigned to one antagonist, and the antagonists were always tested in an ascending dose order. All tests were separated by at least 3 days.

Drug discrimination. Drug discrimination studies were performed using previously described chambers (Coulbourn Instruments Inc., Allentown, PA, USA) and software (Graphic State 3.03, Coulbourn Instruments Inc., Whitehall, PA, USA) (Qiu *et al.*, 2014; Qiu *et al.*, 2015). A modification of previously described training protocols was used (Siemian *et al.*, 2017). Seven rats were trained to discriminate 5.6 mg·kg⁻¹ 2-BFI injected i.p. from saline in a multiple cycle, cumulative-dosing procedure. Each cycle consisted of a 10 min timeout during which the chamber was dark and responses had no programmed consequence, followed by a 5 min response period, during which a house light and a cue light above each lever were illuminated and signalled availability of reinforcers. Ten consecutive responses (fixed ratio 10) on the correct lever resulted in food delivery (45 mg; BioServ Inc., Frenchtown, NJ, USA). The correct lever was predetermined by an injection (e.g. right, saline; left, 2-BFI). Response periods ended after

5 min or after delivery of 10 food pellets, whichever occurred first. Training began with single-cycle sessions, in which saline or 5.6 mg·kg⁻¹ 2-BFI was administered i.p. 10 min before the start of the session. Sessions were conducted 7 days·week⁻¹ according to a roughly double alternation schedule (e.g. saline, saline, drug and drug). Rats had to achieve at least 90% of the total active responses on the correct lever for five consecutive or six out of seven consecutive sessions to progress to multiple cycle training. For multiple cycle training, a saline or 5.6 mg·kg⁻¹ 2-BFI injection was given 10 min before the start of a two- to five-cycle session. Some training days consisted of two cycles in which either the saline lever or the 2-BFI lever was active during both cycles. On other training days, one to three saline training cycles preceded the administration of 2-BFI. These protocols were varied non-systematically; rats needed to pass two consecutive sessions (one saline training session and one 2-BFI training session) by responding at least 90% on the correct lever during each active period before each test. Test sessions lasted up to five cycles and were identical to training sessions except that 10 consecutive responses on either lever delivered a food pellet. During test sessions, vehicle, monoamine receptor antagonists or reuptake inhibitors were administered 10 min before the start of the first cycle followed by increasing cumulative doses of 2-BFI, with the cumulative dose increasing by 0.25 log unit per injection, during the first minute of each subsequent cycle up to doses that occasioned at least 80% responding on the 2-BFI-appropriate lever or suppressed the rate of responding. In tests examining whether reuptake inhibitors substituted for the discriminative stimulus of 2-BFI, the inter-injection time was increased from 15 to 30 min.

Randomization and blinding. Laboratory rats were acquired from Envigo and randomly assigned to experiments and treatment conditions therein. The treatments for all experiments in this study were blinded to minimize experimenter bias with the exception of drug discrimination, for which the data were collected by a computer program.

Data analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). To control for subject-to-subject variation, the antinociceptive effects were normalized and quantified for each rat as % maximal possible effect (MPE) according to the following formula: % MPE = (post-drug PWT – pre-drug PWT)/(pre-CFA PWT – pre-drug PWT) × 100%. These percentages were averaged (±SEM) across rats and plotted as a function of dose. Log(ED₅₀) [±95% confidence limits (CLs)] values were individually calculated from the % MPE values of each rat and averaged within the group to determine the group ED₅₀ values of 2-BFI or CR4056 in each nociception test. Effects were considered significant if 95% CL values from tests with vehicle or test compounds did not overlap. In certain cases, one-way repeated-measures ANOVA was used, and *P* < 0.05 was considered statistically significant. Specific group numbers are given in the figure legends. Raw group mean PWTs are shown in Supporting Information Tables S2–S5.

For body temperature studies, two-way repeated measures ANOVA (treatment dose × 2-BFI dose) was used, and *P* < 0.05 was considered statistically significant. Specific group numbers are given in the figure legends.

For drug discrimination studies, two sets of data were collected for each test: (a) the percentage of responses on the 2-BFI-associated lever, calculated by dividing the number of responses on the 2-BFI-associated lever by the number of total responses on either lever within a cycle and multiplying by 100; and (b) response rate, calculated by dividing the total number of responses made on both levers by the duration of the response period in seconds. When a rat responded at a rate less than 20% of the vehicle control rate (the rate recorded after saline was administered), the percentage of responses on the 2-BFI-associated lever for that rat at that dose was not included in further analyses. The response rate data were still included. 2-BFI ED₅₀ values (±95% CL) were calculated for 2-BFI-associated lever responding (dataset a). Effects were considered significant if 95% CL values from tests with vehicle or test compounds did not overlap. Paired *t*-tests or one- or two-way ANOVA were used to compare response rate data, and *P* < 0.05 was considered statistically significant.

Materials

2-BFI and CR4056 were synthesized according to standard procedures (Ishihara and Togo, 2007) and were provided by Dr. Yanan Zhang. The pharmacological properties of these drugs have been detailed previously (Nutt *et al.*, 1995; Ozaita *et al.*, 1997; McDonald *et al.*, 2010; Ferrari *et al.*, 2011). **MDL100907**, **WAY-100635** maleate, **WB4101** hydrochloride, **yohimbine** methyl ester hydrochloride, **desipramine** hydrochloride, pCPA, fenfluramine hydrochloride and DSP-4 hydrochloride were purchased from Sigma (Sigma-Aldrich). **SB242084** dihydrochloride, **SCH-23390** hydrochloride, **raclopride**, **imipramine** hydrochloride, **fluoxetine** hydrochloride and GBR12909 hydrochloride were purchased from Cayman (Cayman Chemical, Ann Arbor, MI, USA). All drugs were dissolved in 0.9% saline except for SB242084, MDL100907 and CR4056 which were dissolved in 10%, 20% and 20% DMSO in saline respectively. Doses of drugs are expressed in terms of their salt form, and all drugs were administered i.p., except for SCH23390 which was administered s.c., in a volume of 1–2 mL·kg⁻¹. The pharmacological target and property of each drug is given in Supporting Information Table S1.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c).

Results

Prior to CCI surgery, the average PWT was 24.3 ± 0.4 g, which decreased to 5.8 ± 0.2 g following CCI surgery. According to one-way repeated measures ANOVA, fluoxetine (Figure 1A),

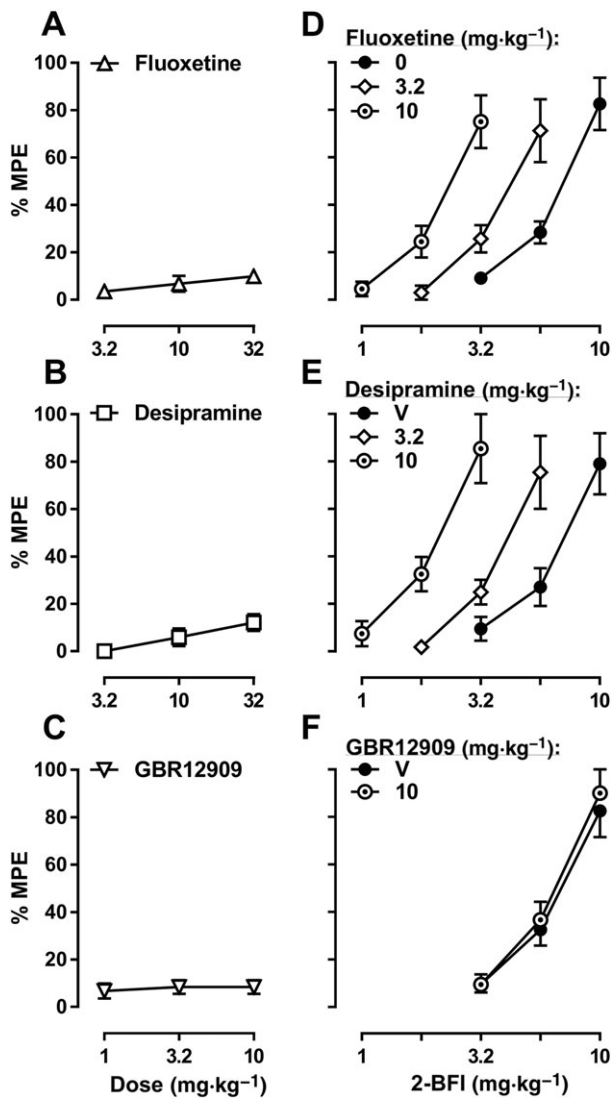


Figure 1

Percentage of the maximum possible effects (%MPE) of fluoxetine (A, $n = 6$), desipramine (B, $n = 5$) or GBR12909 (C, $n = 5$) or 2-BFI alone and in combination with fluoxetine (D), desipramine (E) or GBR12909 (F), on CCI-induced mechanical nociception. Data shown are means \pm SEM.

desipramine (Figure 1B) and GBR12909 (Figure 1C) all produced a significant main effect on PWT [$F(3, 20) = 6.25$ for fluoxetine, $F(3, 16) = 5.21$ for desipramine and $F(3, 16) = 6.48$ for GBR12909]. Bonferroni's post tests revealed that PWTs at 32 mg·kg⁻¹ fluoxetine, 10 mg·kg⁻¹ desipramine and 3.2 and 10 mg·kg⁻¹ GBR12909 were all significantly different as compared to their respective vehicle controls. However, the highest %MPE among these drugs did not exceed 12.1%. When administered prior to 2-BFI, 3.2 and 10 mg·kg⁻¹ fluoxetine produced 1.66- and 2.98-fold leftward shifts of the 2-BFI dose-effect curve (Figure 1D). Likewise, 3.2 and 10 mg·kg⁻¹ desipramine produced 1.56- and 3.05-fold leftward shifts of the 2-BFI dose-response curve (Figure 1E). In contrast, GBR12909 did not significantly affect the 2-BFI dose-effect curve (Figure 1F; see Table 1 for

ED₅₀ values). Prior to 5-HT depletion, the ED₅₀ ($\pm 95\%$ CL) of 2-BFI was 7.43 (5.80, 9.53) mg·kg⁻¹. On the day following the 3 day treatment regimen of pCPA and fenfluramine, the ED₅₀ of 2-BFI was >17.8 mg·kg⁻¹ (Figure 2A). The baseline nociceptive threshold of this group was not significantly different before (6.33 ± 1.09 g) and after depletion (6.66 ± 0.66 g). Likewise, before depletion of nor-adrenaline, the ED₅₀ ($\pm 95\%$ CL) of 2-BFI was 6.63 (4.49, 9.80) mg·kg⁻¹. On the 10th day following DSP-4 treatment, the ED₅₀ of 2-BFI was >17.8 mg·kg⁻¹ (Figure 2B). The baseline nociceptive threshold of this group was not significantly different before (5.66 ± 0.95 g) and after depletion (6.33 ± 0.95 g).

When studied in combination with 2-BFI in the CCI-induced pain model, the 5-HT_{1A} receptor antagonist WAY100635, the 5-HT_{2A} receptor antagonist MDL100907 and the α_1 -adrenoceptor antagonist WB4101 all produced dose-dependent rightward shifts of the 2-BFI dose-effect curve: 0.01, 0.032 and 0.1 mg·kg⁻¹ WAY100635 produced 1.06-, 2.04- and 2.52-fold rightward shifts respectively (Figure 3A); 0.01, 0.032 and 0.1 mg·kg⁻¹ MDL100907 produced 1.00-, 2.08- and 3.25-fold rightward shifts respectively (Figure 3B); and 0.32, 1.0 and 3.2 mg·kg⁻¹ WB4101 produced 0.84-, 1.55- and 2.51-fold rightward shifts respectively (Figure 3D). In contrast, the 5-HT_{2C} receptor antagonist SB242084, the α_2 -adrenoceptor antagonist yohimbine, the dopamine D₁ receptor antagonist SCH23390 and the dopamine D₂ receptor antagonist raclopride did not significantly alter the 2-BFI dose-effect curve (Figure 3C, E-G; see Table 1 for ED₅₀ values). Similar results were found with another I₂ receptor agonist, CR4056, in the CCI-induced pain model: 0.01, 0.032 and 0.1 mg·kg⁻¹ WAY100635 produced 1.11-, 2.15- and 3.90-fold rightward shifts of the CR4056 dose-effect curve respectively (Figure 4A); 0.01, 0.032 and 0.1 mg·kg⁻¹ MDL100907 produced 1.32-, 2.40- and 4.33-fold rightward shifts respectively (Figure 4B); and 0.32, 1.0 and 3.2 mg·kg⁻¹ WB4101 produced 1.08-, 2.09- and 3.73-fold rightward shifts respectively (Figure 4D). In contrast, none of the other antagonists tested significantly altered the CR4056 dose-effect curve (Figure 4C, E-G; see Table 1 for ED₅₀ values). Similar results were also found when 2-BFI was tested in rats with CFA-induced inflammatory pain: 0.01, 0.032 and 0.1 mg·kg⁻¹ WAY100635 produced 1.00-, 1.81- and 2.95-fold rightward shifts of the 2-BFI dose-effect curve respectively (Figure 5A); 0.01, 0.032 and 0.1 mg·kg⁻¹ MDL100907 produced 0.99-, 1.76- and 2.39-fold rightward shifts respectively (Figure 5B); and 0.32, 1.0 and 3.2 mg·kg⁻¹ WB4101 produced 0.98-, 1.45- and 2.52-fold rightward shifts respectively (Figure 5D). In contrast, none of the other antagonists tested significantly altered the 2-BFI dose-effect curve (Figure 5C, E-G; see Table 1 for ED₅₀ values). When tested alone, no antagonist significantly altered PWT over a 90 min period (data not shown).

In the body temperature experiment, when WAY100635 and 2-BFI were studied in combination, both 2-BFI [$F(4, 20) = 52.59$] and WAY100635 [$F(3, 15) = 4.82$] produced significant main effects according to two-way repeated-measures ANOVA, but no significant 2-BFI \times WAY100635 interaction was found [$F(12, 60) = 0.51$] (Figure 6A). When MDL100907 and 2-BFI were studied in combination, both 2-BFI [$F(4, 20) = 38.31$] and MDL100907 [$F(3, 15) = 7.20$] produced significant main effects according to two-way repeated-measures

Table 1

ED₅₀ values (with 95% CL) of 2-BFI or CR4056 alone or in combination with pharmacological manipulations of monoaminergic mechanisms

Dose	V	0.01	0.032	0.1	0.32	1.0	3.2	10
BFI/CCI								
Fluoxetine	7.06 (5.92, 8.44)	-	-	-	-	-	4.25 (3.43, 5.30) ^a	2.37 (1.93, 2.91) ^a
Desipramine	7.05 (5.36, 9.26)	-	-	-	-	-	4.53 (3.29, 6.25)	2.31 (1.48, 3.59) ^a
GBR12909	6.68 (5.37, 8.31)	-	-	-	-	-	-	6.27 (5.57, 7.06)
WAY100635	7.64 (6.17, 9.48)	8.08 (7.19, 9.07)	15.62 (11.62, 21.00) ^b	19.24 (13.38, 27.68) ^b	-	-	-	-
MDL100907	5.90 (4.40, 7.92)	5.88 (4.58, 7.55)	12.27 (9.11, 16.53) ^b	19.18 (13.53, 27.20) ^b	-	-	-	-
SB242084	6.53 (4.91, 8.66)	-	-	-	6.34 (5.09, 7.90)	6.18 (4.64, 8.24)	-	-
WB4101	8.86 (7.27, 10.80)	-	-	-	7.43 (5.46, 10.13)	13.72 (11.17, 16.86) ^b	22.21 (18.63, 26.49) ^b	-
Yohimbine	6.84 (5.82, 8.05)	-	-	-	-	6.65 (5.23, 8.45)	6.63 (4.98, 8.84)	-
SCH23390	6.43 (4.46, 9.29)	-	6.47 (4.51, 9.30)	5.85 (4.16, 8.21)	-	-	-	-
Raclopride	6.50 (4.77, 8.86)	-	-	-	6.93 (4.77, 10.07)	6.09 (4.28, 8.68)	-	-
CR4056/CCI								
WAY100635	10.33 (8.80, 12.12)	11.47 (9.27, 14.19)	22.16 (18.42, 26.66) ^b	40.25 (32.47, 49.88) ^b	-	-	-	-
MDL100907	10.49 (7.72, 14.25)	13.81 (10.45, 18.23)	25.19 (17.70, 35.86) ^b	45.45 (31.12, 66.37) ^b	-	-	-	-
SB242084	10.85 (7.69, 15.30)	-	-	-	10.94 (8.34, 14.35)	12.45 (8.89, 17.42)	-	-
WB4101	11.71 (9.12, 15.04)	-	-	-	12.67 (10.26, 15.66)	24.44 (18.34, 32.57) ^b	43.72 (35.36, 54.07) ^b	-
Yohimbine	12.81 (9.91, 16.57)	-	-	-	-	11.11 (9.11, 13.54)	11.47 (8.09, 16.29)	-
SCH23390	11.10 (8.29, 14.96)	-	10.88 (7.18, 16.49)	11.00 (8.10, 14.96)	-	-	-	-
Raclopride	12.65 (9.50, 16.84)	-	-	-	12.95 (9.50, 17.66)	9.27 (6.79, 12.67)	-	-

continues

Table 1
(Continued)

Dose	V	0.01	0.032	0.1	0.32	1.0	3.2	10
BF1/CFA								
WAY100635	8.46 (7.29, 9.81)	8.46 (7.05, 10.15)	15.34 (13.63, 17.27) ^b	24.99 (21.15, 29.52) ^b	-	-	-	-
MDL100907	8.35 (7.11, 9.82)	8.23 (6.75, 10.04)	14.69 (11.51, 18.74) ^b	19.99 (14.91, 26.81) ^b	-	-	-	-
SB242084	8.33 (7.13, 9.74)	-	-	-	8.49 (7.31, 9.87)	8.52 (6.84, 10.60)	-	-
WB4101	8.59 (6.80, 10.86)	-	-	-	8.40 (6.93, 10.18)	12.47 (10.85, 14.34)	21.68 (17.98, 26.14) ^b	-
Yohimbine	7.78 (6.76, 8.96)	-	-	-	-	6.75 (5.14, 8.88)	7.86 (6.88, 8.97)	-
SCH23390	7.79 (6.72, 9.02)	-	6.38 (4.44, 9.16)	8.29 (7.19, 9.55)	-	-	-	-
Raclopride	7.68 (6.40, 9.20)	-	-	-	8.64 (7.57, 9.86)	7.37 (6.03, 9.00)	-	-

^aCombination ED₅₀ lower than control.

^bCombination ED₅₀ higher than control.

The above indicates that CLs do not overlap with those of the control values (2-BFI or CR4056 alone).

ANOVA, but no significant 2-BFI × MDL100907 interaction was found [$F(12, 60) = 0.68$] (Figure 6B). When SB242084 and 2-BFI were studied in combination, both 2-BFI [$F(4,$

20) = 88.70] and SB242084 [$F(2, 10) = 7.28$] produced significant main effects according to two-way repeated-measures ANOVA, but no significant 2-BFI × SB242084 interaction was found [$F(8, 40) = 1.17$] (Figure 6C). When WB4101 and 2-BFI were studied in combination, both 2-BFI [$F(4, 24) = 99.81$] and WB4101 [$F(3, 18) = 3.99$] produced significant main effects according to two-way repeated-measures ANOVA, but no significant 2-BFI × WB4101 interaction was found [$F(12, 72) = 0.82$] (Figure 6D). When SCH23390 and 2-BFI were studied in combination, 2-BFI [$F(4, 24) = 64.41$] produced a significant main effect according to two-way repeated-measures ANOVA, but no significant main effect of SCH23390 [$F(2, 12) = 1.13$] or 2-BFI × SCH23390 interaction was found [$F(8, 48) = 0.77$] (Figure 6E). When raclopride and 2-BFI were studied in combination, 2-BFI [$F(4, 20) = 49.18$] produced a significant main effect according to two-way repeated-measures ANOVA, but no significant main effect of raclopride [$F(2, 10) = 2.50$] or 2-BFI × raclopride interaction was found [$F(8, 40) = 1.48$] (Figure 6F). Combinations of yohimbine and 2-BFI were not tested in this study since this had been investigated in a previous study (Thorn *et al.*, 2012).

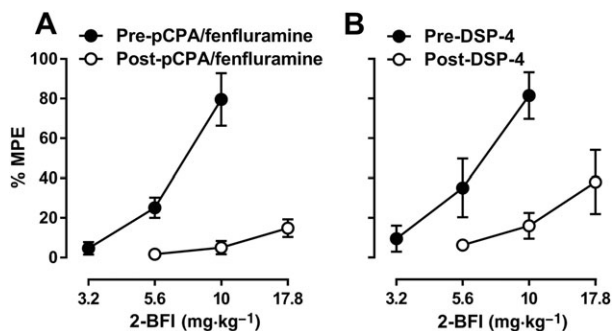


Figure 2

Percentage of the maximum possible effects (%MPE) of 2-BFI on CCI-induced mechanical nociception before and after treatment with pCPA and fenfluramine (A, $n = 6$) or DSP-4 (B, $n = 6$). Data shown are means ± SEM.

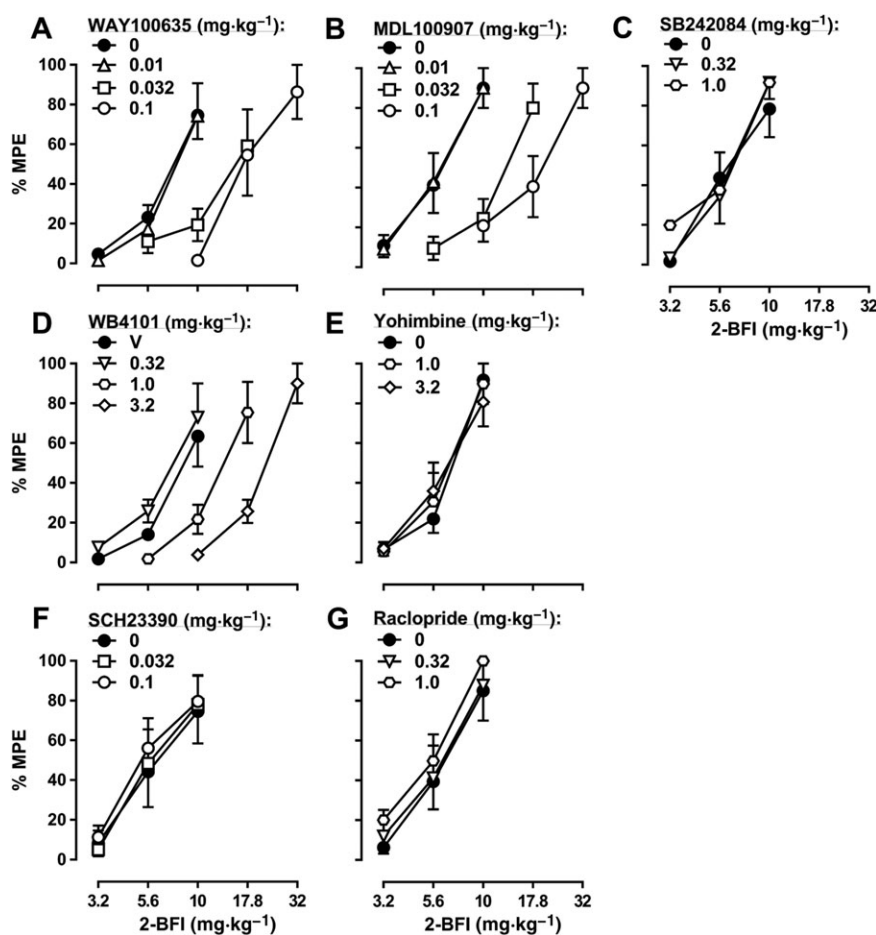


Figure 3

Percentage of the maximum possible effects (%MPE) of 2-BFI alone and in combination with WAY100635 (A, $n = 6$), MDL100907 (B, $n = 5$), SB242084 (C, $n = 6$), WB4101 (D, $n = 5$), yohimbine (E, $n = 6$), SCH23390 (F, $n = 6$) or raclopride (G, $n = 6$) on CCI-induced mechanical nociception. Data shown are means ± SEM.

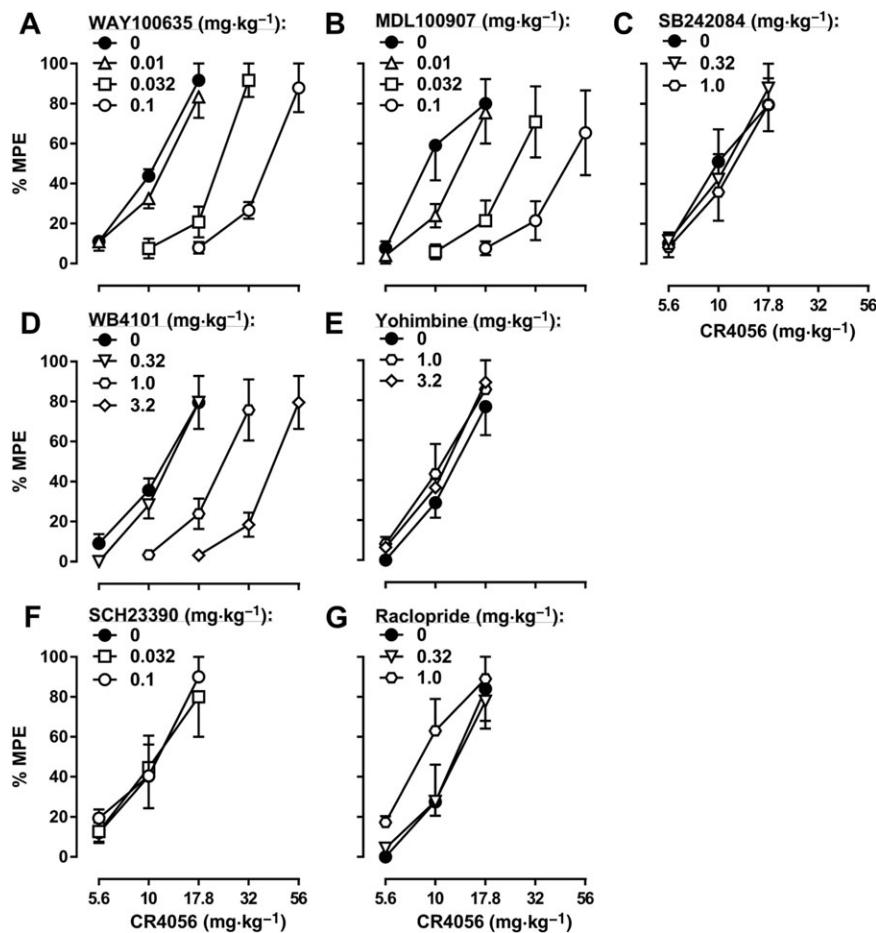


Figure 4

Percentage of the maximum possible effects (%MPE) of CR4056 alone and in combination with WAY100635 (A, $n = 6$), MDL100907 (B, $n = 5$), SB242084 (C, $n = 6$), WB4101 (D, $n = 6$), yohimbine (E, $n = 5$), SCH23390 (F, $n = 5$) or raclopride (G, $n = 5$) on CCI-induced mechanical nociception. Data shown are means \pm SEM.

In the drug discrimination study, rats met test criteria after an average of 41 training sessions (range = 39–44). Saline produced 1.5% 2-BFI-associated lever responding, whereas 2-BFI dose-dependently increased responding on the 2-BFI-associated lever up to a maximum of 98.4% at a dose of 5.6 mg·kg⁻¹. The ED₅₀ value (\pm 95% CL) for 2-BFI substitution was 1.80 (1.35, 2.38) mg·kg⁻¹ (Figure 7A). When the highest dose of each monoamine receptor antagonist used in the nociception studies (or that did significantly affect operant responding) was administered as pretreatments, the ED₅₀ values for 2-BFI substitution did not significantly change. The ED₅₀ values (\pm 95% CL) following antagonist administration are as follows: 0.1 mg·kg⁻¹ WAY100635, 2.14 (1.29, 3.54) mg·kg⁻¹ 2-BFI; 0.1 mg·kg⁻¹ MDL100907, 1.71 (1.01, 2.87) mg·kg⁻¹ 2-BFI; 1.0 mg·kg⁻¹ SB242084, 3.67 (1.57, 8.65) mg·kg⁻¹ 2-BFI; 3.2 mg·kg⁻¹ WB4101, 2.34 (1.41, 3.90) mg·kg⁻¹ 2-BFI; 3.2 mg·kg⁻¹ yohimbine, 1.99 (1.24, 3.21) mg·kg⁻¹ 2-BFI; 0.01 mg·kg⁻¹ SCH23390, 2.96 (1.67, 5.27) mg·kg⁻¹ 2-BFI; and 0.1 mg·kg⁻¹ raclopride, 2.70 (1.67, 4.35) mg·kg⁻¹ 2-BFI (Figure 7A–G). The response rates for all treatments are shown in Figure 7H–N. Two-way ANOVAs

revealed that the following treatments produced significant main effects on response rate: 0.1 mg·kg⁻¹ WAY100635 [$F(1, 58) = 4.50$], 1.0 mg·kg⁻¹ SB242084 [$F(1, 54) = 4.30$], 3.2 mg·kg⁻¹ WB4101 [$F(1, 54) = 8.40$], 3.2 mg·kg⁻¹ yohimbine [$F(1, 58) = 4.66$] and 0.01 mg·kg⁻¹ SCH23390 [$F(1, 54) = 13.81$]. However, in none of these tests was there a significant treatment \times 2-BFI interaction. Paired t -tests showed that 0.032 mg·kg⁻¹ SCH23390 [$t(6) = 10.90$] and 0.32 mg·kg⁻¹ raclopride [$t(6) = 2.38$] significantly reduced response rate when administered alone.

Following these tests, the 2-BFI substitution dose-effect curve was reestablished. Saline produced 0% 2-BFI-associated lever responding, whereas 2-BFI dose-dependently increased responding on the 2-BFI-associated lever up to a maximum of 98.7% at a dose of 5.6 mg·kg⁻¹; the ED₅₀ value (\pm 95% CL) for 2-BFI substitution was 3.17 (2.45, 4.11) mg·kg⁻¹ (Figure 8B). None of the monoamine reuptake inhibitors, when administered alone, induced significant 2-BFI-associated lever responding (Figure 8A); the highest substitution induced by any of these drugs was 33.3% 2-BFI-associated lever responding, produced by 10 mg·kg⁻¹

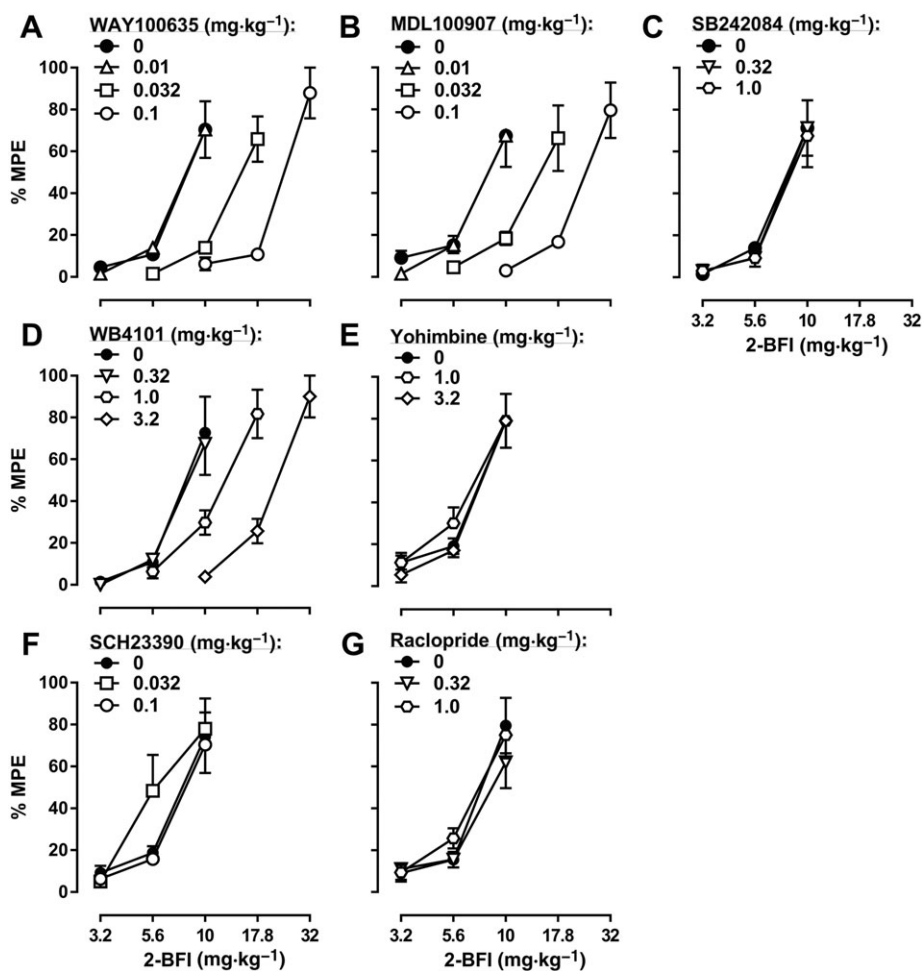


Figure 5

Percentage of the maximum possible effects (%MPE) of 2-BFI alone and in combination with WAY100635 (A, $n = 6$), MDL100907 (B, $n = 6$), SB242084 (C, $n = 6$), WB4101 (D, $n = 6$), yohimbine (E, $n = 5$), SCH23390 (F, $n = 6$) or raclopride (G, $n = 6$) on CFA-induced mechanical nociception. Data shown are means \pm SEM.

GBR12909. One-way ANOVAs revealed that fluoxetine [$F(3, 18) = 8.79$], desipramine [$F(3, 20) = 13.04$], imipramine [$F(3, 19) = 6.33$] and GBR12909 [$F(3, 24) = 7.64$] all produced significant main effects on response rate (Figure 8F). When these reuptake inhibitors were administered as pretreatments, the ED₅₀ values for 2-BFI substitution did not significantly change. The ED₅₀ values ($\pm 95\%$ CL) following reuptake inhibitor administration are as follows: 1.0 mg·kg⁻¹ fluoxetine, 2.51 (1.49, 4.24) mg·kg⁻¹ 2-BFI; 3.2 mg·kg⁻¹ fluoxetine, 2.37 (1.36, 4.14) mg·kg⁻¹ 2-BFI; 3.2 mg·kg⁻¹ desipramine, 3.34 (1.95, 5.70) mg·kg⁻¹ 2-BFI; 3.2 imipramine, 2.69 (1.75, 4.13) mg·kg⁻¹ 2-BFI; 3.2 GBR12909, 3.19 (2.23, 4.56) mg·kg⁻¹ 2-BFI (Figure 8B–E). Two-way ANOVA revealed that the following treatments produced significant main effects on response rate: fluoxetine [$F(2, 88) = 8.84$] and 3.2 mg·kg⁻¹ desipramine [$F(1, 59) = 5.57$] (Figure 8G–H). Paired t -tests showed that 10 mg·kg⁻¹ fluoxetine [$t(6) = 6.91$], 10 mg·kg⁻¹ desipramine [$t(6) = 17.66$], 10 mg·kg⁻¹ imipramine [$t(6) = 17.66$] and 10 mg·kg⁻¹ GBR12909 [$t(6) = 7.04$] significantly reduced response rate when administered alone (Figure 8G–J).

Discussion

The primary findings of this study were that selective 5-HT and noradrenaline reuptake inhibitors each induced significant leftward shifts of the antinociceptive dose-effect curve of the I₂ receptor agonist 2-BFI, whereas specific depletions of either 5-HT or noradrenaline abolished the antinociceptive effect of 2-BFI. Specific antagonists of the 5-HT_{1A}, 5-HT_{2A} and α_1 -adrenoceptors each induced significant, dose-dependent rightward shifts of the antinociceptive dose-effect curves of 2-BFI and another I₂ receptor agonist, CR4056. In contrast, none of the specific antagonists used in this study produced a significant interaction with 2-BFI in an assay measuring 2-BFI-induced hypothermia. Likewise, none of the specific antagonists or monoamine reuptake inhibitors significantly altered the 2-BFI substitution dose-effect curve in rats trained to discriminate 5.6 mg·kg⁻¹ 2-BFI from saline. These results suggest that the antinociceptive effects produced by I₂ receptor agonists involve serotonergic and noradrenergic components but that the hypothermic and discriminative stimulus effects of

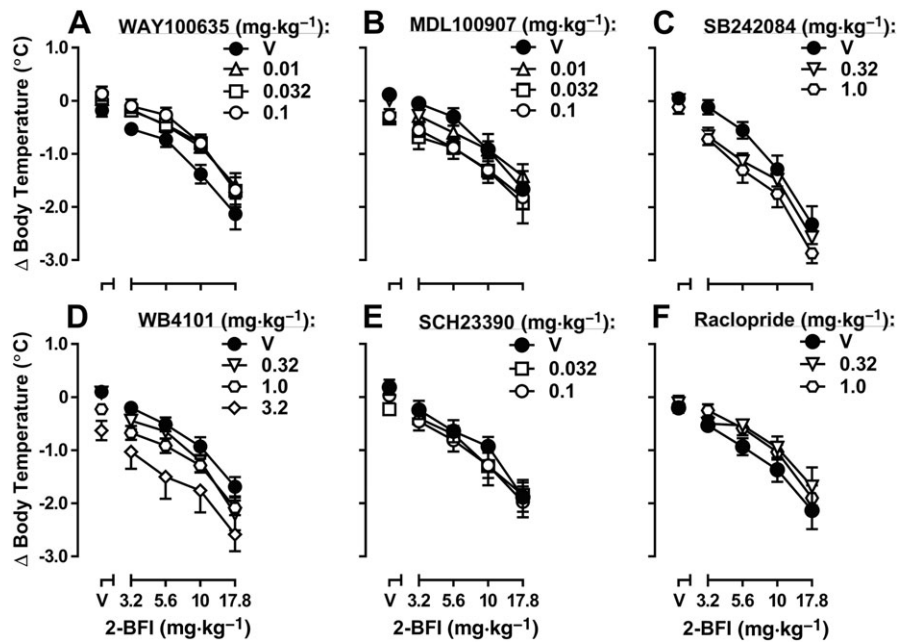


Figure 6

Hypothermic effects of 2-BFI alone and in combination with WAY100635 (A, $n = 6$), MDL100907 (B, $n = 6$), SB242084 (C, $n = 6$), WB4101 (D, $n = 7$), SCH23390 (E, $n = 7$) or raclopride (F, $n = 6$). Data shown are means \pm SEM of body temperature change from baseline.

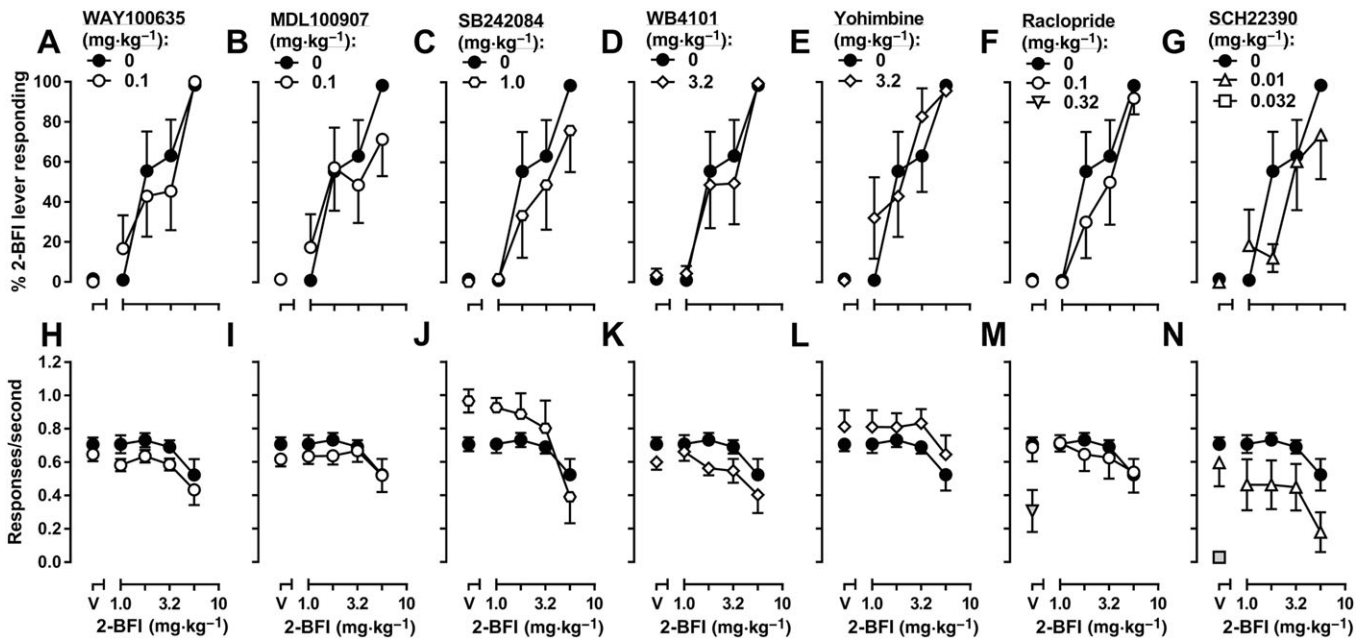


Figure 7

Discriminative stimulus effects of 2-BFI alone or in combination with WAY100635 (A), MDL100907 (B), SB242084 (C), WB4101 (D), yohimbine (E), raclopride (F) or SCH23390 (G) in rats discriminating 5.6 mg·kg⁻¹ 2-BFI from its vehicle ($n = 7$). Upper graphs: substitution profiles of 2-BFI or drug combinations. Data shown are means \pm SEM of percentage of 2-BFI-appropriate lever responding. Lower graphs: response rate. Data shown are means \pm SEM of the response rate (responses·s⁻¹). Shaded symbols are significantly different ($P < 0.05$) from 2-BFI alone.

these drugs are likely to involve different mediators. This finding will increase the understanding of I₂ receptor pharmacology and may help guide the development of novel I₂

receptor ligands towards specifically producing therapeutically important (e.g. analgesic) effects without producing other, unwanted effects.

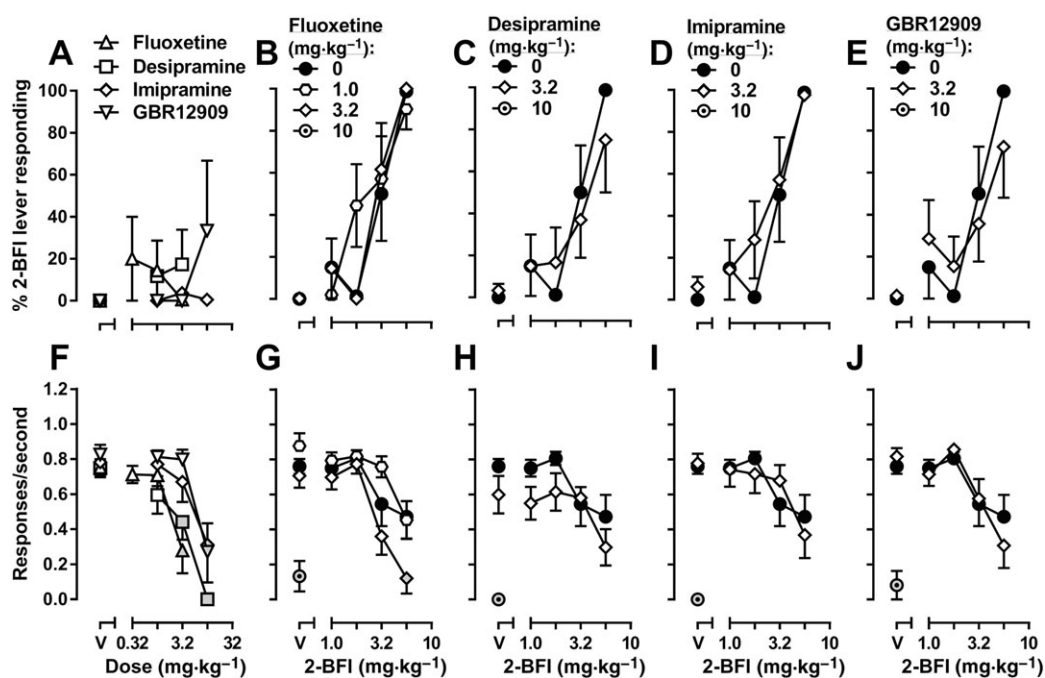


Figure 8

Discriminative stimulus effects of fluoxetine, desipramine, imipramine or GBR12909 alone (A) or 2-BFI alone or in combination with fluoxetine (B), desipramine (C), imipramine (D) or GBR12909 (E) in rats discriminating $5.6 \text{ mg}\cdot\text{kg}^{-1}$ 2-BFI from its vehicle ($n = 7$). Upper graphs: substitution profiles of drugs or drug combinations. Data shown are means \pm SEM of percentage of 2-BFI-appropriate lever responding. Lower graphs: response rate. Data shown are means \pm SEM response rate ($\text{responses}\cdot\text{s}^{-1}$). Shaded symbols are significantly different ($P < 0.05$) from vehicle (A) or 2-BFI alone (B–E).

CCI- and CFA-induced hyperalgesia are well-described and widely used animal models of chronic neuropathic and inflammatory pain respectively. CCI surgery or CFA hind paw injection each induces persistent hypersensitivity to a range of stimuli (Bennett and Xie, 1988; Nagakura *et al.*, 2003; Li *et al.*, 2014), making them appropriate models with which to study potential analgesics. Because the I_2 receptors exist as inhibitory sites on MAO A and B (Jones *et al.*, 2007; McDonald *et al.*, 2010) and because administration of I_2 receptor agonists increases spinal and supraspinal CNS levels of 5-HT, noradrenaline and dopamine (Nutt *et al.*, 1995; Ugedo *et al.*, 1999; Ferrari *et al.*, 2011), we examined whether manipulations of these monoamine systems altered the anti-nociception induced by I_2 receptor agonists, in rats with CCI-induced neuropathic pain. We initially studied fluoxetine, desipramine and GBR12909, selective reuptake inhibitors of 5-HT, noradrenaline and dopamine respectively. While none of these three drugs produced antinociception when administered alone, pretreatments with either fluoxetine or desipramine produced dose-dependent leftward shifts of the 2-BFI dose-effect curve. To verify the importance of 5-HT and noradrenaline in the antinociceptive effects of 2-BFI, we used well-established methods to deplete 5-HT or noradrenaline in separate groups of rats (Jaim-Etcheverry and Zieher, 1980; Prinssen *et al.*, 2002). As compared to dose-effect curves established beforehand, the dose-effect curves of 2-BFI following either 5-HT or noradrenaline depletion were nearly flat, as though the anti-nociceptive effects of

2-BFI had been almost fully abolished. To understand whether particular 5-HT receptors or adrenoceptors were involved in the anti-nociceptive effects of 2-BFI, we investigated a series of specific monoamine receptor antagonists in combination with 2-BFI. The 5-HT $_{1A}$ receptor antagonist WAY100635, the 5-HT $_{2A}$ receptor antagonist MDL100907 and the α_1 -adrenoceptor antagonist WB4101, each dose-dependently shifted the dose-effect curve of 2-BFI rightward, whereas antagonists of other 5-HT, noradrenaline or dopamine receptors had no significant effect, demonstrating pharmacological specificity of these serotonergic and noradrenergic components. This profile of results was reproduced with CR4056 in the CCI model and with 2-BFI in the CFA-induced inflammatory pain model. Importantly, both 2-BFI and CR4056 are remarkably specific for I_2 receptors and do not display appreciable affinity for monoaminergic receptors or transporters (Nutt *et al.*, 1995; Ferrari *et al.*, 2011).

The I_2 receptor agonists produce several behavioural and physiological effects: antinociception (Li *et al.*, 2014; Li, 2017), hypothermia (Thorn *et al.*, 2012) and discriminative stimulus effects (Jordan *et al.*, 1996; MacInnes and Handley, 2002; MacInnes and Handley, 2003; Qiu *et al.*, 2014; Qiu *et al.*, 2015). In order to test the generality of the monoaminergic component observed above, we next tested the role of monoaminergic transmission in the hypothermic effects of 2-BFI. Consistent with the literature, 2-BFI consistently induced hypothermia over a dose range of $3.2\text{--}17.8 \text{ mg}\cdot\text{kg}^{-1}$. Interestingly, none of the specific monoamine receptor

antagonists tested above produced a significant interaction with 2-BFI on hypothermia. This study did not investigate combinations of yohimbine and 2-BFI as data showing that yohimbine did not alter 2-BFI-induced hypothermia had been published previously (Thorn *et al.*, 2012). These data suggest that the hypothermic effects of 2-BFI are not likely to involve serotonergic, noradrenergic or dopaminergic transmission and are instead mediated *via* mechanisms involving other mediators.

We finally tested the role of monoaminergic mechanisms in mediating the discriminative stimulus effects of 2-BFI. Several I₂ receptor agonists have been previously studied as discriminative stimuli including 2-BFI (Jordan *et al.*, 1996; Qiu *et al.*, 2014; Qiu *et al.*, 2015; Siemian *et al.*, 2017). Interestingly, MacInnes and Handley (2002; 2003) reported that serotonergic and noradrenergic components were involved in the discriminative stimulus effects of 2-BFI. For example, monoamine releasers (*d*-amphetamine, fenfluramine), MAO inhibitors (moclobemide, pargyline), as well as 5-HT (clomipramine, citalopram) and noradrenaline (desipramine, reboxetine) reuptake inhibitors all at least partly substituted for 2-BFI. Further, α_1 -adrenoceptor antagonism disrupted the discriminative cue produced by 2-BFI. In the present study, all rats acquired 5.6 mg·kg⁻¹ 2-BFI discrimination at a similar rate as in previous studies where, for example, rats had acquired 7 mg·kg⁻¹ 2-BFI discrimination after 44–64 training sessions (Jordan *et al.*, 1996). However, in contrast to the monoaminergic components suggested by previous studies, none of the monoamine receptor antagonists tested significantly altered the 2-BFI substitution dose-effect curve when administered at the highest dose tested in the nociception studies or at the highest possible dose which did not suppress response rates to the point of obscuring substitution data interpretation (SCH23390 and raclopride). Additionally, among four monoamine reuptake inhibitors tested – fluoxetine (5-HT), desipramine (noradrenaline), imipramine (5-HT/ noradrenaline) and GBR12909 (dopamine) – no drug significantly substituted for 2-BFI or significantly altered the dose-effect curve for 2-BFI substitution when given as a pretreatment. Thus, these results from the current study oppose previous findings that monoaminergic components are important for the discriminative stimulus effects of 2-BFI and suggest that these effects are instead likely to be due to a distinct, independent mechanism. Support for this also comes from a recent finding that the 5-HT_{2A} receptor agonist DOM did not substitute for 2-BFI (Siemian *et al.*, 2017). Moreover, that the same treatments which significantly altered 2-BFI-induced antinociception did not have a significant effect on the discriminative stimulus effects of 2-BFI suggests that these behavioural endpoints are mediated *via* distinct post-I₂ receptor signalling pathways or I₂ receptor populations. Similar divergence was found in a recent study, which showed that I₂ receptor-mediated antinociception and discriminative stimulus effects were differentially sensitive to inhibitors of calcium influx or signalling mechanisms (Siemian *et al.*, 2017), which also lends credence to the present findings, as calcium influx is necessary for vesicular monoamine release (Matthews, 1996).

The populations of I₂ receptors are heterogenous, not only existing as binding sites on MAO but also as several other proteins of 30, 45 and 66 kDa sizes (Keller and Garcia-

Sevilla, 2015). Thus far, only the 45 kDa protein has been identified as brain creatine kinase (Kimura *et al.*, 2009). While the contributions of these different proteins to the behavioural effects of I₂ receptor agonists are unknown, this heterogeneity supports the concept of divergence between different I₂ receptor-mediated behavioural effects. Still, that this phenomenon has only recently begun to be observed makes the results of the current study important. For the population of I₂ receptors that underlie antinociception, the data from the current study support previous theories that I₂ receptors are alternative inhibitory binding sites on MAO. Here, we provide a hypothesis to explain the pharmacological role of these I₂ receptors based on existing evidence as follows. Upon binding to I₂ receptors, the agonists inhibit MAO activity thereby slowing the metabolism of monoamines and increasing their synaptic concentrations. This higher concentration of certain monoamines (e.g. 5-HT and noradrenaline) causes greater activation of certain corresponding receptors (i.e. 5-HT_{1A}, 5-HT_{2A} and α_1 -adrenoceptors) which then produces anti-nociception, presumably through their downstream mechanisms (Figure 9). The involvement of the three receptors implicated in antinociception in this study has previously been demonstrated (Tasker *et al.*, 1992; Bardin, 2011; Valhondo *et al.*, 2013; Wattiez *et al.*, 2013; Sun *et al.*, 2014). Interestingly,

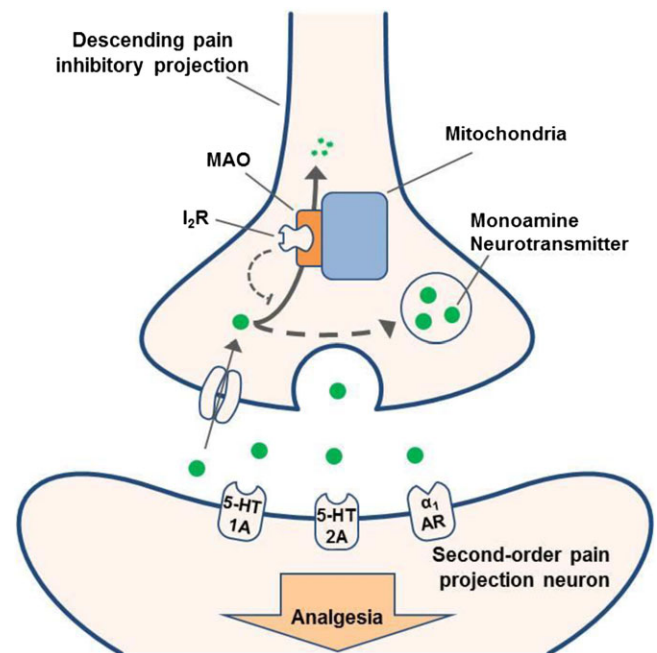


Figure 9

Diagram describing the probable mechanism of action of I₂ receptor agonists. Under normal conditions, monoamines such as 5-HT and noradrenaline undergo reuptake and are metabolized by MAO enzymes (solid arrow). Administration of I₂ receptor agonists, and I₂ receptor activation, inhibits MAO activity and allows monoamine neurotransmitters to be repackaged, increasing their synaptic concentration (dashed arrows). The corresponding increased activation of certain downstream monoaminergic receptors (i.e. 5-HT_{1A}, 5-HT_{2A} and α_1 -adrenoceptors) leads to the production of analgesia.

the anti-nociceptive effects of the reversible MAO_A inhibitor moclobemide appear to be mediated by α_2 -adrenoceptors and not 5-HT receptors or α_1 -adrenoceptors (Schreiber *et al.*, 1998), whereas those of the tricyclic 5-HT/noradrenaline reuptake inhibitor imipramine seem to be mediated by 5-HT_{2A} and α_1 -adrenoceptors but not 5-HT_{1A} receptors (Otsuka *et al.*, 2001). That the mechanistic profile of I₂ receptor agonists overlaps more with reuptake inhibitors than MAO inhibitors is surprising, given the presumptive mechanism of action (i.e. MAO inhibition) of I₂ receptor agonists. However, whether MAO inhibition fully accounts in full for these monoaminergic mechanisms is unknown. Enigmatically, although I₂ receptor ligands appear to saturate their receptors at low nanomolar concentrations, they only inhibit MAO with IC₅₀s in the high nanomolar or low micromolar range *in vitro* (Ozaita *et al.*, 1997). This may result from the competitive inhibition of MAO A, but mixed inhibition of MAO B, wherein a low-affinity I₂ site exists on the enzyme in its active conformation, but a high-affinity site exists during the enzyme in its inactive conformation which occurs during monoamine oxidation (Jones *et al.*, 2007; McDonald *et al.*, 2010). Thus, these inhibition values may be inflated, relative to those actually occurring *in vivo*. No pharmacokinetic/pharmacodynamic studies have been performed on I₂ receptor ligands, so the CNS dose range achieved by systemic administration is currently unknown. In any case, the dose ranges of 2-BFI which produce behavioural effects such as antinociception (3.2–17.8 mg·kg⁻¹) are similar to those which increase monoamines (5–20 mg·kg⁻¹ 2-BFI), supporting the idea that these effects are related (Nutt *et al.*, 1995; Ferrari *et al.*, 2011). Additionally, while the antinociceptive effects of I₂ receptor agonists are known to be centrally mediated, whether monoaminergic effects are more important to antinociception at the spinal or supraspinal levels needs to be further investigated, as there are reports of roles at both levels of the CNS (Diaz *et al.*, 1997; Ugedo *et al.*, 1999; Ruiz-Durantez *et al.*, 2003; Thorn, Qiu, *et al.*, 2016a). Future research, which may elucidate the mechanisms underlying the hypothermic or discriminative stimulus effects as well as the contributions from different proteins or signalling pathways to such functions, should allow the refinement of new I₂ receptor ligands toward the specific production of antinociception while minimizing unwanted side effects.

In summary, this is the first study to demonstrate the direct functional involvement of 5-HT and noradrenaline as well as 5-HT_{1A}, 5-HT_{2A} and α_1 -adrenoceptors in the antinociceptive effects of I₂ receptor agonists. In contrast, the hypothermic and discriminative stimulus effects of 2-BFI appear to be mediated by distinct, unrelated mechanisms. These results support previous findings regarding dissociation between I₂ receptor-mediated behavioural effects (Siemian *et al.*, 2017) and provide further *in vivo* evidence that I₂ receptor populations and/or post-receptor signalling mechanisms are heterogeneous. Some of these mechanisms, such as those that underlie antinociceptive effects, involve 5-HT and noradrenaline and are likely to be related to the presence of I₂ receptors on MAO enzymes, whereas other mechanisms, such as those that underlie hypothermic and discriminative stimulus effects, are more likely to be unrelated to MAO.

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Author contributions

The main idea of this study was from J.S. and J.-X.L. J.S. and K.W. conducted the experiments. J.S. and J.-X.L. designed the study, conducted the data analysis and wrote the first draft of the manuscript. Y.Z. provided 2-BFI and CR4056. All authors contributed to and approved the final version of the manuscript.

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 pre-clinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

- National Research Council (2011). Guide for the Care and Use of Laboratory Animals. edn, 10.17226/12910. National Academy of Sciences: Washington DC.
- Alexander SPH, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA *et al.* (2017a). The Concise Guide to Pharmacology 2017/18: G protein-coupled receptors. *Br J Pharmacol* 174: S17–S129.
- Alexander SPH, Fabbro D, Kelly E, Marrion NV, Peters JA, Faccenda E *et al.* (2017b). The Concise Guide to PHARMACOLOGY 2017/18: Enzymes. *Br J Pharmacol* 174: S272–S359.
- Alexander SPH, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD *et al.* (2017c). The Concise Guide to PHARMACOLOGY 2017/18: Transporters. *Br J Pharmacol* 174: S360–S446.
- Bardin L (2011). The complex role of serotonin and 5-HT receptors in chronic pain. *Behav Pharmacol* 22: 390–404.
- 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.
- Conger JJ (1951). The effects of alcohol on conflict behavior in the albino rat. *Q J Stud Alcohol* 12: 1–29.
- 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.

- Diaz A, Mayet S, Dickenson AH (1997). BU-224 produces spinal antinociception as an agonist at imidazoline I₂ receptors. *Eur J Pharmacol* 333: 9–15.
- Ferrari F, Fiorentino S, Mennuni L, Garofalo P, Letari O, Mandelli S *et al.* (2011). Analgesic efficacy of CR4056, a novel imidazoline-2 receptor ligand, in rat models of inflammatory and neuropathic pain. *J Pain Res* 4: 111–125.
- Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S *et al.* (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucl Acids Res* 46: D1091–D1106.
- Ishihara M, Togo H (2007). Direct oxidative conversion of aldehydes and alcohols to 2-imidazolines and 2-oxazolines using molecular iodine. *Tetrahedron* 63: 1474–1480.
- Jaim-Etcheverry G, Zieher LM (1980). DSP-4: a novel compound with neurotoxic effects on noradrenergic neurons of adult and developing rats. *Brain Res* 188: 513–523.
- Jones TZ, Giurato L, Guccione S, Ramsay RR (2007). Interactions of imidazoline ligands with the active site of purified monoamine oxidase A. *FEBS J* 274: 1567–1575.
- Jordan S, Jackson HC, Nutt DJ, Handley SL (1996). Discriminative stimulus produced by the imidazoline I₂ site ligand, 2-BFI. *Journal of psychopharmacology (Oxford, England)* 10: 273–278.
- Keller B, Garcia-Sevilla JA (2015). Immunodetection and subcellular distribution of imidazoline receptor proteins with three antibodies in mouse and human brains: Effects of treatments with I₁- and I₂-imidazoline drugs. *Journal of psychopharmacology (Oxford, England)* 29: 996–1012.
- 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.
- Kimura A, Tyacke RJ, Robinson JJ, Husbands SM, Minchin MC, Nutt DJ *et al.* (2009). Identification of an imidazoline binding protein: creatine kinase and an imidazoline-2 binding site. *Brain Res* 1279: 21–28.
- Kissin I (2010). The development of new analgesics over the past 50 years: a lack of real breakthrough drugs. *Anesth Analg* 110: 780–789.
- Kudo T, Kushikata T, Kudo M, Kudo T, Hirota K (2011). Antinociceptive effects of neurotrophin in a rat model of central neuropathic pain: DSP-4 induced noradrenergic lesion. *Neurosci Lett* 503: 20–22.
- Li JX (2017). Imidazoline I₂ receptors: an update. *Pharmacol Ther* <https://doi.org/10.1016/j.pharmthera.2017.03.009> 178: 48–56.
- Li JX, Koek W, France CP (2009a). Food restriction and streptozotocin differentially modify sensitivity to the hypothermic effects of direct- and indirect-acting serotonin receptor agonists in rats. *Eur J Pharmacol* 613: 60–63.
- Li JX, Unzeitig A, Javors MA, Rice KC, Koek W, France CP (2009b). Discriminative stimulus effects of 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM), ketanserin, and (R)-(+)-[alpha]-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-pipidinemetha nol (MDL100907) in rats. *J Pharmacol Exp Ther* 331: 671–679.
- Li J-X, Thorn DA, Qiu Y, Peng B-W, Zhang Y (2014). Antihyperalgesic effects of imidazoline I(2) receptor ligands in rat models of inflammatory and neuropathic pain. *Brit J Pharmacol* 171: 1580–1590.
- Lione LA, Nutt DJ, Hudson AL (1998). Characterisation and localisation of [3H]2-(2-benzofuranyl)-2-imidazoline binding in rat brain: a selective ligand for imidazoline I₂ receptors. *Eur J Pharmacol* 353: 123–135.
- MacInnes N, Handley SL (2002). Characterization of the discriminable stimulus produced by 2-BFI: effects of imidazoline I(2)-site ligands, MAOIs, beta-carbolines, agmatine and ibogaine. *Br J Pharmacol* 135: 1227–1234.
- MacInnes N, Handley SL (2003). Potential serotonergic and noradrenergic involvement in the discriminative stimulus effects of the selective imidazoline I₂-site ligand 2-BFI. *Pharmacol Biochem Behav* 75: 427–433.
- MacKinnon AC, Redfern WS, Brown CM (1995). [3H]-RS-45041-190: a selective high-affinity radioligand for I₂ imidazoline receptors. *Br J Pharmacol* 116: 1729–1736.
- Matthews G (1996). Neurotransmitter release. *Annu Rev Neurosci* 19: 219–233.
- McDonald GR, Olivieri A, Ramsay RR, Holt A (2010). On the formation and nature of the imidazoline I₂ binding site on human monoamine oxidase-B. *Pharmacol Res* 62: 475–488.
- 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.
- Nagakura Y, Okada M, Kohara A, Kiso T, Toya T, Iwai A *et al.* (2003). Allodynia and hyperalgesia in adjuvant-induced arthritic rats: time course of progression and efficacy of analgesics. *J Pharmacol Exp Ther* 306: 490–497.
- NIH (2013). Pain in America. In: *NINDS Chronic Pain Information Page*. Bethesda, MD.
- Nutt DJ, French N, Handley S, Hudson A, Husbands S, Jackson H *et al.* (1995). Functional studies of specific imidazoline-2 receptor ligands. *Ann N Y Acad Sci* 763: 125–139.
- Otsuka N, Kiuchi Y, Yokogawa F, Masuda Y, Oguchi K, Hosoyamada A (2001). Antinociceptive efficacy of antidepressants: assessment of five antidepressants and four monoamine receptors in rats. *J Anesth* 15: 154–158.
- Ozaita A, Olmos G, Boronat MA, Lizcano JM, Unzeta M, Garcia-Sevilla JA (1997). Inhibition of monoamine oxidase A and B activities by imidazol(ine)/guanidine drugs, nature of the interaction and distinction from I₂-imidazoline receptors in rat liver. *Br J Pharmacol* 121: 901–912.
- Prinszen EP, Assie MB, Koek W, Kleven MS (2002). Depletion of 5-HT disrupts prepulse inhibition in rats: dependence on the magnitude of depletion, and reversal by a 5-HT precursor. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology* 26: 340–347.
- Qiu Y, He XH, Zhang Y, Li JX (2014). Discriminative stimulus effects of the novel imidazoline I(2) receptor ligand CR4056 in rats. *Sci Rep* 4: 6605.
- Qiu Y, Zhang Y, Li JX (2015). Discriminative stimulus effects of the imidazoline I₂ receptor ligands BU224 and phenylzoline in rats. *Eur J Pharmacol* 749: 133–141.
- Ruiz-Durantez E, Torrecilla M, Pineda J, Ugedo L (2003). Attenuation of acute and chronic effects of morphine by the imidazoline receptor ligand 2-(2-benzofuranyl)-2-imidazoline in rat locus coeruleus neurons. *Br J Pharmacol* 138: 494–500.
- Schreiber S, Getslev V, Weizman A, Pick CG (1998). The antinociceptive effect of moclobemide in mice is mediated by noradrenergic pathways. *Neurosci Lett* 253: 183–186.

Siemian JN, Obeng S, Zhang Y, Zhang Y, Li JX (2016). Antinociceptive interactions between the imidazoline I2 receptor agonist 2-BFI and opioids in rats: role of efficacy at the mu-opioid receptor. *J Pharmacol Exp Ther* 357: 509–519.

Siemian JN, Qiu Y, Zhang Y, Li J-X (2017). Role of intracellular Ca²⁺ signaling in the antinociceptive and discriminative stimulus effects of the imidazoline I2 receptor agonist 2-BFI in rats. *Psychopharmacology (Berl)* 234: 3299–3307.

Sun YH, Li HS, Zhu C, Hu W, Yang J, Zhao GL *et al.* (2014). The analgesia effect of duloxetine on post-operative pain via intrathecal or intraperitoneal administration. *Neurosci Lett* 568: 6–11.

Tasker RA, Connell BJ, Yole MJ (1992). Systemic injections of alpha-1 adrenergic agonists produce antinociception in the formalin test. *Pain* 49: 383–391.

Thorn DA, An XF, Zhang Y, Pignini M, Li JX (2012). Characterization of the hypothermic effects of imidazoline I(2) receptor agonists in rats. *Br J Pharmacol* 166: 1936–1945.

Thorn DA, Siemian JN, Zhang Y, Li JX (2015). Anti-hyperalgesic effects of imidazoline I2 receptor ligands in a rat model of inflammatory pain: interactions with oxycodone. *Psychopharmacology (Berl)* 232: 3309–3318.

Thorn DA, Qiu Y, Jia S, Zhang Y, Li JX (2016a). Antinociceptive effects of imidazoline I2 receptor agonists in the formalin test in rats. *Behav Pharmacol* 27: 377–383.

Thorn DA, Zhang Y, Li JX (2016b). Effects of the imidazoline I2 receptor agonist 2-BFI on the development of tolerance to and behavioural/physical dependence on morphine in rats. *Br J Pharmacol* 173: 1363–1372.

Thorn DA, Zhang Y, Li JX (2017). Tolerance and cross-tolerance to the antinociceptive effects of oxycodone and the imidazoline I2 receptor agonist phenyzoline in adult male rats. *Psychopharmacology (Berl)* 234: 1871–1880.

Ugedo L, Pineda J, Martin-Ruiz R, Ruiz-Ortega JA, Artigas F (1999). Imidazoline-induced inhibition of firing rate of 5-HT neurons in rat

dorsal raphe by modulation of extracellular 5-HT levels. *Ann N Y Acad Sci* 881: 365–368.

Valhondo M, Marco I, Martin-Fontecha M, Vazquez-Villa H, Ramos JA, Berkels R *et al.* (2013). New serotonin 5-HT_{1A} receptor agonists endowed with antinociceptive activity *in vivo*. *J Med Chem* 56: 7851–7861.

Wattiez AS, Pichon X, Dupuis A, Hernandez A, Privat AM, Aissouni Y *et al.* (2013). Disruption of 5-HT_{2A} receptor-PDZ protein interactions alleviates mechanical hypersensitivity in carrageenan-induced inflammation in rats. *PLoS one* 8: e74661.

Zimmermann M (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16: 109–110.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Table S1 Pharmacological properties of drugs used in this study.

Table S2 Group mean PWTs (in g) for the effects of 2-BFI alone and in combination with monoaminergic reuptake inhibitors or before and after monoamine depletion on CCI-induced mechanical nociception.

Table S3 Group mean PWTs (in g) for the effects of 2-BFI alone and in combination with monoaminergic receptor antagonists on CCI-induced mechanical nociception.

Table S4 Group mean PWTs (in g) for the effects of CR4056 alone and in combination with monoaminergic receptor antagonists on CCI-induced mechanical nociception.

Table S5 Group mean PWTs (in g) for the effects of 2-BFI alone and in combination with monoaminergic receptor antagonists on CFA-induced mechanical nociception.