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Neuroanatomical characterization of imidazoline I₂ receptor agonist-induced antinociception.

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Abstract

Chronic pain is a significant public health problem with a lack of safe and effective analgesics. The imidazoline I₂ receptor (I₂R) is a promising analgesic target, but the neuroanatomical structures involved in mediating I₂R-associated behaviors are unknown. I₂Rs are enriched in the arcuate nucleus, dorsal raphe (DR), interpeduncular nucleus, lateral mammillary body, medial habenula, nucleus accumbens (NAc), and paraventricular nucleus, thus this study investigated the antinociceptive and hypothermic effects of microinjections of the I₂R agonist 2-(2-benzofuranyl)-2-imidazoline hydrochloride (2-BFI). In rats, intra-DR microinjections produced antinociception in CFA- and CCI-induced pain models. Intra-NAc microinjections produced antinociception and increased noxious stimulus-associated side time in a place escape/avoidance paradigm. Intra-NAc pretreatment with the I₂R antagonist idazoxan but not the D1 receptor antagonist SCH23390 or the D2 receptor antagonist raclopride attenuated intra-NAc 2-BFI-induced antinociception. Intra-NAc idazoxan did not attenuate systemically-administered 2-BFI-induced antinociception. Microinjections into the other regions did not produce antinociception, and in none of the regions produced hypothermia. These data suggest that I₂R activation in some but not all I₂R-enriched brain regions is sufficient to produce antinociception, and supports the theory that different I₂R-associated effects are mediated via distinct receptor populations, which may in turn be distributed differentially throughout the CNS.

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Conflicts of interest

The authors declare no competing financial interests.

Keywords

2-BFI; pain; neuroanatomy; rat; microinjection

Introduction

Chronic pain is a significant public health problem with a lack of safe and effective pharmacotherapies (Kissin, 2010; NIH, 2013). Opioids are generally effective for treating acute pain, but issues such as tolerance, dependence, overdose, and abuse liability limit their long-term use to manage chronic pain (Okie, 2010). Non-opioid therapies such as acetaminophen and non-steroidal anti-inflammatory drugs are not prone to abuse, but are less effective at managing moderate to severe types of pain and can lead to complications when taken in high doses (Tauben, 2015). Thus, novel analgesics that are both safe and effective are desperately needed in the clinic.

Ligands acting at imidazoline I₂ binding sites, also referred to as imidazoline I₂ receptors (I₂Rs), have been established as such potential candidates (for review, see Li, 2017). Preclinical studies have demonstrated that I₂R agonists are effective in treating several forms of chronic pain as monotherapies, and also additively to synergistically enhance opioid analgesia while reducing opioid tolerance and dependence (Siemian *et al.*, 2016; Thorn *et al.*, 2016b; 2017). However, despite the large literature that report behavioral characterization of I₂R agonist-induced effects, the mechanisms underlying these effects have been less well-studied. Like other imidazoline receptors, I₂Rs have neither yet been molecularly identified nor had their signaling pathways characterized, but evidence exists to suggest that they are heterogeneous in nature and exist as binding sites on multiple proteins (Keller & Garcia-Sevilla, 2015). Interestingly, one of the most prominent roles for I₂Rs appears to be modulation of monoamine oxidase (MAO) A and B activity (Jones *et al.*, 2007; McDonald *et al.*, 2010). *In vivo*, systemic I₂R agonist administration increases dopamine (DA), serotonin (5-HT), and norepinephrine (NE) levels in several CNS regions including striatum, frontal cortex, dorsal raphe, hippocampus, and spinal cord (Nutt *et al.*, 1995; Nutt *et al.*, 1997; Ugedo *et al.*, 1999; Sastre-Coll *et al.*, 2001; Finn *et al.*, 2002; Ferrari *et al.*, 2011). Investigations on the CNS distribution of I₂Rs have shown that I₂Rs are enriched in brain regions including arcuate nucleus of the hypothalamus (ArcN), interpeduncular nucleus (IPN), paraventricular nucleus of the thalamus (PVT), lateral mammillary nucleus (LMN), dorsal raphe (DR), nucleus accumbens (NAc), and medial habenula (MH) in rat and mouse brain (MacKinnon *et al.*, 1995; Lione *et al.*, 1998; MacInnes & Handley, 2005). Yet, while intracerebroventricular injections of 2-BFI produce antinociception (Thorn *et al.*, 2016a), the specific brain regions mediating this effect and other I₂R-associated effects have not yet been demonstrated through functional studies.

To understand the functional neuroanatomy important for I₂R-associated behaviors, this study examined the antinociceptive effects of microinjections of the prototypical I₂R agonist 2-BFI into ArcN, IPN, PVT, LMN, DR, NAc, or MH in rats treated with complete Freund's adjuvant (CFA), a commonly-used and well-established preclinical model of inflammatory pain for the prediction of clinical antinociceptive effects. For some brain areas, 2-BFI

microinjections were tested in rats with chronic constriction injury (CCI)-induced neuropathic pain to verify the generalization of the effect of 2-BFI microinjections found in the CFA model, as differences in the potencies and efficacies of other analgesics have been reported between these models. Body temperatures were also measured to investigate the potential hypothermic effects induced by 2-BFI microinjection.

Methods

Subjects

Male ($n = 77$) and female ($n = 5$) Sprague-Dawley rats (Envigo, Indianapolis, IN) approximately 12 weeks old and weighing approximately 250 g at experiment onset were individually housed in standard rat cages in a laboratory animal facility on a 12/12-hour light/dark cycle. Standard animal husbandry was provided by animal care staff and veterinarians. Behavioral experiments were conducted during the light period between the hours of 9:00 am and 3:00 pm. All rats had free access to water and standard rodent chow in their home cages and were randomly assigned to different study groups. By design, a group size of six rats each was determined by previous studies from our laboratory to ensure sufficient statistical power. Animals were maintained and experiments were conducted in accordance with guidelines of the International Association for the Study of Pain (Zimmermann, 1983), ARRIVE (Kilkenny *et al.*, 2010), and the 2011 Guide for the Care and Use of Laboratory Animals (2011), and were approved by the Institutional Animal Care and Use Committee, University at Buffalo, the State University of New York (Buffalo, NY). Animal welfare was overseen and interventions were recommended by veterinary personnel; rats showing marked signs of discomfort or illness (e.g., due to infection) were sacrificed immediately. Animal numbers were minimized as the pain experiments were predominantly designed to be within-group comparisons, and additionally, some of the rats were also used in the body temperature or locomotion experiments. This eliminated the need for additional rats to test several doses in several experiments.

Stereotaxic surgeries and intracranial injections

The rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (15 mg/kg), a standard anesthetic cocktail, intraperitoneally (i.p.) prior to surgery. Sufficient anesthesia was determined by loss of righting reflex and failure to elicit the toe-pinch reflex. Rats were mounted on a stereotaxic frame and implanted with guide cannulae (23 gauge, Plastics One, Roanoke, VA) above the following brain regions: ArcN [bilateral, 0° angle, anterior/posterior (AP) −3.8 mm, medial/lateral ±0.4 mm, dorsal/ventral (DV) −9 mm, injector +1.0 mm] (Sato *et al.*, 1997), DR [unilateral, 0° angle, AP −7.8 mm, ML ±0.0 mm, DV −4.5 mm, injector +2.0 mm] (Ge *et al.*, 2007), IPN [unilateral, 15° angle, AP −6.3 mm, ML ±2.6 mm, DV −7.7 mm, injector +1.0 mm] (Panchal *et al.*, 2005), LMN [bilateral, 0° angle, AP −4.4 mm, ML ±1.4 mm, DV −8.5 mm, injector +1.0 mm] (Blair *et al.*, 1999), MH [bilateral, 24° angle, AP −4.2 mm, ML ±2.9 mm, DV −3.0 mm, injector +2.0 mm] (Panchal *et al.*, 2005), NAc [bilateral, 10° angle, AP −1.8 mm, ML ±2.1 mm, DV −6.3 mm, injector +1.0 mm] (Liu *et al.*, 2017), and PVT [unilateral, 0° angle, AP −3.3 mm, ML ±0.0 mm, DV −4.1 mm, injector +1.0 mm] (Stratford & Wirtshafter, 2013). The cannulae were anchored to the skull with three or four stainless steel screws and dental cement. A stainless steel stylet was

inserted into each cannula and covered with a dust cap to keep them patent and prevent infection. Rats were given post-operative fluids, antibiotics, and 5 mg/kg carprofen (all subcutaneously, s.c.) and were then allowed to recover for one week before receiving complete Freund's adjuvant (CFA) or chronic constriction injury (CCI) (see next section). 2-BFI or vehicle was freshly prepared before the experiments, and was infused over 1 min in a volume of 1 μ l via microinjection needles, which were then kept in place for an additional 1 min to allow for drug diffusion. Following experiments, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde. Brains were post-fixed and cryoprotected in a 30% sucrose solution, after which they were frozen and sliced in a cryostat. Cannula placements were confirmed in 30 μ m-thick sections using Nissl staining under light microscopy.

Induction of inflammatory pain and neuropathic pain

Inflammatory pain was induced by CFA inoculation as previously described (Li *et al.*, 2014). Briefly, 0.1 ml of CFA (Sigma-Aldrich, St. Louis, MO) containing approximately 0.05 mg of *Mycobacterium butyricum* dissolved in paraffin oil was injected in the right hind foot pad of rats under isoflurane anesthesia (2% isoflurane mixed with 100% oxygen). Sufficient anesthesia was determined by loss of righting reflex and failure to elicit the toe-pinch reflex.

Neuropathic pain was induced by CCI procedure (Bennett & Xie, 1988; Li *et al.*, 2014). Briefly, rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (15 mg/kg) (i.p.) prior to surgery; sufficient anesthesia was determined by loss of righting reflex and failure to elicit the toe-pinch reflex. The right sciatic nerve was exposed and freed from surrounding tissue, and four ligatures (4.0 chromic gut suture, Patterson Veterinary, Devens, MA) were placed around the nerve (approximately 1 mm apart) proximal to the trifurcation. Ligatures were loosely tied so that circulation through the epineural vasculature was uninterrupted. The incisions were closed with surgical clips. Rats received post-operative fluids, antibiotics, and 5 mg/kg carprofen (all s.c.).

Mechanical nociception

64 naive rats were used for these experiments. Mechanical allodynia was measured by the von Frey filament test using equipment and procedures as described in detail previously (Siemian *et al.*, 2016). Briefly, rats were placed in transparent test chambers atop 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 behavioral response in at least two out of three applications. Behavioral testing began one day after CFA treatment or five 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 manipulation thus leading to five rats in certain groups. Each group served as its own control. On test days, one baseline PWT was established, then rats received intracranial vehicle or 2-BFI microinjections before being returned to the test chamber. PWTs were subsequently measured every 15 min for 90 min. All tests were separated by at least three days, and vehicle or drug doses were administered in a random order across tests. In all experiments, experimenters were blind to the treatments, and they received extensive

training with the von Frey procedure to ensure accurate judgment of paw withdrawal responses and minimize experimenter bias.

Body Temperature

35 of the rats previously used in the nociception studies were used in these experiments. 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). Rats were handled and habituated to the procedure for three days before testing began. On test days, one baseline body temperature measurement was taken before rats received intracranial microinjections of vehicle or 2-BFI and were then returned to their homecages. Body temperature measurements in °C were subsequently recorded every 15 min for 90 min. All tests were separated by at least three days, the vehicle or drug doses were administered in a random order across tests, and the experimenter was blinded to the treatment. Data were plotted as the change in °C from the baseline measurement.

Place escape/avoidance paradigm

12 naive rats (two groups of six rats each) were used in these experiments. The place escape/avoidance paradigm (PEAP) has recently been used as a non-reflex measurement of the affective-emotional component of pain (LaBuda & Fuchs, 2000; Fuchs & McNabb, 2012) and was performed using previously described procedures and equipment (Li *et al.*, 2014). Briefly, a rat was placed into an inverted rat cage that was painted half white and half black on top of an elevated metal grid through which the plantar surface of the rat's hind paws could be stimulated with a 60 g von Frey hair every 15 s over a 30 min session. When the rat was in the white side of the chamber, the non-inflamed left paw was mechanically stimulated. When the rat was in the black side of the chamber, the inflamed right paw was mechanically stimulated. The mechanical stimulation was enough to elicit a reflex response in non-inflamed rats. Location in the chamber (black vs. white) at the time of each stimulation was noted. Percentage of the time the rats spent on the white side of the chamber was calculated from each 5 min period and was used as the indication of escape/avoidance learning (observations that rats stay at the white side/total number of observations $\times$ 100). Vehicle or 2-BFI microinjections were administered 10 min before the behavioral testing sessions, and the experimenter was blinded to the treatment.

Locomotion

11 rats were used in these experiments, five of which were previously used for nociception studies. Locomotor activity was monitored by an infrared motor-sensor system (AccuScan Instruments, Columbus, OH) fitted outside clear acrylic chambers (40 $\times$ 40 $\times$ 30 cm). Before tests began, rats were exposed to at least three days of handling by the experimenter. On test day, rats (n = 5–6 per group) received a 10 min microinjection pretreatment of vehicle or 2-BFI before being placed in the chambers and the locomotor activity was recorded for 30 min.

Data analysis

All graphs and statistical analyses were performed using Prism 7.0 (GraphPad Software, Inc., San Diego, CA). Data plotted and reported in the text are group mean $\pm$ SEM. Two-way repeated-measures or mixed-model ANOVA (treatment $\times$ time) was used to analyze all data; the specific tests for each experiment are stated in the Results section. ANOVAs were followed by Bonferroni's post-tests, and $p < 0.05$ was considered statistically significant for all statistical tests.

Drugs

2-(2-benzofuranyl)-2-imidazoline hydrochloride (2-BFI) was synthesized according to standard procedures (Ishihara & Togo, 2007). Idazoxan, raclopride, and SCH23390 were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). All drugs were dissolved in physiologic saline at a concentration of 1.6 – 32 $\mu\text{g}/\mu\text{l}$ and administered in a volume of 1.0 μl (for bilateral injections, half of the dose was administered per side), except for one experiment where 2-BFI was administered i.p. at a dose of 10 mg/kg in a volume of 1 ml/kg.

Results

Prior to manipulations, the average PWT among all the rats was 24.6 ± 0.5 g, which was decreased to 5.2 ± 0.2 g after CFA injection and to 5.6 ± 0.4 g after CCI. The average body temperature among all rats was 37.1 ± 0.1 °C.

In CFA-treated male rats, two-way repeated-measures ANOVA revealed that intra-ArcN 2-BFI microinjections over a dose range of 10 – 32 μg produced no significant 2-BFI $\times$ time interaction on PWT ($F(12, 60) = 1.12$, $p = 0.3587$), with significant main effects of 2-BFI ($F(2, 10) = 4.19$, $p = 0.0476$) but not time ($F(6, 30) = 2.39$, $p = 0.0521$) (Fig. 1A, C). Intra-ArcN 2-BFI microinjections in male rats with CCI also produced no significant 2-BFI $\times$ time interaction on PWT ($F(12, 48) = 1.94$, $p = 0.0521$), with significant main effects of time ($F(6, 24) = 3.78$, $p = 0.0086$) but not 2-BFI ($F(2, 8) = 1.03$, $p = 0.3994$) (Fig. 1B). When the body temperature responses to intra-ArcN 2-BFI microinjections in male rats were measured, two-way repeated-measures ANOVA revealed no significant 2-BFI $\times$ time interaction ($F(12, 36) = 1.88$, $p = 0.0708$), with significant main effects of time ($F(6, 18) = 8.81$, $p = 0.0001$) but not 2-BFI ($F(2, 6) = 4.24$, $p = 0.0712$) (Fig. 1C).

In CFA-treated male rats, two-way repeated-measures ANOVA revealed that intra-DR 2-BFI microinjections over a dose range of 3.2 – 10 μg produced a significant 2-BFI $\times$ time interaction on PWT ($F(12, 48) = 16.04$, $p < 0.0001$), with significant main effects of both 2-BFI ($F(2, 8) = 16.88$, $p = 0.0013$) and time ($F(6, 24) = 14.98$, $p < 0.0001$). Bonferroni's post-test revealed significant differences between 3.2 μg 2-BFI and vehicle treatment at 30 min and significant differences between 10 μg 2-BFI and vehicle treatment at time points 15 through 60 min (Fig. 2A, F). Intra-DR 2-BFI microinjections over a dose range of 3.2 – 10 μg in male rats with CCI also produced a significant 2-BFI $\times$ time interaction on PWT ($F(12, 48) = 19.68$, $p < 0.0001$), with significant main effects of both 2-BFI ($F(2, 8) = 23.41$, $p = 0.0005$) and time ($F(6, 24) = 12.62$, $p < 0.0001$). Bonferroni's post-test revealed significant differences between 3.2 μg 2-BFI and vehicle treatment at time points 15 through 30 min

and significant differences between 10 μ g 2-BFI and vehicle treatment at time points 15 through 45 min (Fig. 2B). In CFA-treated female rats, intra-DR microinjections of 2-BFI over a dose range of 3.2 – 10 μ g produced a significant 2-BFI $\times$ time interaction on PWT ($F(12, 48) = 8.86, p < 0.0001$), with significant main effects of both 2-BFI ($F(2, 8) = 6.95, p = 0.0178$) and time ($F(6, 24) = 12.44, p < 0.0001$). Bonferroni's post-test revealed significant differences between 10 μ g 2-BFI and vehicle treatment at time points 30 through 45 min (Fig. 2C). Two-way mixed-model ANOVA revealed that intra-DR 2-BFI in male rats at a dose of 10 μ g did not affect locomotion with no significant 2-BFI $\times$ time interaction ($F(5, 50) = 1.83, p = 0.1237$), with significant main effects of time ($F(5, 50) = 31.64, p < 0.0001$) but not 2-BFI ($F(1, 10) = 0.16, p = 0.6966$) (Fig. 2D), suggesting that the antinociceptive results were not an artifact of generalized locomotor suppression. When body temperature responses to intra-DR 2-BFI microinjections at doses of 10–32 μ g in male rats were measured, two-way repeated-measures ANOVA revealed a significant 2-BFI $\times$ time interaction ($F(12, 36) = 2.08, p = 0.0445$), with no significant main effects of 2-BFI ($F(2, 6) = 3.44, p = 0.1009$) or time ($F(6, 18) = 2.59, p = 0.0549$). Bonferroni's post-test revealed significant differences between 32 μ g 2-BFI and vehicle treatment at time points 15 through 60 min (Fig. 2E).

In CFA-treated male rats, intra-IPN 2-BFI microinjections produced no significant 2-BFI $\times$ time interaction on PWT ($F(12, 48) = 1.66, p = 0.1059$), with significant main effects of time ($F(6, 24) = 20.45, p < 0.0001$) but not 2-BFI ($F(2, 8) = 0.19, p = 0.8323$) (Fig. 3A, C). Intra-IPN 2-BFI microinjections also produced no significant 2-BFI $\times$ time interaction on body temperature ($F(12, 48) = 1.56, p = 0.1375$), with no significant main effect of 2-BFI ($F(2, 8) = 2.79, p = 0.1204$) or time ($F(6, 24) = 0.50, p = 0.7997$) (Fig. 3B). Intra-LMN 2-BFI microinjections produced no significant 2-BFI $\times$ time interaction on PWT ($F(12, 60) = 1.36, p = 0.2096$), with significant main effects of time ($F(6, 30) = 14.5, p < 0.0001$) but not 2-BFI ($F(2, 10) = 1.83, p = 0.2108$) (Fig. 3D, F). Intra-LMN 2-BFI microinjections also produced no significant 2-BFI $\times$ time interaction on body temperature ($F(12, 60) = 1.44, p = 0.1718$), with significant main effects of time ($F(6, 30) = 10.96, p < 0.0001$) but not 2-BFI ($F(2, 10) = 1.24, p = 0.3310$) (Fig. 3E). Intra-MH microinjections produced no significant 2-BFI $\times$ time interaction on PWT ($F(12, 48) = 1.10, p = 0.3858$), with no significant main effect of 2-BFI ($F(2, 8) = 1.21, p = 0.3466$) or time ($F(6, 24) = 1.89, p = 0.1238$) (Fig. 3G, I). Intra-MH 2-BFI microinjections also produced no significant 2-BFI $\times$ time interaction on body temperature ($F(12, 48) = 0.86, p = 0.5957$), with significant main effects of time ($F(6, 24) = 2.91, p = 0.0281$) but not 2-BFI ($F(2, 8) = 0.72, p = 0.5169$) (Fig. 3H). Intra-PVT microinjections produced no significant 2-BFI $\times$ time interaction on PWT ($F(12, 48) = 1.72, p = 0.0912$), with significant main effects of time ($F(6, 24) = 5.31, p = 0.0013$) but not 2-BFI ($F(2, 8) = 4.05, p = 0.0611$) (Fig. 3J, L). Intra-PVT 2-BFI microinjections also produced no significant 2-BFI $\times$ time interaction on body temperature ($F(12, 36) = 1.64, p = 0.1232$), with no significant main effect of 2-BFI ($F(2, 6) = 0.15, p = 0.8623$) or time ($F(6, 18) = 0.27, p = 0.9441$) (Fig. 3K).

In CFA-treated male rats, intra-NAc 2-BFI microinjection produced a significant 2-BFI $\times$ time interaction ($F(6, 30) = 11.29, p < 0.0001$) with significant main effects of both 2-BFI ($F(1, 5) = 20.68, p = 0.0061$) and time ($F(6, 30) = 8.80, p < 0.0001$) on PWT. Bonferroni's post-test revealed significant differences between 10 μ g 2-BFI and vehicle treatment at time

points 15 through 90 min. Intra-NAc pretreatment with the I₂R antagonist idazoxan before intra-NAc 2-BFI injection produced a significant idazoxan $\times$ time interaction ($F(6, 30) = 7.16, p < 0.0001$) with significant main effects of both idazoxan ($F(1, 5) = 12.49, p = 0.0167$) and time ($F(6, 30) = 12.60, p < 0.0001$). Bonferroni's post-test revealed significant differences between idazoxan/2-BFI and vehicle/2-BFI treatment at time points 15 through 75 min (Fig. 4A, F). However, intra-NAc microinjections of neither the dopamine D1-like receptor antagonist SCH23390 (two-way mixed-model ANOVA: SCH23390 $\times$ time interaction, $F(6, 54) = 0.13, p = 0.9927$) nor the dopamine D2-like receptor antagonist raclopride (raclopride $\times$ time interaction, $F(12, 84) = 0.18, p = 0.9989$) significantly affected intra-NAc 2-BFI-induced antinociception (Fig. 4B). Additionally, intra-NAc microinjection of idazoxan did not significantly alter antinociception induced by 10 mg/kg 2-BFI (i.p.). Two-way repeated measures ANOVA revealed a significant 2-BFI (i.p.) $\times$ time interaction ($F(8, 40) = 14.90, p < 0.0001$) with significant effects of both 2-BFI (i.p.) ($F(1, 5) = 12.35, p = 0.0170$) and time ($F(8, 40) = 14.91, p < 0.0001$). Bonferroni's post-test revealed significant differences between 10 mg/kg 2-BFI and vehicle treatment at time points 15 through 90 min. However, in 2-BFI-treated rats, two-way repeated measures ANOVA revealed no significant idazoxan $\times$ time interaction ($F(8, 40) = 0.65, p = 0.7320$) with a significant effect of time ($F(8, 40) = 25.67, p < 0.0001$) but not idazoxan ($F(1, 5) = 0.49, p = 0.5161$) (Fig. 4C). In the PEAP experiment, two-way mixed-model ANOVA with time as the within-subject factor revealed a significant 2-BFI $\times$ time interaction ($F(5, 50) = 6.61, p < 0.0001$) with a significant main effect of 2-BFI ($F(1, 10) = 5.13, p = 0.0470$) but not time ($F(5, 50) = 1.09, p = 0.3801$). Bonferroni's post-tests revealed significant between-group differences in white-side time at the 15 through 30 min time points (Fig. 4D). Intra-NAc 2-BFI microinjection produced no significant 2-BFI $\times$ time interaction on body temperature ($F(6, 24) = 1.99, p = 0.1063$), with a significant main effect of time ($F(6, 24) = 3.97, p = 0.0067$) but not 2-BFI ($F(1, 4) = 1.73, p = 0.2592$) (Fig. 4E).

Discussion

The primary findings of this study were that microinjections of the I₂R agonist 2-BFI into the DR or NAc produced significant antinociception, whereas microinjections into the ArcN, IPN, LMN, MH, and PVT had no effect. Intra-NAc microinjection of the I₂R antagonist idazoxan attenuated antinociception produced by intra-NAc but not systemically-administered 2-BFI. Microinjections in none of these regions produced significant hypothermia. These data are the first to demonstrate the functional involvement of specific brain nuclei in I₂R agonist-induced antinociception.

Discovered over 20 years ago, I₂Rs have recently been established as promising analgesic targets. I₂R agonists are effective as monotherapy and also produce synergistic antinociception in combination with opioids in several preclinical models of chronic pain (Li *et al.*, 2014; Siemian *et al.*, 2016) and one I₂R agonist, CR4056, has progressed into phase II clinical trials for the treatment of inflammatory pain. However, despite a thorough characterization of I₂R agonist-induced behavioral effects, little is known about the mechanism(s) by which these effects are produced. Molecular evidence suggests that I₂Rs are present on and modulate MAO (Jones *et al.*, 2007; McDonald *et al.*, 2010) and increase monoamine concentrations in various CNS regions (Nutt *et al.*, 1995; Nutt *et al.*, 1997; Finn

et al., 2002; Ferrari *et al.*, 2011), though other I₂R populations exist independent of MAO (Keller & Garcia-Sevilla, 2015). Anatomically, I₂Rs show enrichment in several brain nuclei including ArcN, DR, IPN, LMN, MH, NAc, and PVT (MacKinnon *et al.*, 1995; Lione *et al.*, 1998; MacInnes & Handley, 2005). Yet, while it has been demonstrated that central administration of 2-BFI induces antinociception (Thorn *et al.*, 2016a), the specific nuclei responsible for this effect have not yet been elucidated. To better understand the functional neuroanatomy underlying I₂R-mediated behavioral effects, we investigated the effects of 2-BFI microinjections into several I₂R-enriched brain nuclei.

Interestingly, microinjections of 2-BFI into ArcN, IPN, LMN, MH, or PVT did not produce significant antinociception despite their I₂R density and role in pain processing (Paulson *et al.*, 2000; DeLaTorre *et al.*, 2009; Shelton *et al.*, 2012; Bu *et al.*, 2015). Microinjections into these areas also did not produce hypothermia, another robust behavioral effect produced by systemically-administered I₂R agonists (Thorn *et al.*, 2012). In contrast, intra-DR and intra-NAc microinjections each produced antinociception but not hypothermia; both of these regions are involved in chronic pain processing (Sagheddu *et al.*, 2015). In an effort to examine whether sex plays a role in mediating the effects of intra-DR 2-BFI injection, we used female rats to replicate this finding. Female rats showed similar response to intra-DR injection, suggesting that there is no sex difference in mediating intra-DR-injection induced antinociception. This is not surprising as our previous study reports that there is no sex difference for 2-BFI to produce antinociception in rats in CFA-induced pain when 2-BFI was administered systemically (Siemian *et al.*, 2016). As such, we did not attempt to replicate the results in other brain regions using female rats. Intra-NAc microinjections also decreased aversion to the noxious stimulus-associated side in PEAP, suggesting that this treatment attenuates affective pain, which has been emphasized as an important focus for the validation of novel pain therapies (Fuchs & McNabb, 2012). Subsequent experiments in the NAc showed that while intra-NAc pretreatment with idazoxan attenuated intra-NAc 2-BFI-induced antinociception, it did not attenuate antinociception induced by systemically-administered 2-BFI, indicating that NAc I₂R activation is sufficient but not necessary to produce antinociception. Furthermore, although 95% of NAc neurons are GABAergic medium spiny neurons which primarily express D1 or D2 receptors (Robison & Nestler, 2011), the antinociceptive effects of intra-NAc 2-BFI were unaffected by intra-NAc pretreatment with either dopamine D1- or D2-like receptor antagonists, which suggests that the antinociceptive effects of 2-BFI are not mediated by dopamine in the NAc.

Previous literature on I₂Rs supports the findings of the current study regarding the involvement of NAc and DR. I₂Rs are enriched in the NAc, and systemic administration of I₂R agonists has been shown to increase monoamines and decrease monoamine metabolites in NAc, supporting a mechanism of MAO inhibition (Lalies & Nutt, 1993; Nutt *et al.*, 1995; Sastre-Coll *et al.*, 2001). I₂Rs are also enriched in the DR, yet while 2-BFI increased extracellular 5-HT in DR slice preparations it did not decrease levels of the metabolite 5-HIAA, which suggests a mechanism independent from MAO (Ugedo *et al.*, 1999). Thus, while both MAO-associated and MAO-independent mechanisms increase monoamines and would appear to be involved in antinociception, the relation of I₂Rs to MAO may depend on neuroanatomical location and/or cell type. Additionally, although NAc DA is purported to be important for analgesia in some scenarios (Wood, 2008), it does not seem to be involved in

2-BFI-induced antinociception despite the fact that 2-BFI increases striatal dopamine (Nutt *et al.*, 1995; Sastre-Coll *et al.*, 2001; MacInnes & Duty, 2004). As such, other neurotransmitters within the NAc such as 5-HT may be important for antinociception, or alternatively, I₂R activation on cell bodies in the NAc may affect NAc outputs to other brain areas to mediate antinociception, which would have been unaffected by the pharmacological manipulations in the current study. Non-monoaminergic mechanisms may also be involved, as I₂Rs exist on up to four proteins including brain creatine kinase (Kimura *et al.*, 2009; Keller & Garcia-Sevilla, 2015), but how these proteins contribute to I₂R-mediated behaviors is currently unknown.

Moreover, the CNS cell populations expressing I₂Rs have not been elucidated beyond the general characterization that I₂Rs exist on at least neurons and astrocytes (Regunathan *et al.*, 1993; Keller & Garcia-Sevilla, 2015). Thus, it is possible that functionally opposing neuron populations in certain brain regions (e.g., Agouti-related peptide- and proopiomelanocortin-expressing neurons in the ArcN) may both express I₂Rs, such that concurrent receptor activation in both populations results in no net behavioral effect when administered specifically to that brain region. Alternatively, I₂Rs in some brain regions may not exist on cell populations involved in nociceptive processing by those regions, and instead exist on cell populations involved in other behaviors, such as feeding, which were undetectable by the behavioral assays of this study. I₂R activation in some brain regions could also simply be insufficient to produce behaviorally-relevant effects. Instead, simultaneous activation in two or more brain regions may be required to produce behaviorally relevant effects which may explain the negative results returned from many of the brain regions examined in the current study. Though intra-NAc 2-BFI administration produced antinociception, that intra-NAc idazoxan did not attenuate antinociception by systemic 2-BFI demonstrates that at least one or likely more other brain loci are involved in I₂R-mediated behaviors. These effects may also be mediated by anatomical regions outside of the brain including the spinal cord and primary afferents. The development of molecular tools to characterize the anatomical and cell type distribution of I₂Rs as well as classify potentially distinct I₂R subtype populations will help to clarify this complex picture and understand the mechanisms of I₂Rs. This may allow for the development of new subtype-selective I₂R ligands that produce antinociception and are devoid of undesired side effects such as hypothermia and sedation, which would be ideal analgesics to treat chronic pain in humans.

In conclusion, this was the first report to functionally demonstrate that microinjections of 2-BFI into NAc or DR were sufficient to produce antinociception, but that I₂R activation in the NAc is not necessary for 2-BFI-induced antinociception. In contrast, 2-BFI administration into several other I₂R-enriched brain areas did not produce antinociception. These findings provide evidence that NAc and DR are two brain loci important to I₂R-mediated antinociception, but that activation of I₂Rs in several brain regions likely accounts for I₂R-mediated behavioral effects.

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Abbreviations

2-BFI	2-(2-benzofuranyl)-2-imidazoline hydrochloride
CFA	Complete Freund's adjuvant
CCI	Chronic constriction injury
DR	Dorsal raphe
LMN	Lateral mammillary nucleus
IPN	Interpeduncular nucleus
ArcN	Arcuate nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
PEAP	Place escape/avoidance paradigm
MH	Medial habenula
NAc	Nucleus accumbens
PWT	Paw withdrawal threshold

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Arcuate Nucleus

Figure 1.

Effects of intra-ArcN 2-BFI microinjections on (A) CFA- or (B) CCI-induced mechanical allodynia and (C) body temperature in male rats. Ordinates: A-B, paw withdrawal threshold (g); C, change in body temperature ($^{\circ}\text{C}$). Abscissas: time after vehicle or 2-BFI microinjection. (D) Representative cannula locations above ArcN (arrow). (ArcN, arcuate nucleus; CFA, complete Freund's adjuvant; CCI, chronic constriction injury).

Figure 2.

Effects of intra-DR 2-BFI microinjections on (A) CFA- or (B) CCI-induced mechanical allodynia in male rats or on CFA-induced mechanical allodynia in female rats (C), as well as effects on (D) locomotor activity and (E) body temperature in male rats. Ordinates: A-C, paw withdrawal threshold (g); D, distance traveled per 5 min (cm); E, change in body temperature ($^{\circ}\text{C}$). Abscissas: time after vehicle or 2-BFI microinjection. (F) Representative cannula locations above DR (arrow). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significantly different from vehicle treatment according to Bonferroni's post-tests. (DR, dorsal raphe; CFA, complete Freund's adjuvant; CCI, chronic constriction injury).

Figure 3.

Effects of 2-BFI microinjections in IPN (A-C), LMN (D-F), MH (G-I), or PVT (J-L) on CFA-induced mechanical allodynia (A, D, G, J) and body temperature (B, E, H, K) in male rats. (C, F, I, L) Representative cannula locations above target regions (arrows). (IPN, interpeduncular nucleus; LMN, lateral mammillary nucleus; MH, medial habenula; PVT, paraventricular thalamic nucleus; CFA, complete Freund's adjuvant).

Nucleus Accumbens

Figure 4.

Effects of intra-NAc 2-BFI microinjections alone and in combination with intra-NAc microinjections of the I_2R antagonist idazoxan (A), the dopamine D1 receptor antagonist SCH23390, or the dopamine D2 receptor antagonist raclopride (B) on CFA-induced mechanical allodynia in male rats. (C) Effects of 2-BFI (i.p.) alone and in combination with intra-NAc idazoxan on CFA-induced mechanical allodynia in male rats. (D) Effects of intra-NAc 2-BFI on time spent on the white side of the chamber over the 30 min PEAP session. (E) Effects of intra-NAc 2-BFI on body temperature in male rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different from vehicle treatment; # $P < 0.05$ significantly different from 10 μg intra-NAc 2-BFI according to Bonferroni's post-tests. (F) Representative cannula locations above NAc (arrow). (NAc, nucleus accumbens; CFA, complete Freund's adjuvant; PEAP, place escape/avoidance paradigm).