Title
Analgesia, enhancement of spinal morphine antinociception, and inhibition of tolerance by ultra-low dose of the α2A-adrenoceptor selective antagonist BRL44408

Authors
Milne, Brian
Jhamandas, Khem
Sutak, Maaja
et al.

Publication Date
2014-11-01

DOI
10.1016/j.ejphar.2014.08.040

Peer reviewed

European Journal of Pharmacology 743 (2014) 89–97

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and analgesia

Analgesia, enhancement of spinal morphine antinociception, and inhibition of tolerance by ultra-low dose of the α_{2A}-adrenoceptor selective antagonist BRL44408

Brian Milne [a,*], Khem Jhamandas [a,b], Maaja Sutak [b], Patrick Grenier [b], Catherine M. Cahill [b,c]

[a] Department of Anesthesiology & Perioperative Medicine, Queen's University, 76 Stuart Street, Kingston, Ontario, Canada, K7L 2V7
[b] Department of Biomedical and Molecular Sciences, Queen's University, 18 Stuart Street, Kingston, Ontario, Canada
[c] Department of Anesthesiology and Perioperative Care, University of California, 837 Health Sciences Road, Zot 4265, Irvine, CA, USA

ARTICLE INFO

Article history:
Received 20 June 2014
Received in revised form
27 August 2014
Accepted 28 August 2014
Available online 19 September 2014

Keywords:
BRL44408
Analgesia
Morphine
Norepinephrine
α_{2A}-adrenoceptor
α_2-adrenoceptor antagonist

Chemical compounds studied in this article:
BRL44408 (PubChem CID: 121850)
Morphine (PubChem CID: 5288826)
Norepinephrine (PubChem CID: 439260)
Atipamezole (PubChem CID: 71310)
Clonidine (PubChem CID: 2803)

ABSTRACT

Ultra-low doses of non-selective α_2-adrenoceptor antagonists augment acute spinal morphine antinociception and block morphine tolerance; however, the receptor involved in mediating these effects is currently unknown. Here, we used tail flick and paw pressure tests on the rat to investigate the acute analgesic and tolerance-inducing effects of spinal morphine and norepinephrine alone or in combination with an ultra-low dose of the α_{2A}-adrenoceptor antagonist, BRL44408. We also assessed the potential antinociceptive effects of BRL44408 alone following spinal administration. A spinal dose of BRL44408, over 1000-fold lower than that required to inhibit clonidine-induced antinociception (1.65 ng/10 μL), significantly prolonged morphine and norepinephrine action in both nociception tests. Following repeated morphine or norepinephrine injections, 1.65 ng BRL44408 attenuated both the decline of antinociceptive effect and increase in morphine ED_{50} values, responses indicative of acute morphine tolerance. BRL44408 administered alone produced a delayed antinociceptive effect unrelated to repeated nociceptive testing. This response was partially reduced by the α_2-adrenoceptor antagonist atipamezole (10 μg). Ultra-low dose BRL44408 was able to inhibit the loss of morphine- and norepinephrine-induced antinociceptive response, and prevent the loss of drug potency due to repeated agonist exposure. This implicates the spinal α_{2A}-adrenoceptor subtype in the action of ultra-low dose α_2-adrenoceptor antagonists on morphine and norepinephrine tolerance. The BRL44408-induced analgesia is partially dependent on its interaction with the α_2-adrenoceptors. Thus, this agent class may be useful in pain therapy.

1. Introduction

Spinal administration of morphine and related opioid agonists evokes powerful analgesia; however, repeated exposure to these agents induces tolerance, a phenomenon indicated by reduced potency of the agonist (McNaull et al., 2007). It has long been known that α_2-adrenoceptor agonists are antinociceptive and can enhance the analgesic effects of morphine (Eisenach et al., 1996; Maze and Tranquilli, 1991; Ossipov et al., 1989). More recently, the structurally diverse non-selective α_2-adrenoceptor antagonists atipamezole, yohimbine, mirtazapine, and idazoxan have been shown to augment acute spinal morphine antinociception, block induction of morphine tolerance, and reverse established tolerance when given at ultra-low doses several log units lower than those producing receptor antagonism (Milne et al., 2008). These effects are reminiscent of those produced by ultra-low doses of the competitive opioid receptor antagonists naltrexone (Powell et al., 2002) and naloxone (Mattioli et al., 2010). Some of these observations were recently verified when low doses of intrathecal (i.t.) atipamezole were shown to augment the antinociceptive effect of morphine in opioid naïve and tolerant rats (Lilius et al., 2012).

When administered alone, ultra-low doses of α_2-adrenoceptor antagonists have delayed but sustained weak antinociceptive actions (Milne et al., 2008). Ultra-low doses of atipamezole, influencing spinal morphine actions, similarly augment clonidine- or norepinephrine-induced antinociception, and inhibit acute tolerance to norepinephrine in the spinal model (Milne et al., 2011). These 'pro-opioid' effects of ultra-low dose α_2-adrenoceptor antagonists are stereo-selective, since tolerance to repeated acute injections of spinal morphine is inhibited by an ultra-low dose of

* Corresponding author. Tel.: +1 613 548 7827; fax: +1 613 548 1375.
E-mail address: milneb@queensu.ca (B. Milne).

http://dx.doi.org/10.1016/j.ejphar.2014.08.040
0014-2999/

the active (+) but not the inactive (−) isomer of the α_2-adrenoceptor antagonist efaroxan (Milne et al., 2013). This stereo-selectivity suggests that the observed crossover effects of adrenergic antagonists are specifically mediated via spinal α_2-adrenoceptors.

Considerable evidence supports the existence of three distinct α_2-adrenoceptor subtypes (2A, 2B, and 2C) present in the dorsal spinal cord (Fairbanks et al., 2009), suggesting their potential role in pain modulation. Studies using transgenic mice and pharmacological analyses support a primary role of the α_{2A}-adrenoceptor in pain mediation, although the α_{2C} subtype may also modulate nociceptive transmission (Gentili et al., 2007). In addition, studies in a mouse line expressing a point mutation in the α_{2A}-adrenoceptor indicate that the α_{2A} subtype is the primary mediator of α_2-adrenergic agonist-induced spinal analgesia, and is necessary for analgesic synergy with opioids (Stone et al., 1997). Given the existence of several α_2-adrenoceptors, the role they play in pain modulation, and their interaction with morphine in the analgesia model, it is of interest to determine whether any of these receptor subtypes mediate pain modulation in the presence of antagonists in ultra-low doses.

Recent work has shown that BRL44408, an α_2-adrenoceptor antagonist with high affinity for the α_{2A}-adrenoceptor, has antidepressant action as well as analgesic-like qualities in a model of visceral pain, suggesting that antagonism of this receptor subtype may present an effective treatment strategy for mood disorders and pain (Dwyer et al., 2010). Thus, we sought to investigate whether ultra-low doses of this antagonist influence morphine- and norepinephrine-induced acute antinociception, and whether it modulates analgesic tolerance to norepinephrine and morphine. In addition, we investigated whether ultra-low dose BRL44408 alone can produce characteristic delayed analgesic actions, and whether its effects are influenced by the repeated nociceptive testing involved in the acute tolerance model.

2. Material and methods

This study was approved by the Queen's University Animal Care Committee and conducted under Guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (250–300 g) housed on a normal light-dark cycle were implanted with indwelling i.t. catheters (7.5 cm, PE-10) under isoflurane anesthesia (Yaksh and Rudy, 1976). In each animal, the catheter was inserted through a small slit in the cisterna magna, with the tip terminating at the lumbar enlargement of the spinal cord as in prior experiments (Milne et al., 2011, 2008). Animals had a recovery period of 4–5 days following surgery prior to experimentation. All drugs were injected i.t. via the exteriorized catheter in10 μl volumes with a 10 μl 0.9% saline flush. BRL44408 (Tocris Bioscience, Bristol, United Kingdom), clonidine, norepinephrine (Sigma-Aldrich, St. Louis, MO, USA), morphine (BDH Pharmaceuticals, Toronto, ON, Canada), and atipamezole (Farmos, Turku, Finland) were dissolved in 0.9% saline. Drug combinations were given as one solution after being prepared on the day of experimentation. Analgesia testing was performed between 0800 and 1400 hours with the experimenter being blind to drug treatment. Each animal was used for only one experiment.

In the tail flick test (D'Amour and Smith, 1941), a thermal stimulus from a light source was applied 5 cm from the tail base using an analgesia meter (Owen et al., 1981). The light intensity was adjusted to give baseline latencies of 2–3 s and a cutoff time of 10 s in order to prevent tissue damage. In the paw pressure test, mechanical pressure was applied to the dorsal surface of the hindpaw with an inverted air-filled syringe attached to a pressure gauge (Loomis et al., 1987). A cutoff of 300 mmHg was used in this test, with a baseline of 70–90 mmHg.

2.1. Acute analgesia experiments

To determine the antagonistic effects of BRL44408 on analgesia induced by clonidine (an α_2-adrenoceptor agonist), animals were administered a single injection of clonidine (13.3 μg) or clonidine with a high (antagonist) dose of BRL44408 (16.5 μg), and tail flick and paw pressure responses were assessed for 180 min post-drug injection.

To investigate the effects of ultra-low dose BRL44408 on acute morphine- and norepinephrine-induced analgesia, animals were administered a single injection of morphine (15 μg), morphine plus BRL44408 (1.65 ng), norepinephrine (30 μg), norepinephrine plus BRL44408 (1.65 ng), or BRL44408 (1.65 ng) alone. Tail flick and paw pressure responses were assessed for 180 min post-drug treatment.

2.2. Acute tolerance experiments

To determine the effects of ultra-low dose BRL44408 on the development of acute analgesic tolerance to morphine or norepinephrine, animals were administered three repeated injections of either morphine (15 μg), morphine plus BRL44408 (1.65 ng), BRL44408 alone (1.65 ng), norepinephrine alone, (30 μg), norepinephrine plus BRL44408 (1.65 ng), or saline vehicle every 90 min. Tail flick and paw pressure responses were assessed over 4 h. Twenty-four hours following the three repeated drug injections, cumulative dose response curves were performed to assess morphine or norepinephrine potency (ED_{50} values). Dose response curves were derived using cumulative doses of 2.5–20 μg in the saline group, 12.5–100 μg in the morphine treatment groups, 15–120 μg in the norepinephrine group, and 3.75–45 μg in the norepinephrine plus BRL44408 group.

2.3. Antagonism and limited testing experiments

To determine the potential of an α_2-adrenoceptor antagonist to influence the actions of BRL44408 alone on antinociception, animals were administered a single injection of BRL44408 (1.65 ng), and tail flick and paw pressure responses were assessed for 180 min. At 180 min, animals were administered a single injection of the α_2-adrenoceptor antagonist atipamezole (10 μg) or saline vehicle, and responses were assessed again 30 min later (at 210 min).

To determine if the repeated nociceptive testing in the tail flick and paw pressure tests confounded behavioral nociceptive outcomes, two groups of animals were compared following an injection of BRL44408 (1.65 ng) at 0, 90 and 180 min. One group received regular testing every 30 min for 4 h post-injection, while the other group received only limited testing, with responses assessed only at 30 min post-injection and again at 210 and 240 min.

2.4. Statistical analyses

Percentage of maximum possible effect (M.P.E.) (%MPE = 100 × [postdrug response − baseline response]/[cutoff response − baseline response]) was calculated for the results of both nociceptive tests. Nonlinear regression was used to calculate ED_{50} values, and two-way repeated-measure analysis of variance (ANOVA) using time as a within-subject factor and treatment as a between subjects factor was utilized to factor into account the repeated measures design. Time × treatment interaction tested for longitudinal response pattern differences, and Tukey's post-hoc tests

were utilized where appropriate (Milne et al., 2011). All data are reported as mean ± S.E.M., and the α level was set to 0.05.

3. Results

3.1. Effects of BRL44408 on clonidine-induced antinociception

Intrathecal clonidine (13.3 μg) increased nociceptive thresholds in both the tail flick and paw pressure assays, with peak effects observed 30 min post-injection in both tests (Fig. 1). Clonidine had much greater analgesic effects in the tail flick assay compared to those of the paw pressure assay, reaching approximately 85% MPE and 35% MPE, respectively. In both tests, animals treated with clonidine alone returned to baseline response levels by 180 min. Co-administration of BRL44408 (16.5 μg) blocked the analgesic effects of clonidine and responses were significantly lower over the first 60 min in the tail flick test ($P<0.001$) and over the first 90 min in the paw pressure test ($P<0.001$ from 20–50 min, $P<0.01$ at 60 and 90 min). Two-way ANOVA revealed a significant effect of time ($F_{(8,64)}=51.24$, $P<0.001$), treatment ($F_{(1,8)}=24.79$, $P<0.01$), and interaction ($F_{(8,64)}=58.99$, $P<0.001$) in the tail flick assay. In the paw pressure test, a significant effect of time ($F_{(8,64)}=15.17$, $P<0.001$), treatment ($F_{(1,8)}=54.37$, $P<0.001$), and interaction ($F_{(8,64)}=8.92$, $P<0.001$) was observed.

3.2. Effects of ultra-low dose BRL44408 on acute morphine and norepinephrine antinociception

Intrathecal morphine (15 μg) increased both the thermal latency and mechanical nociceptive threshold, with all animals experiencing nearly 100% MPE in both paradigms with a peak effect at 30 min post-injection (Fig. 2a and b). After 30 min, the antinociception decreased steadily over time, and by 180 min responses returned to pre-injection baselines. Co-administration of ultra-low dose BRL44408 (1.65 ng) with morphine resulted in a slightly delayed antinociceptive effect, with animals reaching peak effect around 50 min post-injection in the tail flick assay and 60 min in the paw pressure test. Despite the delayed peak effects, antinociception was prolonged in the animals co-administered ultra-low dose BRL44408 compared to morphine alone, and responses were significantly higher from 60 min onward in both testing paradigms ($P<0.001$). By 180 min, responses had begun to decrease but were still significantly higher than pre-injection baselines (50–60% MPE), and significantly higher than animals treated with morphine alone. Administration of ultra-low dose BRL44408 alone also produced an increase in nociceptive thresholds, and although the effect was delayed in onset, a significant effect was apparent at 60–80 min post-injection. At 180 min, animals treated with BRL44408 alone had reached nearly 100% MPE in the tail flick test, but only 40% MPE in the paw pressure test. Two-way ANOVA revealed a significant effect of time ($F_{(8,104)}=7.362$, $P<0.001$), treatment ($F_{(2,13)}=4.981$, $P<0.001$), and interaction ($F_{(16,104)}=38.42$, $P<0.001$) for thermal nociceptive testing. In the paw pressure test, a significant effect of time ($F_{(8,104)}=9.663$, $P<0.001$), treatment ($F_{(2,13)}=6.450$, $P<0.001$), and interaction ($F_{(16,104)}=29.03$, $P<0.001$) was observed.

Acute i.t. norepinephrine (30 μg) increased both thermal latency and mechanical nociceptive threshold (Fig. 2c and d). Peak effects were observed in both cases 30 min post-injection, although norepinephrine was more effective in the tail flick compared to the paw pressure test (approximately 80% MPE vs. 65% MPE, respectively). Animals receiving norepinephrine alone did not produce maximal antinociception in either test. Animals that were co-administered BRL44408 (1.65 ng) with norepinephrine experienced delayed peak analgesia in the tail flick assay, which occurred 60 min post-injection compared to 30 min for norepinephrine alone. The peak effect in the paw pressure test, however, occurred at the same time as it did in animals given norepinephrine alone (30–40 min post-injection). Peak MPE in the tail flick test reached almost 100% in rats co-administered norepinephrine with ultra-low dose BRL44408, and prolonged the antinociceptive effects compared to norepinephrine alone. In the tail flick test, significantly higher %MPE was observed from 50–180 min (all $P<0.05$) post-injection in the animals co-administered norepinephrine with ultra-low dose BRL44408 compared to norepinephrine alone. No difference was observed in the paw pressure test in animals co-administered ultra-low dose BRL44408 compared to norepinephrine alone. For norepinephrine (Fig. 2c and d), two-way ANOVA revealed significant effects of time ($F_{(8,80)}=24.49$, $P<0.001$), treatment ($F_{(1,10)}=22.81$, $P<0.001$), and interaction ($F_{(8,80)}=10.68$, $P<0.001$) in the tail flick test, and significant effects of time ($F_{(8,80)}=26.21$, $P<0.001$) and interaction ($F_{(8,80)}=6.552$, $P<0.001$) in the paw pressure test.

3.3. Effects of ultra-low dose BRL44408 on the development of acute morphine and norepinephrine tolerance

Repeated injection of morphine (15 μg) produced a significant decrease in thermal latencies indicative of acute tolerance (Fig. 3a). Compared to morphine alone, animals administered morphine with ultra-low dose BRL44408 had a significantly lower antinociceptive effect at 30 min post-injection ($P<0.001$), but demonstrated attenuation of the acute opioid antinociceptive tolerance. Co-administration of morphine with ultra-low dose BRL44408

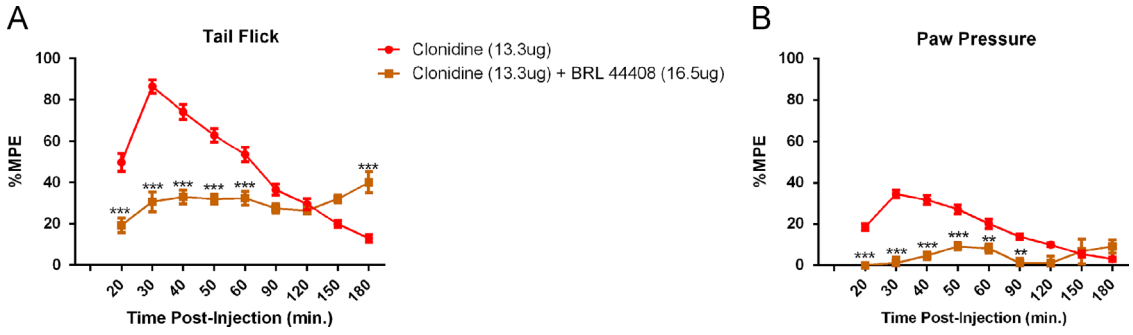

Fig. 1. Animals were administered a single injection of clonidine (13.3 μg, i.t., $n=6$) or clonidine with high dose BRL44408 (16.5 μg, i.t., $n=4$) and tail flick and paw pressure responses were assessed over 180 min to determine the effects of high dose BRL44408 on clonidine antinociception. Intrathecal administration of clonidine alone was analgesic in both nociceptive assays. Co-administration of high dose BRL44408 (16.5 μg) blocked the analgesic effects of clonidine and responses were significantly lower over the first 60 min in the tail flick test ($P<0.001$) and over the first 90 min in the paw pressure test ($P<0.001$ from 20 to 50 min, $P<0.01$ at 60 and 90 min). Two-way ANOVA revealed a significant effect of time ($F_{(8,64)}=51.24$, $^{***}P<0.001$), treatment ($F_{(1,8)}=24.79$, $^{**}P<0.01$), and interaction ($F_{(8,64)}=58.99$, $^{***}P<0.001$) in the tail flick assay. In the paw pressure test, a significant effect of time ($F_{(8,64)}=15.17$, $^{***}P<0.001$), treatment ($F_{(1,8)}=54.37$, $^{***}P<0.001$), and interaction ($F_{(8,64)}=8.92$, $^{***}P<0.001$) was observed.

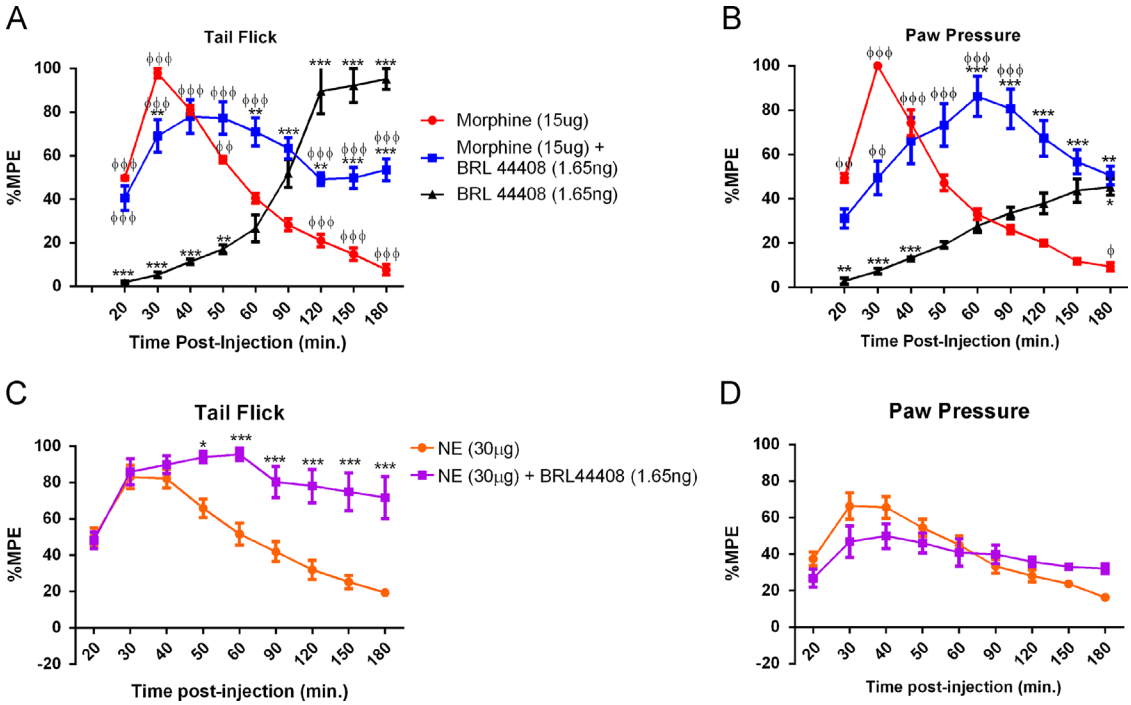

Fig. 2. Effects of ultra-low dose BRL44408 on acute morphine and norepinephrine (NE) antinociception. Morphine administration was analgesic in both the tail flick and paw pressure tests (a, b) ($n=4$). Co-administration of ultra-low dose BRL44408 with morphine ($n=9$) resulted in delayed analgesia with a peak effect slightly lower than the morphine only group. Analgesia was prolonged in the animals co-administered ultra-low dose BRL44408 compared to morphine alone and responses were significantly higher from 60 min onward ($P<0.001$). Administration of ultra-low dose BRL44408 alone demonstrated delayed and increasing analgesia over the time. At 180 min, animals treated with BRL44408 alone ($n=3$) had reached nearly 100% MPE in the tail flick but only around 40% MPE in the paw pressure test, both of which were significantly higher than morphine treated animals ($P<0.001$ and $P<0.05$ respectively). *** indicates significant difference compared to morphine alone. ϕϕϕ indicates significant difference compared to ULD BRL alone. Two-way ANOVA revealed a significant effect of time ($F_{(8,104)}=7.362$, $^{***}P<0.001$), treatment ($F_{(2,13)}=4.981$, $^{***}P<0.001$), and interaction ($F_{(16,104)}=38.42$, $^{***}P<0.001$). In the paw pressure test, a significant effect of time ($F_{(8,104)}=9.663$, $^{***}P<0.001$), treatment ($F_{(2,13)}=6.450$, $^{***}P<0.001$), and interaction ($F_{(16,104)}=29.03$, $^{***}P<0.001$) was observed.

Acute intrathecal norepinephrine (30 μg) was analgesic in both the tail and paw pressure tests (c, d) ($n=7$). Animals co-administered ultra-low dose BRL44408 (1.65 ng, $n=5$) with norepinephrine experienced delayed peak analgesia in the tail flick assay compared to norepinephrine alone. Peak effect in the paw pressure test, however, occurred at the same time as norepinephrine alone. Peak %MPE in the tail flick test reached almost 100% in the rats co-administered norepinephrine with ultra-low dose BRL44408 and animals were still experiencing significant analgesia even 180 min post-injection, whereas animals receiving norepinephrine alone were not. In the tail flick test, significantly higher %MPE was observed at 50 ($P<0.05$), 60 ($P<0.001$), 90 ($P<0.01$), 120 ($P<0.001$), 150 ($P<0.001$), and 180 ($P<0.001$) min post-injection in the animals co-administered norepinephrine with ultra-low dose BRL44408 compared to norepinephrine alone. No difference was observed in the paw pressure test in animals co-administered ultra-low dose BRL44408 compared to norepinephrine alone. For norepinephrine (c, d), two-way ANOVA revealed significant effects of time ($F_{(8,80)}=24.49$, $^{***}P<0.001$), treatment ($F_{(1,10)}=22.81$, $^{***}P<0.001$), and interaction ($F_{(8,80)}=10.68$, $^{***}P<0.001$) in the tail flick test, and significant effects of time ($F_{(8,80)}=26.21$, $^{***}P<0.001$) and interaction ($F_{(8,80)}=6.552$, $^{***}P<0.001$) in the paw pressure test.

(1.65 ng), however, resulted in significantly higher antinociception (at nearly 100% MPE) from 150–240 min (all $P<0.001$) compared to morphine alone. In the tail flick test, ultra-low dose BRL44408 administered alone significantly increased thermal nociceptive thresholds beginning 90 min post-injection. Similar effects were observed in the paw pressure test, with the first injection of morphine alone producing 100% MPE but decreasing over time and following repeated injections as tolerance developed. At the end of the time course (240 min), animals that had received morphine alone were not experiencing any analgesia, and their responses were not significantly different from the responses of the saline-treated controls. The decline in morphine-induced antinociception was significantly attenuated by co-administration of ultra-low dose BRL44408. As in the tail flick test, BRL44408 alone increased mechanical nociceptive thresholds in the paw pressure assay, but the effects were delayed. At 240 min post-injection, paw pressure responses in animals given an ultra-low dose of BRL44408 alone were not different from those in animals co-administered morphine with ultra-low dose BRL44408. A two-way ANOVA revealed significant effects of time ($F_{(7,133)}=42.41$, $P<0.001$), treatment ($F_{(3,19)}=231.9$, $P<0.001$), and interaction ($F_{(21,133)}=72.18$, $P<0.001$) in the tail flick test. In the paw pressure test, a significant effect of time ($F_{(7,133)}=6.425$, $P<0.001$), treatment ($F_{(3,19)}=38.04$, $P<0.001$) and interaction ($F_{(21,133)}=27.32$, $P<0.001$) was observed.

Assessment of morphine potency 24 h following the acute tolerance paradigm revealed a rightward shift in the dose response curves in the animals that had been repeatedly treated with morphine compared to those treated with saline. Ultra-low dose BRL44408 prevented the rightward shift in morphine dose response curve in both the tail flick (Fig. 3c) and paw pressure (Fig. 3d) assays. Calculation of morphine ED_{50} from the dose response curves revealed that animals that had received morphine alone had significantly higher ED_{50} values in both the tail flick and paw pressure tests compared to animals that had received saline, morphine with ultra-low dose BRL44408, or BRL44408 alone ($P<0.001$ for all three), suggesting that ultra-low dose BRL44408 prevented the decline in morphine potency (Fig. 5a). There was no difference in morphine ED_{50} values between morphine plus ultra-low dose BRL44408, BRL44408 alone, or saline in either test, and values for all groups were similar in both tests (Fig. 5a).

Acute antinociceptive tolerance to repeated norepinephrine injections (30 μg) was observed in both the tail flick and paw pressure tests (Fig. 4a and b). After the first injection of norepinephrine, thermal latencies increased to 80% MPE and mechanical thresholds increased to 50–60% MPE, with peak effect observed 30 min post-injection. Co-administration of norepinephrine with ultra-low dose BRL44408 (1.65 ng) attenuated the development of

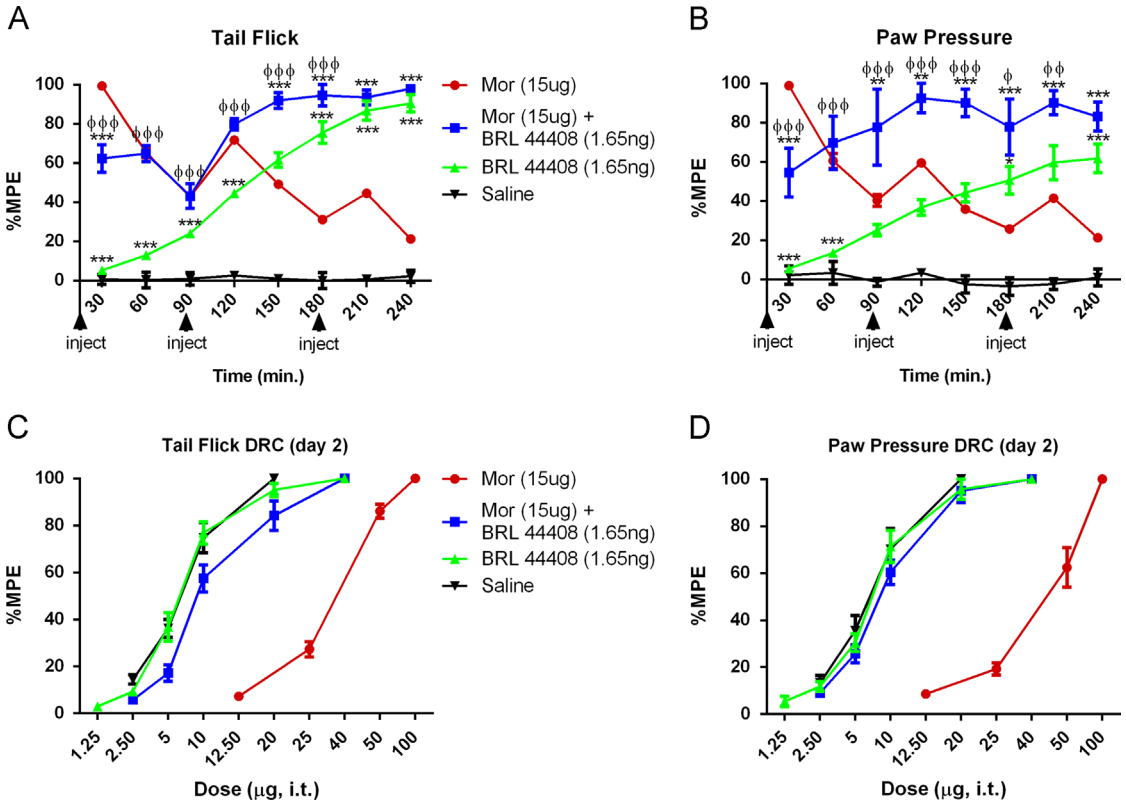

Fig. 3. Effects of ultra-low dose BRL44408 on the development of acute morphine (Mor) tolerance and cumulative dose response curves (DRC) 24 h following acute tolerance testing. Drugs were injected at 0, 90 and 180 min to induce tolerance (a, b). Compared to morphine alone ($n=4$), animals co-administered morphine with ultra-low dose BRL44408 ($n=4$) displayed a delayed effect early on and the antinociception was significantly lower at 30 min than morphine alone ($P<0.001$), but higher than BRL44408 alone ($P<0.001$, $n=9$), and there was no difference in %MPE at 60 or 90 min compared to morphine alone. After the second injection of morphine with ultra-low dose BRL44408, attenuation of the development of acute opioid tolerance was observed and the antinociception increased steadily over the remainder of the time course. Co-administration of morphine with ultra-low dose BRL44408 resulted in significantly higher antinociception (at nearly 100% MPE) at 150, 180, 210 and 240 min ($P<0.001$ for all those time points) compared to morphine alone. In the tail flick and paw pressure tests, ultra-low dose BRL44408 administered alone demonstrated delayed sustained analgesia. *** indicates significant difference compared to morphine alone. ϕϕϕ indicates significant difference compared to ULD BRL alone. Two-way ANOVA revealed significant effects of time ($F_{(7,133)}=42.41$, $^{***}P<0.001$), treatment ($F_{(3,19)}=231.9$, $^{***}P<0.001$), and interaction ($F_{(21,133)}=72.18$, $^{***}P<0.001$) in the tail flick test. In the paw pressure test, a significant effect of time ($F_{(7,133)}=6.425$, $^{***}P<0.001$), treatment ($F_{(3,19)}=38.04$, $^{***}P<0.001$) and interaction ($F_{(21,133)}=27.32$, $^{***}P<0.001$) was observed. Assessing morphine potency 24 h later revealed a leftward shift in the dose response curves in the animals that had been previously treated with ultra-low dose BRL44408 (1.65 ng) alone or morphine plus ultra-low dose BRL44408 compared to morphine alone in both the tail flick (c) and paw pressure (d) assays.

acute tolerance to norepinephrine in both the tail flick and paw pressure tests. Thermal latencies were significantly higher in animals co-administered ultra-low dose BRL44408 compared to norepinephrine alone (60–240 min, all $P<0.05$). Similarly, in the paw pressure test, co-administration of ultra-low dose BRL44408 with norepinephrine compared to norepinephrine alone resulted in significantly higher nociceptive thresholds from 120–240 min (all $P<0.001$). In the tail flick test, statistical analysis by two-way ANOVA revealed a significant effect of time ($F_{(7,77)}=16.16$, $P<0.001$), treatment ($F_{(1,11)}=169.8$, $P<0.001$), and interaction ($F_{(7,77)}=13.73$, $P<0.001$). In the paw pressure test, a significant effect of time ($F_{(7,77)}=12.30$, $P<0.001$), treatment ($F_{(1,11)}=39.61$, $P<0.001$), and interaction ($F_{(7,77)}=20.05$, $P<0.001$) was observed.

Norepinephrine dose–response curves, assessed 24 h following the acute tolerance paradigm, revealed a leftward shift in the curves of the animals that had received norepinephrine with ultra-low dose BRL44408 (1.65 ng) on day one compared to norepinephrine alone in both the tail flick and paw pressure tests (Fig. 4c and d). Calculating the ED_{50} from the dose response curves (Fig. 5b) showed that animals treated with norepinephrine and ultra-low dose BRL44408 on day one had ED_{50} values nearly seven-fold lower than those treated with norepinephrine alone on day one (approximately 5 μg i.t. compared to 35 μg i.t., respectively, $P<0.001$) in the tail flick test, and around 50% lower in the paw pressure test (20 μg i.t. compared to 45 μg i.t., respectively, $P<0.001$).

3.4. Effects of the α_2-adrenoceptor antagonist atipamezole on ultra-low dose BRL44408 antinociception

BRL44408 antinociception peaked at 180 min post-injection in both the tail flick and paw pressure tests (Fig. 6). At this time point, animals were injected with either saline or atipamezole (10 μg). Following injection of the antagonist, there was a significant decrease in BRL44408 antinociception in both the tail flick and paw pressure tests compared to saline controls ($P<0.001$). However, the reversal was only partial in both tests, atipamezole reducing %MPE by approximately 20% in the tail flick test and by approximately 30% in the paw pressure test. The BRL44408 antinociception continued to increase in the control animals that were injected with saline at 180 min. Two-way ANOVA revealed significant effects of time ($F_{(9,198)}=701.0$, $P<0.001$), treatment ($F_{(3,22)}=3.246$, $P<0.05$), and interaction ($F_{(27,198)}=7.422$, $P<0.001$). In the paw pressure test, a significant effect of time ($F_{(9,189)}=435.1$, $P<0.001$) and interaction ($F_{(27,189)}=12.49$, $P<0.001$) was observed.

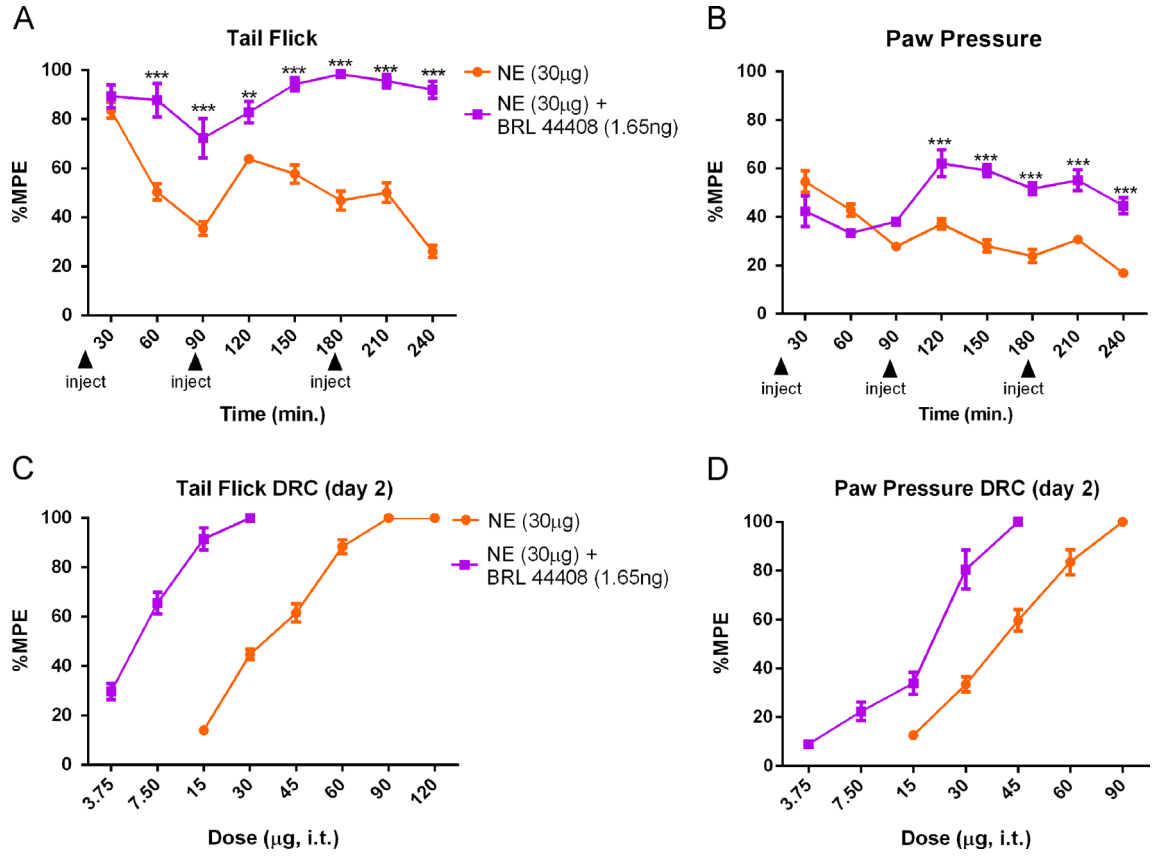

Fig. 4. Effects of ultra-low dose BRL 44408 on the development of acute norepinephrine (NE) tolerance and cumulative dose response curves (DRC) 24 h after acute tolerance testing. Acute tolerance to repeated norepinephrine injections (arrows) was observed in both the tail flick and paw pressure tests (a, b) ($n=8$). Compared to norepinephrine alone, co-administration of norepinephrine with ultra-low dose BRL44408 (1.65 ng, $n=5$) completely attenuated the development of acute tolerance to norepinephrine. Thermal response thresholds were significantly higher in animals co-administered ultra-low dose BRL44408 compared to norepinephrine alone at 60 ($P<0.001$), 90 ($P<0.001$), 120 ($P<0.05$), 150 ($P<0.001$), 180 ($P<0.001$), 210 ($P<0.001$) and 240 ($P<0.001$) min. Similarly, in the paw pressure test co-administration of ultra-low dose BRL44408 with norepinephrine compared to norepinephrine alone resulted in significantly higher response thresholds at 120 ($P<0.001$), 150 ($P<0.001$), 180 ($P<0.001$), 210 ($P<0.001$) and 240 ($P<0.001$) min. Unlike norepinephrine alone, %MPE did not decrease following repeated injections in the animals co-administered ultra-low dose BRL4408, but the peak effect increased after each subsequent injection in both tail flick and paw pressure tests. In the tail flick test, statistical analysis by two-way ANOVA revealed a significant effect of time ($F_{(7,77)}=16.16$, $^{***}P<0.001$), treatment ($F_{(1,11)}=169.8$, $^{***}P<0.001$), and interaction ($F_{(7,77)}=13.73$, $^{***}P<0.001$). In the paw pressure test, a significant effect of time ($F_{(7,77)}=12.30$, $^{***}P<0.001$), treatment ($F_{(1,11)}=39.61$, $^{***}P<0.001$), and interaction ($F_{(7,77)}=20.05$, $^{***}P<0.001$) was observed. Plotting the norepinephrine dose–response curves 24 h later revealed a leftward shift in the curves of the animals that had received on day one norepinephrine with ultra-low dose BRL44408 (1.65 ng) compared to norepinephrine alone in both the tail flick and paw pressure tests (c, d).

3.5. Effects of repeated vs. limited testing on ultra-low dose BRL44408 antinociception

There was no significant difference at any time point between the repeated or limited testing groups in either the tail flick (Fig. 7a) or paw pressure (Fig. 7b) test. For both testing paradigms, BLR44408 significantly increased thermal and mechanical nociceptive thresholds throughout the entire testing period and did not show evidence of decline at 240 min. Assessing morphine potency 24 h following injection of BRL44408 for repeated and limited testing (Fig. 7c) revealed that there was no difference in the morphine ED_{50} values for either the regular or limited testing groups that had received ultra-low dose BRL44408 the previous day.

4. Discussion

Previous studies from our laboratory have provided evidence that extremely low doses of several non-selective α_2-adrenoceptor antagonists, well below those producing the adrenoceptor blockade, can augment spinal morphine antinociception and inhibit the development of tolerance (Milne et al., 2008). Such effects of low dose antagonists also extend to the acute antinociception and tolerance produced by the spinal injections of the α_2-adrenoceptor agonists clonidine or norepinephrine (Milne et al., 2011). These actions of the adrenoceptor antagonists on morphine-induced responses thus parallel the previously documented actions of ultra-low dose competitive opioid receptor antagonists, such as naltrexone (McNaull et al., 2007; Powell et al., 2002) and naloxone (Mattioli et al., 2014, 2010). While the mechanisms underlying the crossover effects of the adrenergic antagonists on opioid agonist-induced responses remain unclear, they are not without precedence. Similar effects have been observed in other models, most notably in the peripheral nociception model (Aley and Levine, 1997). The effects observed in the spinal model used in the present study apparently involve a specific interaction of antagonists with the spinal α_2-adrenoceptors, since extremely low doses of the enantiomers of efaroxan, a non-selective α_2-adrenoceptor antagonist, have recently been reported to exhibit a stereo-selective enhancement of morphine antinociception and inhibition of acute tolerance to repeated doses of i.t. morphine (Milne et al., 2013). Since all the antagonists tested in previous experiments are known to be non-selective α_2-adrenoceptor ligands, the nature of the α_2-adrenoceptor subtype mediating their unusual pro-opioid actions at the spinal level remains unclear. The availability of BRL44408, a receptor antagonist that is highly selective for the α_{2A}-adrenoceptor type (Young et al., 1989), prompted us to determine in the present

study whether this receptor has a role in expression of the low dose effects of antagonists on the antinociception and acute tolerance produced by the spinal administration of morphine or norepinephrine.

The present study established that i.t. BRL44408 behaves as an α_2-adrenergic antagonist since at a higher dose it effectively blocked the antinociceptive actions of clonidine. However, when administered at an ultra-low dose, BRL44408 effectively prolonged morphine antinociception in tests of thermal and mechanical nociception, and inhibited the acute morphine tolerance produced by three successive maximal doses of spinal morphine. The action of BRL44408 on morphine tolerance was reflected in: a) a significant attenuation of the progressive decline of the peak pharmacological response, and b) a marked inhibition of the agonist potency loss, as evidenced by a significant increase in the morphine ED_{50} values derived from the dose–response curves obtained 24 h post-drug treatment. The results of this study also showed low dose BRL44408 to exert similar effects on the responses produced by i.t. norepinephrine, although in this case its action on antinociception was significant only in the tail flick test. As observed in our previous work (Milne et al., 2013), the paw pressure test demonstrated reduced sensitivity to the spinal actions of adrenergic agonists when compared with the effectiveness of morphine, and this factor might partly contribute to the poor actions of BRL44408 observed in this test. However, this discrepancy notwithstanding the ability of BRL44408 to largely replicate the effects of non-selective adrenoceptor antagonists observed in earlier studies (Milne et al., 2013, 2008) suggests that the α_{2A}-adrenoceptor subtype in the spinal cord likely plays a major role in expression of the modulatory actions of such antagonists on opioid antinociception and tolerance.

Consistent with the actions of non-selective antagonists, the ultra-low dose of BRL44408 in the present study showed intrinsic activity, eliciting a slowly developing antinociceptive response that attained peak levels towards the end of the 240 min testing period. However, in contrast with the low level response produced by non-selective antagonists (Milne et al., 2008), BRL44408 produced a stronger response that reached a near maximal value in the tail flick test. The basis of this incremental response over the test period remains unclear. We sought to determine if the application of a repeated test stimulus, entailed in derivation of the time–response relationship of the drug effect over a 240 min period, could be a factor in the production of this antinociceptive effect. Such application of a repeated test stimulus could lead to a local release of endogenous factors (for example, opioid or adrenergic transmitters), and the ultra-low dose of BRL44408 might augment their action to yield an antinociceptive response. Thus, the action of BRL44408 was re-examined using only limited

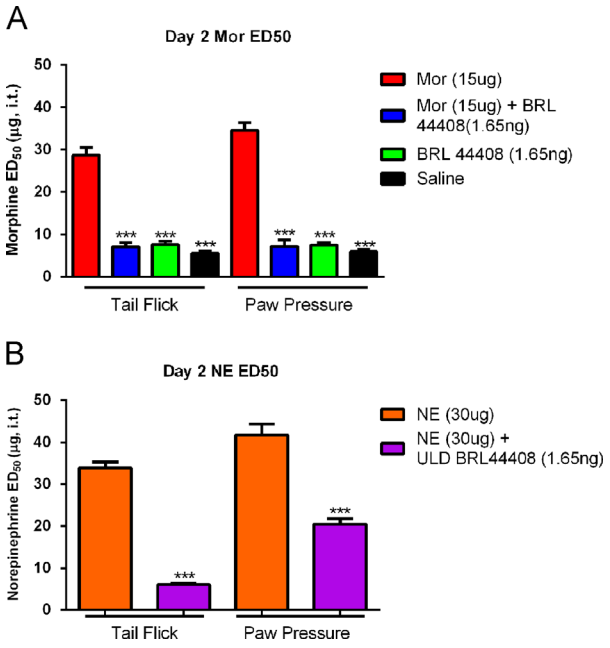

Fig. 5. Morphine (Mor) and norepinephrine (NE) ED_{50} values following acute tolerance testing. Calculation of morphine ED_{50} from the dose response curves revealed that animals that had received morphine alone on day one had significantly higher ED_{50} values in both the tail flick and paw pressure tests compared to animals that had received morphine with ultra-low dose (ULD) BRL44408 or BRL44408 alone on day one ($^{***}P<0.001$, a). There was no difference in morphine ED_{50} values between morphine plus ultra-low dose BRL44408 or BRL44408 alone in either test (a). Calculating the ED_{50} from the dose response curves (b) showed that animals treated with norepinephrine and ultra-low dose BRL44408 on day one had ED_{50} values nearly seven-fold lower than those treated with norepinephrine alone on day one (approximately 5 µg, i.t. compared to 35 µg, i.t. respectively, $^{***}P<0.001$) in the tail flick test, and around 50% lower in the paw pressure test (20 µg, i.t. compared to 45 µg, i.t. respectively, $^{***}P<0.001$). ED_{50} values for both groups were significantly lower in the tail flick compared to the paw pressure test.

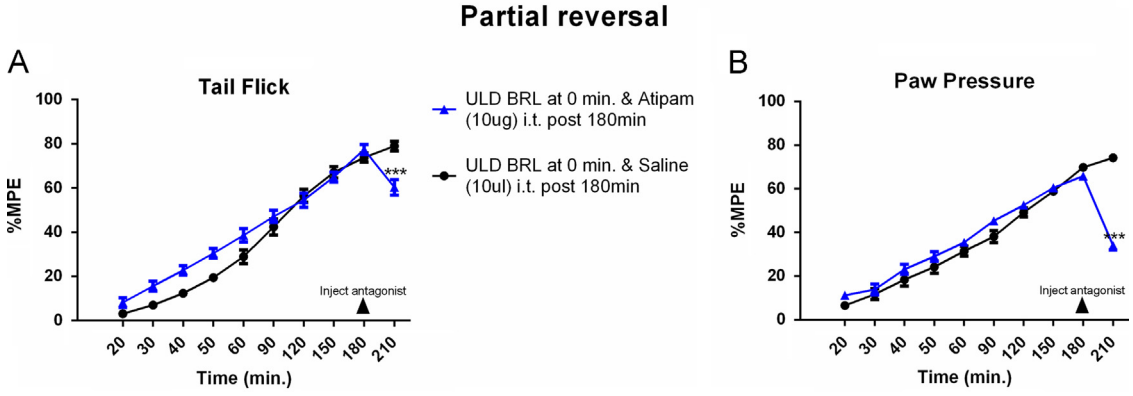

Fig. 6. Effects of the α_2-adrenergic antagonist atipamezole (Atipam) on ultra-low dose BRL44408 antinociception. BRL44408 (1.65 ng, given at time zero) antinociception increased steadily, and at 180 min, just prior to antagonist ($n=7$) or saline (arrow, $n=4$) administration, there was no difference in BRL44408 antinociception in either of the groups. Following injection of atipamezole there was a significant decrease in BRL44408 antinociception in both the tail flick and paw pressure tests ($P<0.001$ compared to saline). Two-way ANOVA revealed significant effects of time ($F_{(9,198)}=701.0$, $^{***}P<0.001$), treatment ($F_{(3,22)}=3.246$, $^{*}P<0.05$), and interaction ($F_{(27,198)}=7.422$, $^{***}P<0.001$). In the paw pressure test, a significant effect of time ($F_{(9,189)}=435.1$, $^{***}P<0.001$) and interaction ($F_{(27,189)}=12.49$, $^{***}P<0.001$) was observed.

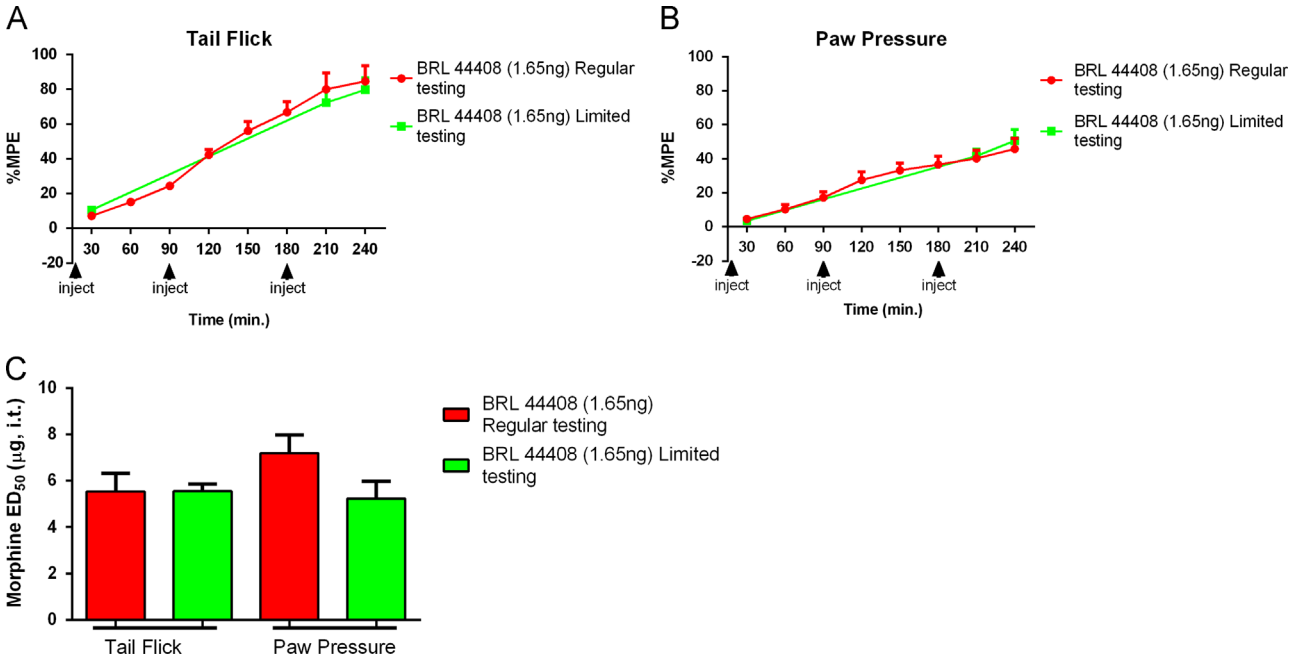

Fig. 7. Effects of regular vs. limited testing on ultra-low dose BRL44408 antinociception. Animals received a single injection of BRL 44408 (1.65 ng) at time zero and had antinociception testing every 30 min (regular) for 4 h or limited testing at 30, 210 and 240 min post-injection. There was no significant difference at any time point for either the regular ($n=4$) or limited ($n=5$) testing group in either the tail flick (a) or paw pressure (b) assay. Assessing morphine potency 24 h later (c), there was no difference in the morphine ED_{50} values for either the regular or limited testing groups that had received ultra-low dose BRL44408 the previous day, either in the tail flick or paw pressure tests.

nociceptive testing. Interestingly, the timing of the stimulus completely failed to influence the action of BRL44408, suggesting that repeated stimulation was not a contributory factor in the intrinsic activity of BRL44408. Also, neither mode of stimulation influenced the potency of morphine determined 24 h after the low dose BRL44408 treatment. This suggests that the specific mode of stimulation is an unlikely factor in the intrinsic actions of BRL44408. We also considered that such actions of BRL44408 may originate from its interaction with α_{2A}-adrenoceptors, either via a direct action on the receptor or via an indirect release of norepinephrine from terminals of the noradrenergic neurons (Umeda et al., 1997). This possibility was suggested by the observation that the antinociceptive action of BRL44408 alone, like those of the adrenergic agonists (clonidine and norepinephrine), was weaker in the paw pressure than in the tail flick test. Indeed, administration of the non-selective antagonist atipemazole, at a dose shown to produce adrenergic receptor blockade in analgesia tests (Milne et al., 2008), partially reversed the delayed antinociceptive action of BRL44408 in both tests. However, the reversal of BRL44408 action was incomplete, suggesting that some other factors also contribute to its origin. Thus, analysis of its action merits exploration in future tests. Although BRL44408 can elicit analgesic-like activity in the visceral nociception model (Dwyer et al., 2010), its action has been attributed to blockade of the α_{2A}-adrenoceptor. In the current study the ultra-low dose BRL44408 did not produce such blockade, as evident by its inability to antagonize the norepinephrine-induced responses.

An interesting question raised by the intrinsic actions of BRL44408 is whether its additivity or synergy with morphine is the basis of its pro-opioid actions. While this possibility cannot be excluded in the acute antinociception experiments, it is unlikely to explain BRL44408 effects on the development of tolerance, especially its ability to influence the loss of agonist potency, a hallmark indicator of this phenomenon. As indicated, low dose BRL44408 produced a strong inhibition of the loss of morphine potency, the estimates of which were made 24 h after the drug treatment, a time point at which BRL44408 is likely to be absent from the spinal space. Thus, it would appear that BRL44408 likely induces a change in the processes that contribute to the genesis of tolerance in response to repeated opioid agonist exposure.

While it is known that the α_{2A}-adrenoceptor subtype is the primary mediator of α_2 spinal analgesia, and is necessary for analgesia synergy with opioids (Stone et al., 1997), the mechanism of action of the α_{2A}-adrenoceptor antagonist BRL44408 on morphine antinociception and tolerance is unknown. Considering the co-localization of α_2-adrenoceptors and opioid receptors in the spinal cord (Jordan et al., 2003), it is likely that conformational cross-talk between α_{2A}-adrenergic and µ opioid receptors controlling cell signaling with the G protein-coupled receptor heterodimers may facilitate these changes (Vilardaga et al., 2008). Other possibilities include the ability of these α_2-adrenoceptor antagonists to counteract the stimulatory hyperalgesic-like responses of opioid agonists (Crain and Shen, 2000, 1995; Milne et al., 2013), or to influence glial activation since ultra-low doses of the opioid antagonist naltrexone affect activation of glia by morphine (Mattioli et al., 2010). Similarly, prolonged spinal delivery of the α_2-adrenoceptor agonists such as clonidine, resulting in loss of the antinociceptive response, produces a hyperalgesic thermal hypersensitivity (Quartilho et al., 2004) and thus BRL44408 may be acting to influence this response.

5. Conclusion

In summary, ultra-low doses of BRL44408 were found to significantly augment acute morphine and norepinephrine analgesia, and to inhibit the loss of drug potency from repeated exposure, implicating involvement of the α_{2A}-adrenoceptor in the action of low-dose α_2-adrenoceptor antagonists on morphine and norepinephrine antinociception and tolerance. BRL44408 produces significant analgesia that is unrelated to testing frequency and is only partially dependent on its interaction with α_2-adrenoceptors,

suggesting that this agent class may be potentially useful in the treatment of pain.

Role of the funding source

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and the Canada Research Chairs Program. These funding agencies were not involved in any aspect of this study, including its design, the collection, analysis, and interpretation of data, the writing of this manuscript, or in the decision to submit this manuscript for publication.

Acknowledgments

This work was supported by grants from CIHR (Grant nos. FRN5960 and MOP123298) and the Canada Research Chairs Program. The authors would like to thank Joelle B. Thorpe, PhD for her assistance with the preparation of this manuscript.

References

Aley, K.O., Levine, J.D., 1997. Different mechanisms mediate development and expression of tolerance and dependence for peripheral μ-opioid antinociception in the rat. J. Neurosci. 17, 8018–8023.

Crain, S.M., Shen, K.F., 2000. Antagonists of excitatory opioid receptor functions enhance morphine's analgesic potency and attenuate opioid tolerance/dependence liability. Pain 84, 121–131.

Crain, S.M., Shen, K.F., 1995. Ultra-low concentrations of naloxone selectively antagonize excitatory effects of morphine on sensory neurons thereby increasing its anti-nociceptive potency and attenuating tolerance/dependence during chronic cotreatment. Proc. Natl. Acad. Sci. USA 92, 10540–10544.

D'Amour, F.E., Smith, D.L., 1941. A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72, 74–79.

Dwyer, J.M., Platt, B.J., Sukoff Rizzo, S.J., Pulicicchio, C.M., Wantuch, C., Zhang, M.-Y., Cummons, T., Leventhal, L., Bender, C.N., Zhang, J., Kowal, D., Lu, S., Rajarao, J.R., Smith, D.L., Shilling, A.D., Wang, J., Butera, J., Resnick, L., Rosenzweig-Lipson, S., Schechter, L.E., Beyer, C.E., 2010. Preclinical characterization of BRL 44408: antidepressant- and analgesic-like activity through selective alpha2A-adrenoceptor antagonism. Int. J. Neuropsychophysiol. 13, 1193–1205.

Eisenach, J.C., De Kock, M., Klimscha, W., 1996. α_2-adrenergic agonists for regional anesthesia: a clinical review of clonidine (1984–1995). Anesthesiology 85, 655–674.

Fairbanks, C.A., Stone, L.S., Wilcox, G.L., 2009. Pharmacological profiles of alpha 2 adrenergic receptor agonists identified using genetically altered mice and isobolographic analysis. Pharmacol. Ther. 123, 224–238.

Gentili, F., Pigini, M., Piergentili, A., Giannella, M., 2007. Agonists and antagonists targeting the different α_2-adrenoceptor subtypes. Curr. Top. Med. Chem. 7, 163–186.

Jordan, B.A., Gomes, I., Rios, C., Filipovska, J., Devi, L.A., 2003. Functional interactions between mu opioid and alpha 2A-adrenergic receptors. Mol. Pharmacol. 64, 1317–1324.

Lilius, T.O., Rauhala, P.V., Kambur, O., Rossi, S.M., Vaananen, A.J., Kalso, E.A., 2012. Intrathecal atipamezole augments the antinociceptive effect of morphine in rats. Anesth. Analg. 114, 1353–1358.

Loomis, C.W., Milne, B., Cervenko, F.W., 1987. Determination of cross tolerance in rat spinal cord using intrathecal infusion via sequential mini-osmotic pumps. Pharm. Biochem. Behav. 26, 131–139.

Mattioli, T.A., Leduc-Pessah, H., Skelhorne-Gross, G., Nicol, C.J.B., Milne, B., Trang, T., Cahill, C.M., 2014. Toll-like receptor 4 mutant and null mice retain morphine-induced tolerance, hyperalgesia, and physical dependence. PLoS One 9 (5), e97361.

Mattioli, T.A., Milne, B., Cahill, C.M., 2010. Ultra-low dose naltrexone attenuates chronic morphine-induced gliosis in rats. Mol. Pain 6, 22.

Maze, M., Tranquilli, W., 1991. Alpha-2 adrenoceptor agonists: defining the role in clinical anesthesia. Anesthesiology 74, 581–605.

McNaull, B., Trang, T., Sutak, M., Jhamandas, K., 2007. Inhibition of tolerance to spinal morphine antinociception by low doses of opioid receptor antagonists. Eur. J. Pharmacol. 560, 132–141.

Milne, B., Jhamandas, K., Sutak, M., Grenier, P., Cahill, C.M., 2013. Stereo-selective inhibition of spinal morphine tolerance and hyperalgesia by an ultra-low dose of the alpha-2-adrenoceptor antagonist efaroxan. Eur. J. Pharmacol. 702, 227–234.

Milne, B., Sutak, M., Cahill, C.M., Jhamandas, K., 2011. Low dose alpha-2 antagonist paradoxically enhances rat norepinephrine and clonidine analgesia. Anesth. Analg. 112, 1500–1503.

Milne, B., Sutak, M., Cahill, C.M., Jhamandas, K., 2008. Low doses of α_2-adrenoceptor antagonists augment spinal morphine analgesia and inhibit development of acute and chronic tolerance. Br. J. Pharmacol. 155, 1264–1278.

Ossipov, M.H., Suarez, L.J., Spaulding, T.C., 1989. Antinociceptive interactions between alpha$_2$-adrenergic and opiate agonists at the spinal level in rodents. Anesth. Analg. 68, 194–200.

Owen, J.A., Milne, B., Jhamandas, K., Nakatsu, K., 1981. Assembly of an inexpensive tail flick analgesia meter. J. Pharmacol. Methods 6, 33–37.

Powell, K.J., Abul-Husn, N.S., Jhamandas, A., Olmstead, M.C., Beninger, R.J., Jhamandas, K., 2002. Paradoxical effects of the opioid antagonist naltrexone on morphine analgesia, tolerance and reward in rats. J. Pharmacol. Exp. Ther. 300, 588–596.

Quartilho, A., Mata, H.P., Ibrahim, M.M., Vanderah, T.W., Ossipov, M.H., Lai, J., Porreca, F., Malan, T.P., 2004. Production of paradoxical sensory hypersensitivity by α_2-adrenoreceptor agonists. Anesthesiology 100, 1538–1544.

Stone, L.S., MacMillan, L.B., Kitto, K.F., Limbird, L.E., Wilcox, G.L., 1997. The α_{2a} adrenergic receptor subtype mediates spinal analgesia evoked by α_2 agonists and is necessary for spinal adrenergic-opioid synergy. J. Neurosci. 17, 7157–7165.

Umeda, E., Satoh, T., Nagashima, H., Potter, P.E., Tarkovacs, G., Vizi, E.S., 1997. α_{2A} subtype of presynaptic α_2-Adrenoceptors modulates the release of [^{3}H]-noradrenaline from rat spinal cord. Brain Res. Bull. 42, 129–132.

Vilardaga, J.P., Nikolaev, V.O., Lorenz, K., Ferrandon, S., Zhuang, Z., Lohse, M.J., 2008. Conformational cross-talk between α_{2A}-adrenergic and μ-opioid receptors controls cell signaling. Nat. Chem. Biol. 4, 126–131.

Yaksh, T.L., Rudy, T.A., 1976. Chronic catheterization of the spinal subarachnoid space. Physiol. Behav. 17, 1031–1036.

Young, P., Berge, J., Chapman, H., Cawthorne, M.A., 1989. Novel alpha 2-adrenoceptor antagonists show selectivity for alpha 2A- and alpha 2B-adrenoceptor subtypes. Eur. J. Pharmacol. 168, 381–386.