RESEARCH PAPER

δ-Opioid receptor agonists inhibit migraine-related hyperalgesia, aversive state and cortical spreading depression in mice

Amynah A Pradhan[1,2,3,4], Monique L Smith[1,2,3], Jekaterina Zyuzin[2] and Andrew Charles[2,3]

[1]Semel Institute for Neuropsychiatry & Human Behavior, University of California, Los Angeles (UCLA), Los Angeles, CA, USA, [2]Headache Research and Treatment Program, Department of Neurology David Geffen School of Medicine, UCLA, Los Angeles, CA, USA, [3]Shirley and Stefan Hatos Center for Neuropharmacology, UCLA, Los Angeles, CA, USA, and [4]Department of Psychiatry, University of Illinois at Chicago (UIC), Chicago, IL, USA

BACKGROUND AND PURPOSE

Migraine is an extraordinarily common brain disorder for which treatment options continue to be limited. Agonists that activate the δ-opioid receptor may be promising for the treatment of migraine as they are highly effective for the treatment of chronic rather than acute pain, do not induce hyperalgesia, have low abuse potential and have anxiolytic and antidepressant properties. The aim of this study was to investigate the therapeutic potential of δ-opioid receptor agonists for migraine by characterizing their effects in mouse migraine models.

EXPERIMENTAL APPROACH

Mechanical hypersensitivity was assessed in mice treated with acute and chronic doses of nitroglycerin (NTG), a known human migraine trigger. Conditioned place aversion to NTG was also measured as a model of migraine-associated negative affect. In addition, we assessed evoked cortical spreading depression (CSD), an established model of migraine aura, in a thinned skull preparation.

KEY RESULTS

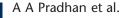NTG evoked acute and chronic mechanical and thermal hyperalgesia in mice, as well as conditioned place aversion. Three different δ-opioid receptor agonists, SNC80, ARM390 and JNJ20788560, significantly reduced NTG-evoked hyperalgesia. SNC80 also abolished NTG-induced conditioned place aversion, suggesting that δ-opioid receptor activation may also alleviate the negative emotional state associated with migraine. We also found that SNC80 significantly attenuated CSD, a model that is considered predictive of migraine preventive therapies.

CONCLUSIONS AND IMPLICATIONS

These data show that δ-opioid receptor agonists modulate multiple basic mechanisms associated with migraine, indicating that δ-opioid receptors are a promising therapeutic target for this disorder.

Abbreviations

ARM390, N,*N*-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide; CSD, cortical spreading depression; JNJ20788560, 9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9*H*-xanthene-3-carboxylic acid diethylamide; NTG, nitroglycerin; SNC80, (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl benzamide

Introduction

Migraine is a highly prevalent neurological disorder that affects over 800 million people worldwide (Stovner *et al.*, 2007; Bigal *et al.*, 2008; Stewart *et al.*, 2008; Victor *et al.*, 2010). The basic mechanisms underlying migraine remain inadequately understood, and while available therapies are effective for some, there are a large number of patients for whom current treatments are ineffective or poorly tolerated (Stovner *et al.*, 2007; Bigal *et al.*, 2008).

Most opioid analgesics in common clinical use for migraine and other pain conditions primarily target the μ-opioid receptor (receptor nomenclature follows Alexander *et al.*, 2013). However, these μ-receptor agonists have relatively poor efficacy as analgesics for migraine headache, and can contribute to progression of migraine from an episodic phenomenon to a frequent or daily condition that is refractory to other therapies (Bigal and Lipton, 2008; Bigal *et al.*, 2008). This 'medication overuse headache' is a particularly difficult clinical problem and is a common pathway to abuse of prescription μ-opioid receptor agonists.

The δ-opioid receptor agonists have markedly different properties from those of the μ-agonists. While compounds that selectively activate δ-opioid receptors have been found to be less effective as analgesics in acute pain models, they have shown efficacy in assays of chronic inflammatory (Fraser *et al.*, 2000; Cahill *et al.*, 2003; Petrillo *et al.*, 2003; Pradhan *et al.*, 2009; 2010) and neuropathic (Petrillo *et al.*, 2003; Kabli and Cahill, 2007; Gaveriaux-Ruff *et al.*, 2011) pain. In addition, δ-agonists do not induce the same types of adverse events as μ-agonists, such as respiratory depression and constipation (Negus *et al.*, 1994; Gallantine and Meert, 2005). Further, we have shown that unlike pharmacotherapies targeting the μ-opioid receptor, hyperalgesia does not occur following chronic administration with δ-agonists (Pradhan *et al.*, 2010). As an additional advantage, activation of the δ-opioid receptor does not appear to be highly rewarding, thus decreasing the abuse potential of δ-opioid receptor agonists. Finally δ-opioid receptors positively modulate emotional state. Genetic deletion of either the δ-opioid receptor or its endogenous ligand, enkephalin, results in anxiogenic and depressive-like behaviours (Konig *et al.*, 1996; Filliol *et al.*, 2000). Conversely, δ-agonists produce anxiolytic and antidepressant effects (Saitoh *et al.*, 2004; Perrine *et al.*, 2006). This emotional modulation may be particularly important for the treatment of migraine pain, as there is a high co-morbidity of migraine with depression and anxiety (Silberstein *et al.*, 2007). Although their characteristics seem well suited for treatment of migraine, δ-agonists have not yet been systematically investigated as a therapy for this disorder.

The aim of this study was to investigate the potential of δ-opioid receptor agonists as anti-migraine therapies. We found that δ-opioid receptor agonists given to mice modulate several basic mechanisms associated with migraine, indicating that these receptors are a promising therapeutic target for this disorder.

Methods

Animals

All animal care and experimental procedures were approved by the University Of California Los Angeles Office of Animal Research, in accordance with AALAC guidelines. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques, if available. A total of 225 animals were used in the experiments described here.

We used male and female C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME, USA), and δ-opioid receptor knockout mice (backcrossed onto a C57Bl6 background; Brigitte Kieffer, IGMBC, Illkirch, France) weighing 20–30 g. Animals were housed in a 12 h light–dark cycle, and food was available *ad libitum*. For experiments using δ-opioid receptor knockout mice, heterozygous pairs were bred to obtain wild-type and knockout animals (Filliol *et al.*, 2000).

Drug administration

Nitroglycerin (NTG) was prepared from a stock solution of 5.0 mg·mL^{-1} NTG in 30% alcohol, 30% propylene glycol and water (American Regent, Shirely, NY, USA). NTG was freshly diluted in 0.9% saline to a dose of 10 mg·kg^{-1}. The vehicle used in these experiments was 0.9% saline. We found that there was no significant difference in mechanical thresholds between 0.9% saline and the solution in which NTG was dissolved (6% propylene glycol, 6% alcohol, 0.9% saline). All injections were administered as a 10 mL·kg^{-1} volume. Unless otherwise noted, animals were tested for baseline responses immediately before i.p. injection with NTG. Animals were injected i.p. with vehicle, SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl benzamide) or sumatriptan, or *per os* with vehicle, ARM390 (N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide) or JNJ20788560 (9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9*H*-xanthene-3-carboxylic acid diethylamide), 1 h 15 min–1 h 30 min following NTG injection and were tested for mechanical or thermal sensitivity 30–45 min later (2 h post-NTG).

Sensory sensitivity testing

To determine mechanical sensitivity, the threshold for responses to punctate mechanical stimuli (mechanical hyperalgesia) was tested according to the up-and-down method (Chaplan *et al.*, 1994). In brief, the plantar surface of the animal hindpaw was stimulated with a series of eight von Frey filaments (bending force ranging from 0.008 to 2 g). A response was defined as a lifting or shaking of the paw upon stimulation. The first filament tested was 0.4 g. In the absence of a response, a heavier filament (up) was tried, and in the presence of a response, a lighter filament (down) was tested. This pattern was followed for a maximum of four filaments following the first response. To assess thermal responses, heat sensitivity was assessed by immersing the tail (5 cm from the tip) into a 46°C water bath. Tail withdrawal latencies were determined, and a cut-off of 40 s was established. For all pain experiments, groups were counterbalanced based on their naïve baselines (basal responses taken on day 1).

Conditioned place aversion

Independent groups of mice were trained for NTG-induced place conditioning and tested either treatment-free or under treatment effects (state dependency). We used a two-chamber conditioned place paradigm, which is described in detail by Tzschentke (2007). This consists of two identical Plexiglas shuttle boxes divided into two distinct compartments. The shuttle boxes consist of two large compartments separated by a plastic guillotine door. The compartments differ in floor texture (metal grated floors with a horizontal or vertical pattern) and wall pattern (horizontal or vertical black and white stripes). Live video tracking was used to collect data on time spent in each chamber and analysed using Noldus Etho-vision software (Leesburg, VA, USA).

NTG conditioning consisted of three phases. For the pre-conditioning phase, on day 1, naïve mice were allowed to freely explore the apparatus for 15 min. Based on the results of this preconditioning, the drug-paired chambers were assigned in a counterbalanced and unbiased manner to saline and NTG groups. During the conditioning phase, on days 2, 4, 6 and 8, animals were injected with saline and restricted to one of the conditioning chambers 1 h 50 min to 2 h 10 min post-injection. On days 3, 5, 7 and 9, mice were injected with saline (VEH group) or 10 mg·kg^{-1} i.p. NTG (NTG group), and were restricted to the other conditioning chamber 1 h 50 min to 2 h 10 min post-injection. For experiments where animals received a second treatment of vehicle (0.9% saline) or SNC80 (10 mg·kg^{-1} SNC80 i.p.), these compounds were administered 35 min prior to the conditioning session. For the post-conditioning test phase, on day 10, animals were allowed free access to both chambers in a drug/hyperalgeisa-free state and the time spent in each chamber was recorded (PAIN/DRUG-FREE). On day 11, animals were given their assigned drug treatments and given free access to the entire chamber as a test of state-dependent aversion (STATE-DEPENDENT).

Cortical spreading depression

Mice were randomly assigned to treatment or control groups. Each animal was transferred to the surgical area and weighed prior to the start of the experiment. Gas mixture of 2:1 nitrogen to oxygen was used throughout the duration of the experiment. Anaesthesia was induced with 5% isoflurane, which was immediately lowered to ~2% for the placement into the stereotaxic frame and the surgery. During recordings of cortical spreading depression (CSD), isoflurane was maintained at ~1.5%. To insure anaesthesia level was stable, we waited at least 1 h between the surgery and the start of the experiment. Anaesthesia level was assessed throughout the experiment by monitoring breathing rate (80 to 110 times per minute) and response to tail pinch. Temperature was established with the rectal probe and maintained at 37 ± 0.7°C.

After induction of anaesthesia, the skin from the skull was detached and a rectangular region of ~2.5 × 3.3 mm^2 (~0.5 mm from sagittal, and ~1.4 from coronal and lamboid sutures) of the right parietal bone was thinned to transparency with a micro drill (Fine Science Tools, Inc., Foster City, CA, USA). To improve transparency, a thin layer of silicon oil was applied over the skull. The brain was illuminated with a green LED (540 nm). Images were captured with a resolution of 1040 × 1392 at 1 Hz with a High-Sensitivity USB Monochrome CCD Camera (Mightex Systems, Pleasanton, CA, USA) through a 4× objective (UPlanSApo; Olympus, Center Valley, PA, USA).

The burr hole was placed outside of and lateral to the midpoint of the thinned rectangular region. A MicroFil syringe needle with 1 M KCl was placed into the burr hole, avoiding contact with brain or bone. The initial drop of KCl stimulation was formed by using a pneumatic pico-pump with the pressure at ~20 psi. Afterward, even flow of KCl was obtained using constant holding pressure of ~0.8 psi, to maintain a pool of 1 M KCl for the duration of the experiment. This resulted in a CSD frequency of 10–14 CSDs per hour in control animals. Excess liquid was removed with tissue paper applied next to the burr hole. The first CSD was elicited within 80 s of the recording. At ~400 s, mice were injected with either saline or saline plus SNC80 (10.0 mg·kg^{-1}, administered 1 mg·mL^{-1} i.p.), and the recording was continued for an additional 3600 s, resulting in a total recording duration of 4000 s. Experiments were included in analysis only if at least two CSD events occurred during the first 400 s (prior to administration of either saline or SNC80).

Data analysis

Data are expressed as mean ± SEM. All statistical analyses were performed by Sigmastat software (Systat Software Inc., San Jose, CA, USA). For all acute pain experiments, one-way ANOVA was performed. For mutant mouse experiments, a two-way ANOVA was used, and for chronic pain experiments, a two-way repeated measures ANOVA was performed. For conditioned place aversion, CPA magnitude was defined as the difference between time spent on the treatment-paired and vehicle-paired sides on the test days, and a t-test was performed for the NTG-induced CPA experiment, while a two-way ANOVA was performed to determine the effect of δ-agonists on NTG-induced CPA. Unless otherwise noted, all experiments were further analysed using Holm–Sidak post hoc analysis.

Materials

The drugs used in these experiments were supplied as follows; ARM390 provided by AstraZeneca R&D (Montreal, Canada); isoflurane by Piramal Critical Care Inc (Bethlehem, PA); JNJ20788560 provided by Johnson & Johnson (Spring House, PA); SNC80 by Tocris (Bristol, UK); sumatriptan by Zogenix (San Diego, CA)

Results

Mechanical and thermal hyperalgesia induced by acute NTG is reversed by selective δ-agonists

NTG is a known migraine trigger in humans, and acute systemic administration of NTG has been shown to produce mechanical and heat hyperalgesia in mice that is inhibited by the anti-migraine drug sumatriptan (Bates et al., 2010; Pradhan et al., 2013). We first determined whether selective δ-agonists could reverse acute NTG-evoked hyperalgesia. Two selective δ-agonists were tested: SNC80 and ARM390. Both compounds show similar receptor binding and G protein activation properties, and produce comparable anti-hyperalgesic effects in models of inflammatory pain.
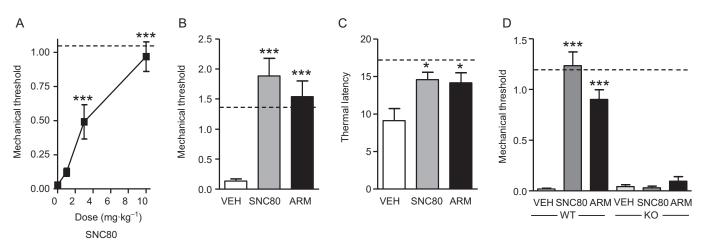
Figure 1

Acute treatment with NTG produced mechanical and thermal hyperalgesia, which was reversed by δ-opioid receptor agonists. In all cases, before NTG (10 mg·kg⁻¹, i.p.) administration, baseline mechanical or thermal responses were determined (dashed lines and below mean ± SEM). δ-Agonists were administered 1 h 15 min after NTG injection, and animals were tested 45 min later (2 h post-NTG). (A) The selective δ-agonist, SNC80, dose dependently reversed NTG-induced mechanical hyperalgesia. $n = 8$–10 per group, ***$P < 0.001$, signifcantly different from vehicle controls, one-way ANOVA. Naïve baseline (1.30 ± 0.12). At maximal doses, both SNC80 (10 mg·kg⁻¹), and another δ-agonist, ARM390 (10 mg·kg⁻¹), produced comparable reversals of NTG-induced mechanical (B, naïve baseline 1.36 ± 0.09) and thermal hyperalgesia (C, naïve baseline 17.16 ± 0.59) $n = 6$ per group, *$P < 0.05$, ***$P < 0.001$; signifcantly different from vehicle controls, one-way ANOVA. (D) Neither SNC80 nor ARM390 (10 mg·kg⁻¹) were effective in δ-opioid receptor knockout mice. $n = 4$–5 per group, ***$P < 0.001$, two-way ANOVA. Naïve baseline (1.2 ± 0.13). For mechanical responses, the Y axis indicates the 50% mechanical threshold to respond.

However, the two drugs produce distinct forms of analgesic tolerance, correlated with distinct receptor internalizing properties (see Pradhan *et al.*, 2009).

NTG (10 mg·kg⁻¹, i.p.) produced marked mechanical hypersensitivity in mice compared with baseline responses (Figure 1A, vehicle – 0 mg·kg⁻¹ vs. dashed line), and SNC80 dose dependently reversed this hyperalgesia (Figure 1A). In a separate group of animals, a maximal doses of ARM390 (10 mg·kg⁻¹) also reversed NTG-induce mechanical hyperalgesia, comparable to the effect of SNC80 (10 mg·kg⁻¹, Figure 1B). In addition, systemic administration of NTG also induced heat hyperalgesia (Figure 1C, VEH vs. dashed line), which was also reversed by both SNC80 and ARM390 (Figure 1C). We further examined the selectivity of both δ-agonists in the NTG model, by testing δ-opioid receptor wild-type and knockout mice. We found that the effects of SNC80 and ARM390 in reversing NTG-induced mechanical hyperalgesia in wild-type mice were absent in the δ-receptor knockout animals (Figure 1D). In addition, the δ-opioid receptor antagonist, naltrindole, blocked the anti-hyperalgesic effects of SNC80 and ARM390 in wild-type mice (Supporting Information Fig. S1). Thus, selective δ-opioid receptor agonists reverse sensory hypersensitivity in the NTG model of migraine pain.

δ-Opioid receptor agonists alleviate chronic NTG-evoked hyperalgesia

As with other types of pain, migraine can progress from an episodic to a chronic condition. We therefore examined the effectiveness of repeated administration of δ-opioid receptor agonists in the treatment of hyperalgesia evoked by chronic

intermittent administration of NTG. To model the progression to chronic migraine, we administered NTG (10 mg·kg⁻¹, i.p.) every second day for 9 days, resulting in a total of five NTG injections/test days. Each injection of NTG produced marked mechanical hyperalgesia, as observed in the vehicle treated groups (Figure 2A and B). Both SNC80 and ARM390 inhibited the hyperalgesia that followed each dose of NTG (Figure 2A). There appeared to be tolerance to SNC80 and ARM390 following day 1; however, this day appeared to be unusually high, and there was no significant difference of the effect of these compounds days 2–5. We also tested the novel δ-opioid receptor agonist, JNJ20788560 at a dose that was previously shown to inhibit CFA-induced hyperalgesia (Codd *et al.*, 2009). As with the other two δ-agonists, JNJ20788560 significantly inhibited the NTG-evoked hyperalgesia (Figure 2B). Similar results were observed in response to treatment with sumatriptan, a prototypic anti-migraine mediation (Figure 2C). These results indicate that δ-agonists maintain efficacy in reversing the effects of NTG, even with repeated administration.

δ-Agonists alleviate the aversive experience induced by NTG

We tested the ability of SNC80 and ARM390 to alleviate the aversive experience produced by NTG by using a conditioned place aversion paradigm. The concept underlying these experiments is that if pain/discomfort is consistently paired with a certain location, that location ultimately becomes aversive.

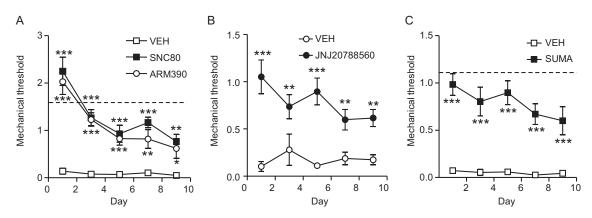We first determined whether NTG produced a conditioned place aversion. Animals were tested in a two-chamber

Figure 2

Chronic NTG-evoked hyperalgesia is attenuated by δ-opioid receptor agonists. C57Bl/6J mice were treated every second day with NTG (10 mg·kg^{-1}, i.p.) for 9 days. NTG injection produced mechanical hyperalgesia (VEH), which was significantly attenuated by SNC80 and ARM390 (A, 10 mg·kg^{-1}), JNJ20788560 (B, 30 mg·kg^{-1}) and the anti-migraine drug, sumaptriptan (C, 600 μg·kg^{-1}). n = 5–11 per group, *P < 0.05, **P < 0.01, ***P < 0.001, two-way repeated measures ANOVA. Dashed lines represent mean basal responses as determined on day 1 (1.5 ± 0.10, 1.17 ± 0.08 and 1.11 ± 0.14 for panels A, B and C respectively). The Y axis indicates the 50% mechanical threshold to respond.
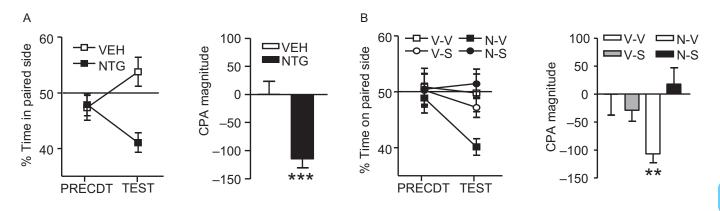

Figure 3

Chronic NTG produced conditioned place aversion, which was reversed by treatment with SNC80. Mice were tested following the conditioning phase in a state-dependent test. (A) NTG treated animals showed a clear (left panel) and significant (right panel) conditioned place aversion n = 8 per group, ***P < 0.001, Student's t-test. CPA magnitude was defined as the difference between time spent on the NTG-paired and VEH-paired sides on the test days. (B) Animals were conditioned with vehicle-vehicle (V-V), vehicle-SNC80 (10 mg·kg^{-1} i.p., V-S), NTG (10 mg·kg^{-1}, i.p.) -vehicle (N-V) or NTG-SNC80 (10 mg·kg^{-1}, N-S). In a state-dependent test, NTG produced a significant conditioned place aversion, which was reversed by SNC80 (right panel). n = 8–9 per group, **P < 0.01, two-way ANOVA. CPA magnitude was defined as the difference between time spent on the treatment-paired and VEH-VEH-paired sides on the test days.

conditioning paradigm. On the preconditioning day, no significant preference for either chamber was observed, and mice were assigned to either NTG (10 mg kg^{-1} i.p.) or vehicle treatment groups (Figure 3A left panel, PRECDT). Following the conditioning sessions, animals were tested in treatment/drug-free state, and neither an aversion nor preference was observed following chronic NTG or vehicle treatment (Supporting Information Fig. S2A). We then determined if an aversion could be observed during a state-dependent test. In this case, animals were tested following treatment with either NTG or vehicle, and given free access to both compartments. NTG-conditioned animals showed a significant aversion to the treatment-paired compartment during this state-dependent test (Figure 3A, right panel). Thus, NTG produces

a state-dependent aversion within the conditioned place aversion paradigm.

We next tested if a δ-agonist could reverse NTG-induced aversion. Again, no initial preference was observed during the preconditioning test (Figure 3B left panel, PRECDT). During the conditioning period, animals were injected twice: first with NTG or vehicle and 1.15 h later with SNC80 (10 mg·kg^{-1} i.p.) or vehicle. During the treatment-free test, neither aversion nor preference was observed in any of the treatment groups (Supporting Information Fig. S2B). However, during the state-dependent test, NTG animals subsequently treated with vehicle showed a significant conditioned place aversion, which was not observed in mice treated with NTG followed by SNC80 (Figure 3B, right panel).

CSD is inhibited by a δ-opioid receptor agonist

To investigate the effects of δ-opioid receptor agonists on mechanisms of cortical excitability underlying migraine, we quantified the effects of SNC80 on repetitive CSD evoked by continuous application of KCl, a model that has previously been shown to have predictive value for migraine preventive medications (Ayata *et al.*, 2006; Bogdanov *et al.*, 2011). CSD events were visualized as waves of optical intrinsic signals emanating from the site of KCl application. In previous studies, these were shown to be invariably associated with electrophysiological changes consistent with CSD (Brennan *et al.*, 2007a,b; Chang *et al.*, 2010). Either saline or SNC80 were injected 400 s after the initiation of KCl application, and recordings were continued for an additional 3600 s. Neither SNC80 nor saline resulted in any significant change in respiratory rate or pulse rate (consistent with previous studies).

The number of CSD events before and after administration of SNC80 was counted based on kymographs of optical intrinsic signal and verified by evaluation of original video recordings. The number of CSD events occurring in the 1 h time interval following injection of SNC80 was significantly less than the number of events that occurred in the same time period following saline (Figure 4). We did not observe any significant differences in the amplitude, duration, or propagation rate of CSD events following SNC80 versus saline injection (not shown). We also investigated if acute or chronic NTG treatment altered the frequency of repetitive CSDs evoked by continuous KCl stimulation. Previous studies indicated that acute NTG did not alter the threshold for KCl-evoked stimulation (Bates *et al.*, 2010). Consistent with this result, we did not observe any significant differences in the frequency of CSD events in mice treated acutely with NTG or chronically with NTG using the protocol described above (Supporting Information Fig. S3).
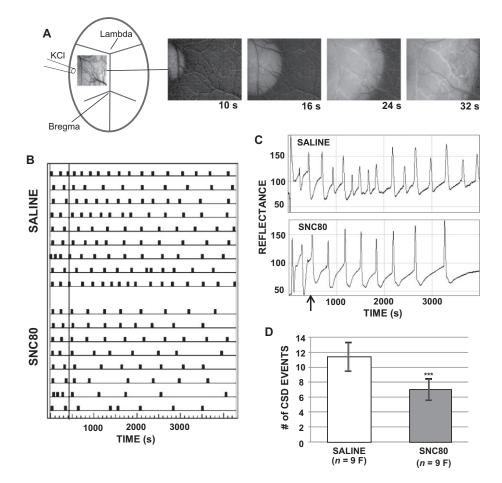

Figure 4

SNC80 inhibits CSD. (A) The schematic drawing on the left indicates the window of thinned skull through which CSD was imaged and the location of KCl injection. The image sequence on the right shows the wave of change in reflectance associated with a typical CSD event. (B) Data from all imaging experiments showing the occurrence of CSD events over time. Each horizontal line represents the results from an individual mouse, with CSD events indicated by the vertical bars. Either saline or SNC80 were injected i.p. at 400 s (indicated by vertical line), and the recording was then continued for an additional 10 min. (C) Line tracings of reflectance versus time for typical individual experiments shown in B, with injection of either saline or SNC80 at 400 s (indicated by arrow). Following injection of SNC80, there is a reduced number of CSD events as compared with following injection of saline. (D) Graph shows the average number of CSD events over 10 min following injection of saline or SNC80. Treatment with SNC80 resulted in a significant reduction in the number of CSD events ($P < 0.001$, Student's *t*-test).

Discussion

Our results show that δ-opioid receptor agonists are promising potential therapies for migraine. In an acute and chronic model of migraine pain, SNC80 and ARM390 effectively reversed hyperalgesia evoked by the known migraine trigger NTG. In addition, SNC80 prevented NTG-induced conditioned place aversion suggesting that δ-agonists may also have the potential to treat the negative emotional state induced by migraine. Furthermore, in a well-established model of migraine aura, SNC80 caused a decrease in the number of CSD events, a characteristic shared with migraine prophylactics. Together, these results make a strong case for the development of δ-agonists for the treatment of migraine.

Previous characterization of δ-agonists show that they are effective in many other models of chronic pain (Fraser et al., 2000; Cahill et al., 2003; Petrillo et al., 2003; Kabli and Cahill, 2007; Pradhan et al., 2009; 2010), and do not produce opioid-induced hyperalgesia (Pradhan et al., 2010). Furthermore, δ-opioid receptor activation positively modulates emotional tone, and δ-agonists are being tested clinically for the treatment of anxiety and depression. Considering the high level of co-morbidity between migraine and emotional disorders (Merikangas et al., 1993; Breslau et al., 1994), δ-agonists may be well positioned as novel migraine therapies.

NTG is a reliable migraine trigger in susceptible patients (Christiansen et al., 1999; Afridi et al., 2005), and NTG-evoked sensory hypersensitivity in rodents has been developed as a model for the cutaneous allodynia (Bates et al., 2010; Pradhan et al., 2013), photophobia and vasodilation of meningeal blood vessels (Greco et al., 2011; Markovics et al., 2012) associated with migraine. In addition, in a transgenic mouse model of familial migraine, mice expressing a human migraine gene showed an even greater sensitivity to NTG-evoked hyperalgesia (Brennan et al., 2013). Furthermore, the prototypic anti-migraine drug, sumatriptan, was shown to inhibit acute NTG-induced hyperalgesia (Bates et al., 2010; Pradhan et al., 2013), and we further validated its effects in our chronic NTG model. Similar to sumatriptan, three distinct δ-agonists SNC80, ARM390, and JNJ20788560, all inhibited hyperalgesia evoked by acute and chronic intermittent NTG administration. There is the possibility that the anti-hyperalgesic effects of δ-agonists may be mediated only through the spinal cord and dorsal root ganglia. However, δ-opioid receptors are expressed in numerous supraspinal regions within the pain matrix, including the trigeminal nucleus caudalis, the amygdala and the cortex (Mansour et al., 1988; Pradhan and Clarke, 2005; Pradhan et al., 2011), indicating that these receptors can regulate pain processing at a number of different levels. Importantly, the ability of SNC80 to reduce NTG-induced conditioned place aversion and CSD events suggest that δ-agonists are acting on multiple anatomical sites regulating migraine.

In this study, we tested agonists with differing abilities to induce δ-opioid receptor internalization (Pradhan et al., 2009). Both the high-internalizing agonist (SNC80) and the low internalizing agonist (ARM390) equally reduced hyperalgesia in a peripheral inflammatory pain model (Pradhan et al., 2009; 2010), and our results show that this is also the case in a model of migraine pain. These results would suggest that the anti-hyperalgesic effects of δ-agonists are due to

G-protein-dependent signalling, and not to signalling cascades activated following receptor sequestration. We have also previously shown that repeated treatment with δ-agonists results in tolerance (Pradhan et al., 2010), which would limit their use for chronic disorders. However, in those experiments, SNC80 and ARM390 were administered daily for 5 days. In this manuscript, animals were treated every second day, and tolerance was attenuated. Although we observed a drop in effectiveness following day 1, responses on this day appeared to be unusually high, and there was no significant decrease in drug effect on days 2–5. This differing response could reflect experimenter or environmental factors particular to that day, or habituation to the test apparatus following the first treatment day. Further, we did not observe tolerance to the δ-agonist JNJ20788560. Overall, it appears that giving the animals a 48 h drug-free period between injections may be sufficient to prevent adaptations that result in tolerance to δ-agonists. In addition, tolerance was not observed to δ-opioid receptor agonists following chronic treatment within other behavioural paradigms (Brandt et al., 2001; Petrillo et al., 2003; Beaudry et al., 2009; Codd et al., 2009; Pradhan et al., 2010), indicating that both the drug, the drug regimen and the disease model are important aspects to consider in the development of δ-agonists for chronic use.

We have also extended the characterization of the nociceptive effects of NTG administration by demonstrating that it conditions an aversive association from which an animal learns to avoid associated contextual cues. Measurement of the response evoked by heat or mechanical stimulation is a commonly used pain model, but this type of measurement does not consistently correlate with analgesic efficacy (Vierck et al., 2008; King et al., 2009) nor does it capture the emotional dysfunction that is characteristic of chronic pain states (Merikangas et al., 1993; Breslau et al., 1994). The conditioned place aversion assay is designed to address these issues (Hummel et al., 2008). We found that mice showed significant state-dependent place aversion following administration of NTG. State-dependent learning refers to the facilitation of a preference or aversion response during the time in which the animal is in the same state that has been used to produce the learned response (see (Tzschentke, 2007)). The δ-agonist, SNC80, abolished NTG-evoked place aversion. SNC80 alone has been previously reported to produce conditioned place preference (Longoni et al., 1998); or under other circumstances, conditioned taste aversion (Hutchinson et al., 2000). In our study, however, SNC80 (vehicle-SNC80 group, Figure 3) produced neither preference nor aversion, most likely due to differences in experimental design. There is also the possibility that the locomotor stimulant effect of SNC80 obscured the expression of conditioned place aversion to NTG. However, previous work has shown that the locomotor stimulants, morphine and cocaine, both showed enhanced conditioned place preference in state-dependent tests (Bespalov et al., 1999; Dockstader and van der Kooy, 2001; Harris et al., 2001). Together with our results, this data suggests that hyperlocomotion does not override state-dependent learning and retrieval.

Multiple migraine preventive medications with diverse pharmacological mechanisms have been shown to inhibit CSD (Ayata et al., 2006). Other studies indicate that the CSD model may only be predictive of medications effective in

migraine with aura, rather than migraine in general (Bogdanov et al., 2011). The effects of SNC80 on CSD could be the result of direct action in the cortex, on sub-cortical modulators of cortical excitability, or both. δ-Opioid receptors are widely expressed in the cortex (Mansour et al., 1988; Pradhan and Clarke, 2005; Pradhan et al., 2011), but their cortical function is unclear. Enkephalins, endogenous agonists at the δ-opioid receptor, have been reported to have both excitatory and inhibitory effects on cortical excitability and CSD in rodents (Sprick et al., 1981; Oitzl et al., 1985). Dual effects of δ-agonists on cortical excitability could be explained by direct inhibition of the cortex versus an indirect excitation mediated by inhibition of either cortical or subcortical inhibitory neurons. Regardless of the underlying mechanism, the inhibition of CSD by SNC80 in our studies suggests that δ-agonists may have migraine preventive properties, in addition to the acute therapeutic properties indicated by the NTG models.

CSD and NTG-induced behaviours likely represent distinct migraine mechanisms. In this study, and in another (Bates et al., 2010), NTG did not alter CSD measurements. CSD is a well-established model of migraine aura, a condition experienced only by a subset of migraineurs. NTG administration to patients with migraine, even those who have migraine with aura, does not typically induce aura. It is therefore not necessarily surprising that NTG does not alter CSD propensity, and we suggest that NTG activates migraine pain pathways through mechanisms that are distinct from changes in cortical excitability that cause migraine aura. Migraine is a multidimensional phenomenon that affects multiple brain and peripheral regions (Charles, 2013).

μ-Opioid receptor agonists such as codeine, hydrocodone, oxycodone, meperidine and morphine, are commonly used in North America as 'rescue' therapy for migraine (Bigal and Lipton, 2009). In Europe and elsewhere in the world, the use of μ-agonists for migraine has been significantly curtailed because of the recognition that these medications not only have relatively poor efficacy as acute migraine therapies, but that they also have significant abuse potential and can play a role in progression of migraine to a chronic state (Evers et al., 2009). In contrast to μ-agonists, δ-agonists do not produce adverse effects such as respiratory suppression, sedation or reduction in gastric motility (Gallantine and Meert, 2005; see Pradhan et al., 2011). To date, there are no human studies examining the abuse liability of δ-agonists. In non-human primates, however, δ-agonists appear to have low abuse liability as SNC80 is not self-administered (Negus et al., 1998; Stevenson et al., 2005) nor does it cause dependence (Brandt et al., 2001). In addition, in a rodent model of abuse liability, SNC80 did not alter intracranial self-stimulation responses (Do Carmo et al., 2009). δ-Agonists are therefore an appealing alternative to the μ-agonists currently being used to treat migraine.

There is a significant need for new acute and preventive treatments for migraine, and there is an additional need for better translational models to identify novel treatments. Given the complexity and heterogeneity of migraine, it is unlikely that any single model will adequately predict efficacy of therapies. Our studies using several behavioural and physiological models identify the δ-opioid receptor as a promising therapeutic target for migraine. Several δ-opioid receptor compounds are currently in development for clinical use and our results indicate that clinical trials of these compounds are warranted for this highly prevalent and frequently disabling condition.

Acknowledgements

Conflicts of interest

The authors declare no conflicts of interest.

References

Afridi SK, Matharu MS, Lee L, Kaube H, Friston KJ, Frackowiak RS et al. (2005). A PET study exploring the laterality of brainstem activation in migraine using glyceryl trinitrate. Brain 128 (Pt 4): 932–939.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M et al. (2013). The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Ayata C, Jin H, Kudo C, Dalkara T, Moskowitz MA (2006). Suppression of cortical spreading depression in migraine prophylaxis. Ann Neurol 59: 652–661.

Bates EA, Nikai T, Brennan KC, Fu YH, Charles AC, Basbaum AI et al. (2010). Sumatriptan alleviates nitroglycerin-induced mechanical and thermal allodynia in mice. Cephalalgia 30: 170–178.

Beaudry H, Proteau-Gagne A, Li S, Dory Y, Chavkin C, Gendron L (2009). Differential noxious and motor tolerance of chronic delta opioid receptor agonists in rodents. Neuroscience 161: 381–391.

Bespalov AY, Tokarz ME, Bowen SE, Balster RL, Beardsley PM (1999). Effects of test conditions on the outcome of place conditioning with morphine and naltrexone in mice. Psychopharmacology (Berl) 141: 118–122.

Bigal ME, Lipton RB (2008). Excessive acute migraine medication use and migraine progression. Neurology 71: 1821–1828.

Bigal ME, Lipton RB (2009). Excessive opioid use and the development of chronic migraine. Pain 142: 179–182.

Bigal ME, Serrano D, Reed M, Lipton RB (2008). Chronic migraine in the population: burden, diagnosis, and satisfaction with treatment. Neurology 71: 559–566.

Bogdanov VB, Multon S, Chauvel V, Bogdanova OV, Prodanov D, Makarchuk MY *et al.* (2011). Migraine preventive drugs differentially affect cortical spreading depression in rat. Neurobiol Dis 41: 430–435.

Brandt MR, Furness MS, Rice KC, Fischer BD, Negus SS (2001). Studies of tolerance and dependence with the delta-opioid agonist SNC80 in rhesus monkeys responding under a schedule of food presentation. J Pharmacol Exp Ther 299: 629–637.

Brennan KC, Beltran-Parrazal L, Lopez-Valdes HE, Theriot J, Toga AW, Charles AC (2007a). Distinct vascular conduction with cortical spreading depression. J Neurophysiol 97: 4143–4151.

Brennan KC, Romero RM, Lopez Valdes HE, Arnold AP, Charles AC (2007b). Reduced threshold for cortical spreading depression in female mice. Ann Neurol 61: 603–606.

Brennan KC, Bates EA, Shapiro RE, Zyuzin J, Hallows WC, Huang Y *et al.* (2013). Casein kinase idelta mutations in familial migraine and advanced sleep phase. Sci Transl Med 5: 183ra156.

Breslau N, Merikangas K, Bowden CL (1994). Comorbidity of migraine and major affective disorders. Neurology 44 (10 Suppl. 7): S17–S22.

Cahill CM, Morinville A, Hoffert C, O'Donnell D, Beaudet A (2003). Up-regulation and trafficking of delta opioid receptor in a model of chronic inflammation: implications for pain control. Pain 101: 199–208.

Chang JC, Shook LL, Biag J, Nguyen EN, Toga AW, Charles AC *et al.* (2010). Biphasic direct current shift, haemoglobin desaturation and neurovascular uncoupling in cortical spreading depression. Brain 133 (Pt 4): 996–1012.

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

Charles A (2013). Migraine: a brain state. Curr Opin Neurol 26: 235–239.

Christiansen I, Thomsen LL, Daugaard D, Ulrich V, Olesen J (1999). Glyceryl trinitrate induces attacks of migraine without aura in sufferers of migraine with aura. Cephalalgia 19: 660–667.

Codd EE, Carson JR, Colburn RW, Stone DJ, Van Besien CR, Zhang SP *et al.* (2009). JNJ-20788560 [9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide], a selective delta opioid receptor agonist, is a potent and efficacious antihyperalgesic agent that does not produce respiratory depression, pharmacologic tolerance, or physical dependence. J Pharmacol Exp Ther 329: 241–251.

Do Carmo GP, Folk JE, Rice KC, Chartoff E, Carlezon WA Jr, Negus SS (2009). The selective non-peptidic delta opioid agonist SNC80 does not facilitate intracranial self-stimulation in rats. Eur J Pharmacol 604: 58–65.

Dockstader CL, van der Kooy D (2001). Mouse strain differences in opiate reward learning are explained by differences in anxiety, not reward or learning. J Neurosci 21: 9077–9081.

Evers S, Afra J, Frese A, Goadsby PJ, Linde M, May A *et al.* (2009). EFNS guideline on the drug treatment of migraine – revised report of an EFNS task force. Eur J Neurol 16: 968–981.

Filliol D, Ghozland S, Chluba J, Martin M, Matthes HW, Simonin F *et al.* (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. Nat Genet 25: 195–200.

Fraser GL, Gaudreau GA, Clarke PB, Menard DP, Perkins MN (2000). Antihyperalgesic effects of delta opioid agonists in a rat model of chronic inflammation. Br J Pharmacol 129: 1668–1672.

Gallantine EL, Meert TF (2005). A comparison of the antinociceptive and adverse effects of the mu-opioid agonist morphine and the delta-opioid agonist SNC80. Basic Clin Pharmacol Toxicol 97: 39–51.

Gaveriaux-Ruff C, Nozaki C, Nadal X, Hever XC, Weibel R, Matifas A *et al.* (2011). Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. Pain 125: 1238–1248.

Greco R, Meazza C, Mangione AS, Allena M, Bolla M, Amantea D *et al.* (2011). Temporal profile of vascular changes induced by systemic nitroglycerin in the meningeal and cortical districts. Cephalalgia 31: 190–198.

Harris GC, Altomare K, Aston-Jones G (2001). Preference for a cocaine-associated environment is attenuated by augmented accumbal serotonin in cocaine withdrawn rats. Psychopharmacology (Berl) 156: 14–22.

Hummel M, Lu P, Cummons TA, Whiteside GT (2008). The persistence of a long-term negative affective state following the induction of either acute or chronic pain. Pain 140: 436–445.

Hutchinson AC, Simpson GR, Randall JF, Zhang X, Calderon SN, Rice KC *et al.* (2000). Assessment of SNC 80 and naltrindole within a conditioned taste aversion design. Pharmacol Biochem Behav 66: 779–787.

Kabli N, Cahill CM (2007). Anti-allodynic effects of peripheral delta opioid receptors in neuropathic pain. Pain 127: 84–93.

King T, Vera-Portocarrero L, Gutierrez T, Vanderah TW, Dussor G, Lai J *et al.* (2009). Unmasking the tonic-aversive state in neuropathic pain. Nat Neurosci 12: 1364–1366.

Konig M, Zimmer AM, Steiner H, Holmes PV, Crawley JN, Brownstein MJ *et al.* (1996). Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. Nature 383: 535–538.

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.

Longoni R, Cadoni C, Mulas A, Di Chiara G, Spina L (1998). Dopamine-dependent behavioural stimulation by non-peptide delta opioids BW373U86 and SNC 80: 2. Place-preference and brain microdialysis studies in rats. Behav Pharmacol 9: 9–14.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ (1988). Anatomy of CNS opioid receptors. Trends Neurosci 11: 308–314.

Markovics A, Kormos V, Gaszner B, Lashgarara A, Szoke E, Sandor K *et al.* (2012). Pituitary adenylate cyclase-activating polypeptide plays a key role in nitroglycerol-induced trigeminovascular activation in mice. Neurobiol Dis 45: 633–644.

Merikangas KR, Merikangas JR, Angst J (1993). Headache syndromes and psychiatric disorders: association and familial transmission. J Psychiatr Res 27: 197–210.

Negus SS, Butelman ER, Chang KJ, DeCosta B, Winger G, Woods JH (1994). Behavioral effects of the systemically active delta opioid agonist BW373U86 in rhesus monkeys. J Pharmacol Exp Ther 270: 1025–1034.

Negus SS, Gatch MB, Mello NK, Zhang X, Rice K (1998). Behavioral effects of the delta-selective opioid agonist SNC80 and related compounds in rhesus monkeys. J Pharmacol Exp Ther 286: 362–375.

Oitzl MS, Koroleva VI, Bures J (1985). D-ala2-Metenkephalinamide blocks the synaptically elicited cortical spreading depression in rats. Experientia 41: 625–627.

Perrine SA, Hoshaw BA, Unterwald EM (2006). Delta opioid receptor ligands modulate anxiety-like behaviors in the rat. Br J Pharmacol 147: 864–872.

Petrillo P, Angelici O, Bingham S, Ficalora G, Garnier M, Zaratin PF *et al.* (2003). Evidence for a selective role of the delta-opioid agonist [8R-(4bS*,8aalpha,8abeta, 12bbeta)]7,10-Dimethyl-1-methoxy-11-(2-methylpropyl)oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzofuro[3,2-e]pyrrolo[2,3-g]iso quinoline hydrochloride (SB-235863) in blocking hyperalgesia associated with inflammatory and neuropathic pain responses. J Pharmacol Exp Ther 307: 1079–1089.

Pradhan AA, Clarke PB (2005). Comparison between delta-opioid receptor functional response and autoradiographic labeling in rat brain and spinal cord. J Comp Neurol 481: 416–426.

Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A *et al.* (2009). In vivo delta opioid receptor internalization controls behavioral effects of agonists. PLoS ONE 4: e5425.

Pradhan AA, Walwyn W, Nozaki C, Filliol D, Erbs E, Matifas A *et al.* (2010). Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance. J Neurosci 30: 16459–16468.

Pradhan AA, Befort K, Nozaki C, Gaveriaux-Ruff C, Kieffer BL (2011). The delta opioid receptor: an evolving target for the treatment of brain disorders. Trends Pharmacol Sci 32: 581–590.

Pradhan AA, Smith ML, McGuire B, Tarash I, Evans CJ, Charles A (2013). Characterization of a Novel Model of Chronic Migraine. Pain 155: 269–274.

Saitoh A, Kimura Y, Suzuki T, Kawai K, Nagase H, Kamei J (2004). Potential anxiolytic and antidepressant-like activities of SNC80, a selective delta-opioid agonist, in behavioral models in rodents. J Pharmacol Sci 95: 374–380.

Silberstein SD, Dodick D, Freitag F, Pearlman SH, Hahn SR, Scher AI *et al.* (2007). Pharmacological approaches to managing migraine and associated comorbidities – clinical considerations for monotherapy versus polytherapy. Headache 47: 585–599.

Sprick U, Oitzl MS, Ornstein K, Huston JP (1981). Spreading depression induced by microinjection of enkephalins into the hippocampus and neocortex. Brain Res 210: 243–252.

Stevenson GW, Folk JE, Rice KC, Negus SS (2005). Interactions between delta and mu opioid agonists in assays of schedule-controlled responding, thermal nociception, drug self-administration, and drug versus food choice in rhesus monkeys: studies with SNC80 [(+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenz yl]-N,N-diethylbenzamide] and heroin. J Pharmacol Exp Ther 314: 221–231.

Stewart WF, Wood C, Reed ML, Roy J, Lipton RB (2008). Cumulative lifetime migraine incidence in women and men. Cephalalgia 28: 1170–1178.

Stovner L, Hagen K, Jensen R, Katsarava Z, Lipton R, Scher A *et al.* (2007). The global burden of headache: a documentation of headache prevalence and disability worldwide. Cephalalgia 27: 193–210.

Tzschentke TM (2007). Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict Biol 12: 227–462.

Victor TW, Hu X, Campbell JC, Buse DC, Lipton RB (2010). Migraine prevalence by age and sex in the United States: a life-span study. Cephalalgia 30: 1065–1072.

Vierck CJ, Hansson PT, Yezierski RP (2008). Clinical and pre-clinical pain assessment: are we measuring the same thing? Pain 135: 7–10.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12591

Figure S1 The anti-hyperalgesic effects of SNC80 and ARM390 were blocked by the δ-antagonist, naltrindole. NTG (10 mg·kg⁻¹ i.p.) was injected immediately after baseline mechanical responses were determined (dashed lines, 1.07 ± 0.11), and 15 min later mice were injected with vehicle (V) or naltrindole (N, 10 mg·kg⁻¹ s.c.). SNC80 (S) or ARM390 (A) (10 mg·kg⁻¹) was administered 1 h 15 min post-NTG and mice were injected 45 min later. n = 4–6 mice per group, *$P <$ 0.05, **$P <$ 0.01, t-test with Bonferroni correction. SNC80 and ARM390 are selective for the δ-opioid receptor.

Figure S2 Chronic NTG did not produce a significant condition place aversion in a treatment-free test. Mice were tested following the conditioning phase treatment-free. (A) In a treatment-free test, conditioning with vehicle or NTG (10 mg·kg⁻¹, i.p.) did not produce a significant change in % time spent on the paired side (left panel) or conditioned place aversion (CPA) magnitude (right panel). CPA magnitude was defined as the difference between time spent on the NTG-paired and VEH-paired sides on the test days. (B) In a treatment-free test, conditioning with vehiclevehicle (V-V), vehicle-SNC80 (10 mg·kg⁻¹ i.p., V-S), NTG (10 mg·kg⁻¹, i.p.) -vehicle (N-V) or NTGSNC80 (10 mg·kg⁻¹, N-S) did not produce a significant change in % time spent on the paired side (left panel) or CPA magnitude (right panel). CPA magnitude was defined as the difference between time spent on the treatment-paired and VEH-VEH-paired sides on the test days.

Figure S3 Graph shows the average number of CSD events over 10 min in saline-treated mice versus mice treated with chronic nitroglycerin (10 mg·kg⁻¹ i.p.) over 9 days (as in hyperalgesia assays) versus acute nitroglycerin (10 mg kg⁻¹ i.p.) during an imaging experiment. There was no significant difference between the number of CSD events under these conditions.