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RESEARCH PAPER

In vivo properties of KNT-127, a novel δ opioid receptor agonist: receptor internalization, antihyperalgesia and antidepressant effects in mice

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BACKGROUND AND PURPOSE

Activation of δ opioid (DOP) receptors regulates pain and emotional responses, and also displays ligand-biased agonism. KNT-127 (1,2,3,4,4a,5,12,12a-octahydro-2-methyl-4aβ,1β-([1,2]benzenomethano)-2,6-diazanaphthacene-12aβ,17-diol) is a novel DOP receptor agonist inducing analgesia and antidepressant effects in mice. Here, we have assessed KNT-127 for (i) analgesia against chronic inflammatory pain; (ii) effects on depression, locomotion and DOP receptor internalization; and (iii) for cross-tolerance to analgesic and antidepressant effects of acute treatment by other DOP receptor agonists.

EXPERIMENTAL APPROACH

Inflammatory pain was induced by complete Freund's adjuvant injection into tail or hindpaw, and thermal and mechanical sensitivities were determined in mice. Locomotor and antidepressant-like effects were measured using actimetry and forced swim test respectively. *In vivo* KNT-127 selectivity and internalization were assessed using DOP receptor knockout mice and knock-in mice expressing fluorescent-tagged DOP receptors. KNT-127 was injected acutely at 0.1–10.0 mg·kg^{-1} or administered chronically at 5 mg·kg^{-1} daily over 5 days.

KEY RESULTS

Acute treatment with KNT-127 reversed inflammatory hyperalgesia, produced an antidepressant-like effect but induced neither hyperlocomotion nor receptor sequestration. Chronic treatment with KNT-127 induced tolerance and cross-tolerance to SNC80-induced analgesia, but no tolerance to SNC80-evoked hyperlocomotor or antidepressant-like effects.

CONCLUSIONS AND IMPLICATIONS

The DOP receptor agonist KNT-127 induced agonist-specific acute and chronic responses, at both behavioural and cellular levels. It displays activities similar to the other recently reported DOP agonists, AR-M1000390, ADL5747 and ADL5859, and differs from SNC80. SNC80 differs from the other DOP receptor agonists including KNT-127, by exhibiting ligand-biased tolerance at this receptor.

Abbreviations

ADL5747, N,N-diethyl-3-hydroxy-4-(spiro[chromene-2,4′-piperidine]-4-yl)benzamide; ADL5859, N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4′-piperidine]-4-yl) benzamide; AR-M1000390, N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide; CFA, complete Freund's adjuvant; DOP, δ opioid; DOP–eGFP, mouse line with functional fluorescent-tagged δ opioid receptors; DRG, dorsal root ganglia; KNT-127, 1,2,3,4,4a,5,12,12a-octahydro-2-methyl-4aβ,1β-([1,2]benzenomethano)-2,6-diazanaphthacene-12aβ,17-diol; SNC80, 4-[(R)-[(2S,5R)-4-allyl-2,5-dimethyl-piperazin-1-yl]-(3-methoxyphenyl)methyl]-N,N-diethyl-benzamide

Tables of Links

TARGETS	LIGANDS
DOP receptor	SNC80
KOP receptor	
MOP receptor	

These Tables lists key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

GPCRs are the largest family of membrane receptors (Lagerstrom and Schioth, 2008) and are therapeutically essential, representing targets for 50% of marketed drugs. Opioid receptors are GPCRs classified into three subgroups, μ, δ and κ receptors. These receptors are abundantly expressed in the nervous system and regulate numerous physiological processes such as pain, emotions and reward. The δ opioid (DOP) receptors play roles highly distinct from those of μ (MOP) or κ (KOP) receptors (Pradhan *et al.*, 2011), and represent promising targets for the treatment of chronic pain (Gaveriaux-Ruff and Kieffer, 2011) without abuse liability (Chu Sin Chung and Kieffer, 2013), considered as a hallmark of MOP receptor agonists (Lutz and Kieffer, 2013). In dorsal root ganglia (DRGs) containing cell bodies of somatosensory neurons, DOP receptors are expressed mainly in large and medium diameter neurons with some small and medium diameter neurons co-expressing both DOP and MOP receptors (Scherrer *et al.*, 2009; Wang *et al.*, 2010; Gaveriaux-Ruff and Kieffer, 2011; Bardoni *et al.*, 2014). Additionally, DOP receptors have emerged as potential targets for the treatment of several neurological and psychiatric diseases (Chu Sin Chung and Kieffer, 2013), including notably mood disorders.

It was previously demonstrated that distinct agonists acting at the DOP receptor engage a different set of physiological responses at cellular and behavioural levels, a concept known as biased agonism in the GPCR field (Pradhan *et al.*, 2009; 2010; 2012; Audet and Bouvier, 2012; Audet *et al.*, 2012; Tudashki *et al.*, 2014). Specifically, we used a knock-in mouse line expressing functional fluorescent-tagged DOP receptor (DOP–eGFP; Scherrer *et al.*, 2006) to show that DOP receptor agonists may show similar binding properties but different receptor internalization potencies *in vivo*. Thus, systemic administration of SNC80 (4-[(R)-[(2S,5R)-4-allyl-2,5-dimethyl-piperazin-1-yl]-(3-methoxyphenyl)methyl]-N,N-diethyl-benzamide), considered as the prototypic DOP receptor agonist, induced robust receptor internalization in regions of the nervous system where DOP–eGFP receptor is highly visible (striatum, hypothalamus, spinal cord and DRGs) whereas AR-M1000390 (N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide) (Pradhan *et al.*, 2009), ADL5747 (N,N-diethyl-3-hydroxy-4-(spiro[chromene-2,4′-piperidine]-4-yl)benzamide) and ADL5859 (N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4′-piperidine]-4-yl) benzamide) (Nozaki *et al.*, 2012) were unable to trigger receptor internalization in these tissues. Further, only SNC80 produced locomotor activation in these animals at doses where all the compounds efficiently reduced inflammatory pain (Scherrer *et al.*, 2006; Pradhan *et al.*, 2009; 2010; Nozaki *et al.*, 2012). Finally, we demonstrated that chronic treatment with either internalizing or non-internalizing agonist leads to two distinct forms of tolerance that appeared generalized or pain specific respectively (Pradhan *et al.*, 2010). Other studies have also reported differential effects of DOP receptor agonists (Aguila *et al.*, 2012; Audet and Bouvier, 2012; Audet *et al.*, 2012; Pradhan *et al.*, 2012). Altogether, accumulating data lead to categorize both existing and novel drugs targeting the DOP receptor, in order to better predict *in vivo* effects and utility of δ drugs *in vivo*.

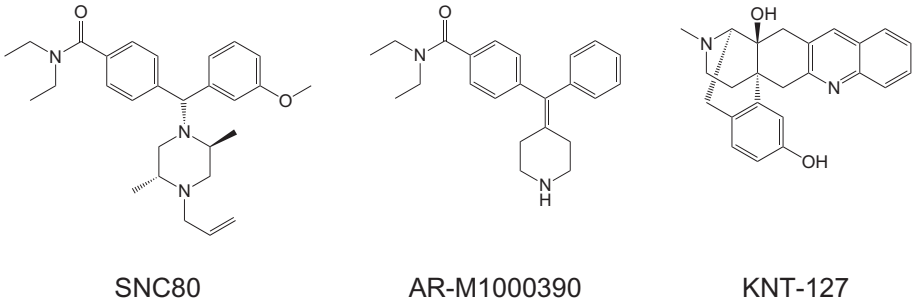

Figure 1
Chemical structures of SNC80, AR-M1000390 and KNT-127.

KNT-127 (1,2,3,4,4a,5,12,12a-octahydro-2-methyl-4aβ,1β-([1,2]benzenomethano)-2,6-diazanaphthacene-12aβ,17-diol) is a DOP receptor agonist recently produced by Nagase *et al.* (2010) (Figure 1) with a chemical structure different from those of other non-peptidic DOP receptor agonists. The compound shows high *in vitro* affinity for the DOP (K_i = 0.16 nM) and low affinity for MOP and KOP receptors (K_i = 21.3 and 153 nM each), indicating a two-order magnitude for DOP receptor selectivity. KNT-127 also induces a strong analgesia in mouse chemical pain assays (Saitoh *et al.*, 2011). Finally, systemic KNT-127 induced antidepressant effects comparable with the clinically used tricyclic antidepressant imipramine (Saitoh *et al.*, 2011; Saitoh and Yamada, 2013) or other DOP receptor agonists (Pradhan *et al.*, 2011; Chu Sin Chung and Kieffer, 2013). Interestingly, KNT-127 did not induce any convulsion or locomotor activation typically observed in SNC80-treated mice (Saitoh *et al.*, 2011), nor did it produce amnesia or coordination deficits (Saitoh and Yamada, 2013).

The purpose of this study was to classify KNT-127 among DOP receptor agonists, as we had previously performed for AR-M1000390 (Pradhan *et al.*, 2009; 2010), ADL5747 and ADL5859 (Nozaki *et al.*, 2012). To this aim, we compared *in vivo* effects of SNC80 and KNT-127 in DOP receptor mouse mutants at both cellular and behavioural levels. We found that, as for AR-M1000390 and ADL compounds, KNT-127 produced strong antihyperalgesic and antidepressant effects but did not induce receptor internalization in striatum, hippocampus, spinal cord or DRG neurons nor locomotor activation. The prototypical DOP receptor agonist SNC80, therefore, appears as a unique agonist compared with several more recently developed compounds.

Methods

Animals

All experimental procedures and animal husbandry were carried out in accordance with the European Communities Council Directive of 22 September 2010 (directive 2010/63/UE), with the guidelines of the Committee for Research and Ethical issues of IASP published in *Pain*, 1983; 16:109–110, and were approved by the local ethical committee (Com'Eth, Comité d'Ethique pour l'Expérimentation Animale IGBMC-ICS, license no. 2010-003). All studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Particular efforts were made to minimize the number of mice and the pain they experienced. A total of 217 animals were used in the experiments described here.

Mice were bred and maintained at IGBMC, housed in a temperature (21 ± 1°C) and humidity (55 ± 10%) controlled room with a 12 h light:12 h dark cycle (light on between 08:00 and 20:00 h). Food and water were available *ad libitum* except during behavioural observations. Mice were habituated to their new experimental environment and handled for 1 week before starting the experiments. Different groups of male and female DOP–eGFP mice (50% C57BL/6J-50% SV129Pas) (Scherrer *et al.*, 2006), DOP receptor knockout mice (50% C57BL/6J-50% SV129Pas) (Filliol *et al.*, 2000) or male C57BL/6J mice (Charles River, L'Arbresle, France) weighing 23–29 g at the beginning of the experiments were used. Animals were randomly assigned to experimental groups and blinded for genotype and treatment.

Induction of inflammatory pain

Complete Freund's adjuvant (CFA) was used to induce the inflammatory pain on the hindpaw or tail of mice. Hindpaw and tail CFA models were used to evaluate the thermal and mechanical analgesic tolerance of KNT-127 in DOP–eGFP mice. Tail CFA mice were used to examine the effect of KNT-127 on emotional behaviour in analgesia-tolerated animals. Baseline nociceptive thresholds were measured before CFA injection (dashed lines on Figures 2–4). Following a previous report (Pradhan *et al.*, 2010), 15 or 20 μL of CFA was injected s.c. into the plantar surface of the left hindpaw or 3 cm from the tip of the tail under inhalation anaesthetic (2.5% isoflurane) respectively. Pain testing was conducted at 48 h after CFA injection (Figure 3) and every day from day 1 to day 6 (Figure 4).

Behavioural assessment

The nociceptive thresholds, locomotor activity and depressive-like behaviour were evaluated throughout the present study. All experiments were performed between 9:00 and 15:00 h, without knowledge of the treatment groups. In all cases, animals were habituated to the testing area for 20 min before the testing. Separate groups of mice were used for each end point.

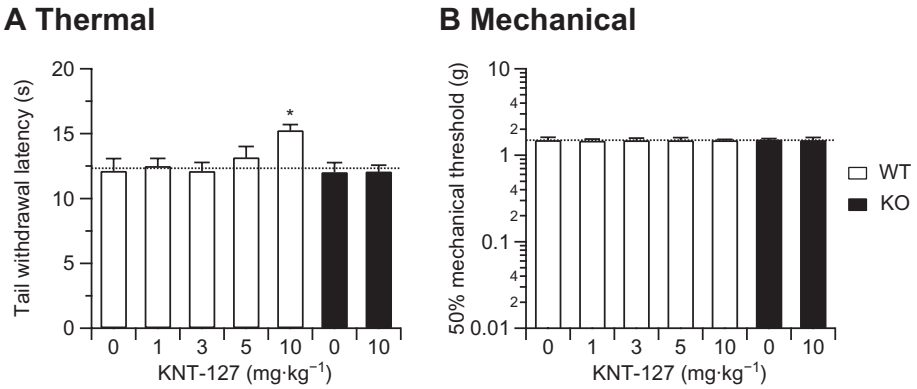

Figure 2

KNT-127 induced antinociception in naïve mice. Nociceptive thresholds were determined in WT or DOP receptor KO mice, by the tail immersion test for thermal sensitivity (A) or von Frey test for mechanical sensitivity (B), 30 min after saline or KNT-127 administration. Broken lines indicate basal nociceptive thresholds before CFA injection. KNT-127 induced slight but significant inhibition with dose of 10 mg·kg^{-1} on thermal nociception, whereas it showed no effect on tactile stimulation. Further, analgesic effect of high-dose KNT-127 was abolished in KO mice. Data are expressed as means ± SEM of eight to nine mice per group. *$P < 0.05$, significant effects of drug treatments; two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test.

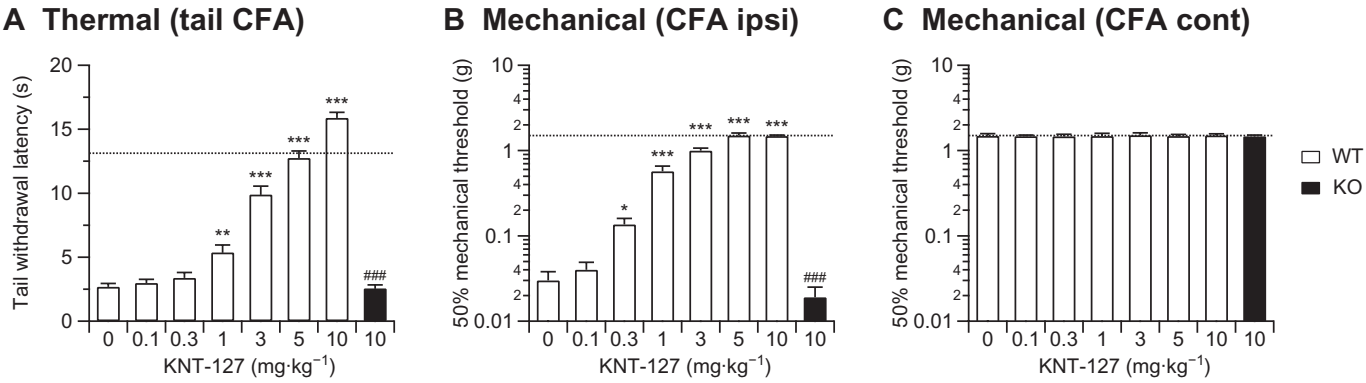

Figure 3

KNT-127 induced antihyperalgesia in CFA-inflamed mice. CFA was injected in tail or plantar surface of hindpaw. Nociceptive thresholds were determined in WT or DOP receptor KO mice, by the tail immersion test for thermal sensitivity (A) or von Frey test for mechanical sensitivity (B, CFA-treated ipsilateral paw; C, contralateral paw), 30 min after saline or KNT-127 administration. Broken lines indicate basal nociceptive thresholds before CFA injection. KNT-127 significantly and dose dependently reversed the CFA-induced thermal and mechanical hypersensitivity in WT mice, which was abolished in KO mice. Data are expressed as means ± SEM of eight to nine mice per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant effect of drug treatments; ###$P < 0.001$, significantly different from WT mice; two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test.

Tail immersion test. Thermal sensitivity was measured by immersing the tail (5 cm from the tip) into a water bath at 46°C. Each individual mouse was lightly restrained in a 50 mL cylinder and habituated twice daily from 3 days prior to testing. Tail withdrawal latencies were determined, and a cut-off of 30 s was established.

von Frey test. von Frey filament with up-down method (Chaplan *et al.*, 1994) was used to assess the mechanical sensitivity. In this test, the hindpaw plantar surface was gently probed with a series of eight von Frey filaments with logarithmically incremental stiffness (0.008, 0.04, 0.07, 0.16, 0.40, 0.60, 1.00 and 2.00 g) (Stoelting, Wood Dale, IL, USA). Stimuli were presented by probing at intervals of 5–10 s, and sharp withdrawal or flinching was indicated as the positive response. The threshold of response (50% mechanical threshold) was calculated using the up-down Excel program based on the equation formula described before (Chaplan *et al.*, 1994), generously provided by Allan Basbaum's laboratory (UCSF, San Francisco, CA, USA).

Locomotor activity. Locomotor activity was determined during exploratory behaviour in the acrylic cage (21 × 11 × 17 cm) on an actimetry platform. Assessment of locomotor activity was carried out for 1 h pre-injection (habituation to the cage) and 2 h post-injection, and locomotion (total distance of the movement) was measured in 5 min windows automatically by the video camera with the automatic

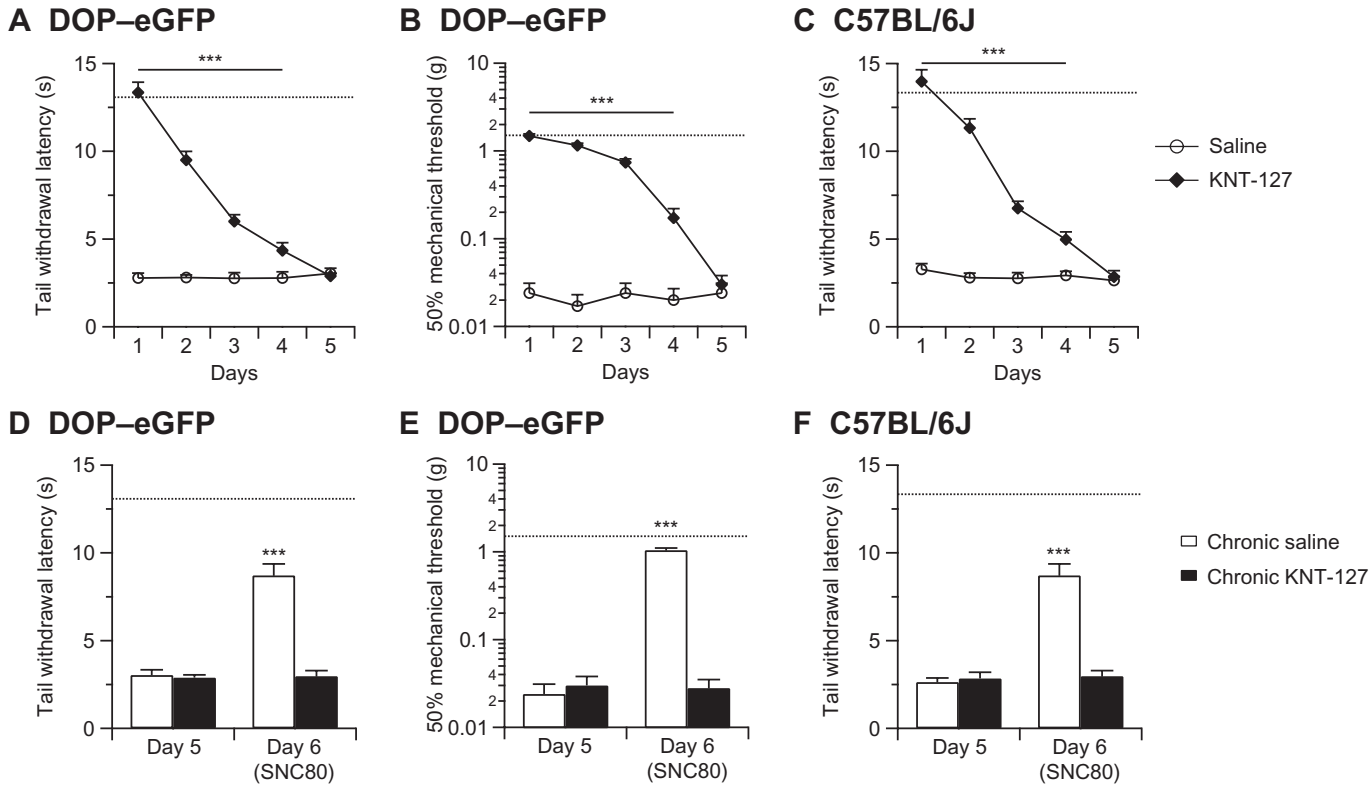

Figure 4

Chronic KNT-127 administration produces analgesic tolerance. Inflammatory pain was induced by CFA injection to tail (A and C) or hindpaw (B) in DOP–eGFP (A and B) or C57BL/6J mice (C). Mice received saline control or KNT-127 (5 mg·kg^{-1}, s.c.) for 5 days after the induction of CFA inflammatory pain. Thermal (A and C) or mechanical (B) sensitivity was determined 30 min after daily injection. Broken lines represent baseline thermal or mechanical nociceptive thresholds before CFA injection. After 5 days of treatment, KNT-127 produced full analgesic tolerance independent of mouse strain or nociceptive end point. Data are expressed as means ± SEM of seven to eight mice per group. A ***P < 0.001, significantly different from vehicle-treated group; one-way ANOVA followed by Bonferroni *post hoc* test. The acute analgesic effect of SNC80 (10 mg·kg^{-1}, i.p.) was determined at day 6 on chronic 5 day treated KNT-127 or saline control mice. Both tolerance and cross-tolerance on thermal (D, F) and mechanical (E) sensitivity in DOP–eGFP mice (D, E) or C57BL/6J mice (F) are shown. Broken lines represent baseline thermal or mechanical nociceptive thresholds before CFA injection. Acute SNC80 administration on day 6 did not produce any analgesia in KNT-127-tolerant animals (black columns on day 6) as compared with significant analgesia in chronic saline animals (white columns), which indicates that cross-tolerance occurred, and this occurred independently of genetic background. Data are expressed as means ± SEM of seven to eight mice per group. For KNT-127, ***P < 0.001, significantly different from chronic vehicle-treated group; one-way ANOVA followed by Bonferroni *post hoc* test. For SNC80, ***P < 0.001, day 6 significantly different from day 5 for corresponding treatment group; Student's *t*-test.

tracking system during that time. To measure the effect of KNT-127 chronic treatment, mice were repeatedly tested in the tail CFA model to ensure that tolerance to KNT-127 was established before testing.

Forced swim test. Forced swim test was conducted as described (Saitoh *et al.*, 2011) to measure the antidepressant effect of DOP receptor agonists. A 5 L glass cylinder (diameter, 20 cm; height, 30 cm) filled with water (25 ± 1°C) to a depth of 20 cm was used as a swimming apparatus. Mice were trained 24 h before the measurement by putting them individually in the swimming apparatus for 10 min. Twenty-four hours later, the animals were placed in the swimming apparatus and recorded by a video camera for 10 min. Duration of immobility was measured at the last 5 min in whole recording as the test session. After each recording, the cylinder was rinsed with clean water to avoid the influence of alarm substances. The apparatus was placed in indirect light (20 lx). SNC80 (3 mg·kg^{-1}) was injected 60 min before the testing to avoid the locomotive stimulation. KNT-127 (1 mg·kg^{-1}) was injected 30 min before the testing.

Perfusion and microscopy

Mice were anaesthetized with a ketamine/xylazine mixture and intracardially perfused with 9.25% sucrose in double distilled water, followed by 4% paraformaldehyde/0.1 M phosphate buffer (PB) as described previously (Scherrer *et al.*, 2006). Perfusion was conducted 30 min (KNT-127) or 45 min (SNC80) after drug administration. Brain, spinal cord and DRGs were dissected, cryoprotected in 30% sucrose/0.1 M PB solution and cut to 30 µm thick sections in a cryostat. After mounting the sections on glass slides, DOP–eGFP receptor distribution in striatum, hippocampus, spinal cord and DRG

was observed under a Leica SP2UV confocal microscope, and the LCS (Leica) software (Leica, Wetzlar, Germany) was used for image acquisition.

Quantification of cell surface mean fluorescence intensity was determined using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). Nuclear fluorescence defined the background level. Fluorescence densities of cell surface (Df surf) and cytoplasm (Df cyto) was calculated from cell membrane or cytosolic fluorescence intensity (Scherrer *et al.*, 2006), and internalization index was obtained by the following equation: *Internalization index* = 1 – (*Df surf*/*Df cyto*). In total, two to three neurons per region per mouse were analysed, and there were four mice per group.

Data analysis

All data are presented as means ± SEM. Pharmacokinetics of drug were analysed using repeated-measures ANOVA followed by Student's *t*-test for individual time points when appropriate. The analysis of pharmacological effect was performed using two-way ANOVA for drug effect and genotype, followed by Bonferroni–Dunn test to determine statistically significant differences.

Materials

KNT-127 (Nagase *et al.*, 2010) was synthesized at Kitasato University, Japan, and administered s.c. SNC80 was purchased from Tocris Co. (Bristol, UK) and administered i.p. SNC80 was dissolved in 0.9% saline with 2 μL of 1 M HCl solution per mg SNC80. KNT-127 was dissolved in saline. The vehicle used was 0.9% saline. Acute DOP receptor agonist effects were determined 30 min after KNT-127 injection (s.c.) or 45 min after SNC80 injection (i.p.), except in the forced swim test (see above). For chronic administration, mice received the drug once a day for 5 days, starting from 1 day after inflammation induction, and tested 24 h after the latest injection.

Results

KNT-127 produces analgesia in the CFA inflammatory pain model in a DOP receptor selective manner

KNT-127 was previously shown to induce analgesia in both acute formalin and acetic acid pain models (Saitoh *et al.*, 2011). Here, we first investigated whether KNT-127 would show antinociceptive activities in thermal and mechanical pain assays. In naïve wild-type C57BL/6J (WT) mice, KNT-127 administered s.c. had no effect on mechanical sensitivity while it inhibited thermal nociception at 10 mg·kg^{-1}, indicating thermal analgesic property at this dose (Figure 2). The analgesic effects of KNT-127 against chronic inflammatory pain was then tested in the tail and hindpaw CFA models as described previously for other DOP receptor agonists (Pradhan *et al.*, 2010; Nozaki *et al.*, 2012). Administration of KNT-127 significantly reduced tail CFA-induced thermal hyperalgesia on tail at the 1–10 mg·kg^{-1} doses (Figure 3A) and mechanical hindpaw allodynia at 0.3–10 mg·kg^{-1} (Figure 3B), with best efficacy within the 5–10 mg·kg^{-1} dose range. In addition, the highest analgesic dose of KNT-127 (10 mg·kg^{-1}) had no effect in either naïve or CFA-inflamed DOP–KO mice (Figures 2 and 3), demonstrating the clear DOP receptor selectivity of KNT-127. Moreover, nociceptive thresholds were unchanged in contralateral paws following CFA administration in both WT and KO mice, and KNT-127 had no effect to the contralateral mechanical thresholds (Figure 3C). The best effective and selective 5–10 mg·kg^{-1} dose range of KNT-127 was then used for further pain experiments.

Chronic administration of KNT-127 produces full analgesic tolerance and cross-tolerance

We then examined whether KNT-127 induces analgesic tolerance and cross-tolerance with SNC80, as in our previous study comparing analgesic effects of AR-M1000390 and SNC80 (Pradhan *et al.*, 2010). DOP–eGFP mice were tested in both tail–CFA (Figure 4A) and hindpaw–CFA (Figure 4B) assays, and C57BL/6J mice were also tested using tail–CFA (Figure 4C). One day following CFA, KNT-127 was administered systemically every day for 5 days and KNT-127-evoked analgesia was determined every day by pain score measurement. For all the chronically saline-treated control groups, heat hyperalgesia and mechanical allodynia were stable over 5 days. KNT-127 (5 mg·kg^{-1}) fully reversed both thermal hyperalgesia and mechanical allodynia at first administration, and this effect gradually diminished over 5 days (Figure 4). The loss of KNT-127 antihyperalgesic effect was observed in both DOP–eGFP and WT C57BL/6J mice, and together the data indicate that tolerance to analgesic effects of KNT-127 develops independently from pain modality and mouse strain.

We further examined the analgesic effects of SNC80 in KNT-127 tolerant animals using a SNC80 (10 mg·kg^{-1}) challenge on the sixth experimental day, in all the groups. SNC80 was ineffective in reducing thermal (Figure 4D,F) or mechanical (Figure 4E) hypersensitivity in either DOP–eGFP (Figure 4D,E) or C57BL/6J (Figure 4F) mice repeatedly treated with KNT-127. This result shows that chronic KNT-127 induces *in vivo* tolerance to DOP receptor analgesia, and in different mouse genetic backgrounds, which operates for another agonist with highly differing properties.

Effect of chronic KNT-127 treatment in the forced swim depression test

Low doses of KNT-127 (0.3–1.0 mg·kg^{-1}) in mice have been reported to produce an antidepressant effect comparable with the clinically used tricyclic antidepressant imipramine (Saitoh *et al.*, 2011). To investigate whether the KNT-127 chronic treatment that produces analgesic tolerance would also induce tolerance to DOP receptor agonist-induced antidepressant action, we tested the acute effect of SNC80 (1 mg·kg^{-1}) or KNT-127 (3 mg·kg^{-1}) in mice that repeatedly received 5 mg·kg^{-1} KNT-127 or saline for 5 days. In control chronic saline-treated groups, both acute KNT-127 and SNC80 decreased immobility (Figure 5A), in accordance with previous studies (Saitoh *et al.*, 2004; 2011). Furthermore, acute SNC80 induced an increase in climbing behaviour (Figure 5B), whereas acute KNT-127 produced an elevation of swimming (Figure 5C). Although chronic KNT-127 administration did not modify acute KNT-127 effects on immobility and swimming, it moderately attenuated acute SNC80 effect on immobility and induced a change in SNC80 activity from climbing to swimming. Thus, repeated KNT-127 treatment

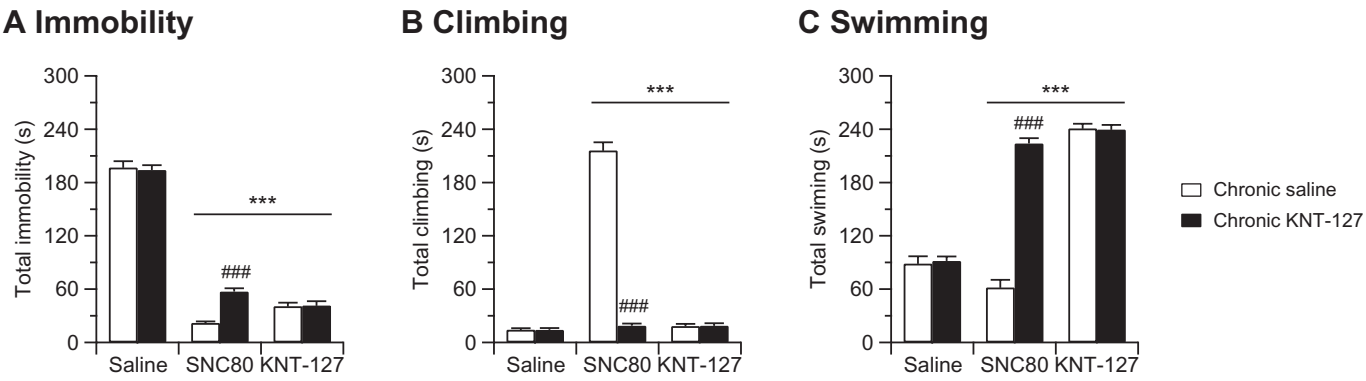

Figure 5

KNT-127 enhances swimming behaviour in the forced swim test while SNC80 increases climbing behaviour. WT C57BL/6J mice were treated with KNT-127 or vehicle for 5 days, following which they were challenged with SNC80 (3 mg·kg^{-1}, i.p.), KNT-127 (1 mg·kg^{-1}, s.c.) or saline (s.c.). Although acute administration of both SNC80 and KNT-127 drastically reduced immobility (A), SNC80 enhanced climbing (B) whereas KNT-127 increased swimming (C). Repeated KNT-127 did not modify acute KNT-127 effect on immobility and swimming, whereas it modulated acute SNC80 activity, leading to a smaller decrease in immobility and to suppression of climbing and enhancement of swimming. ***P < 0 .001 significantly different from acute saline group (left columns); ###P < 0 .001 significantly different from chronic saline-treated group; repeated-measures two-way ANOVA with Bonferroni *post hoc* test, n = 7 per group.

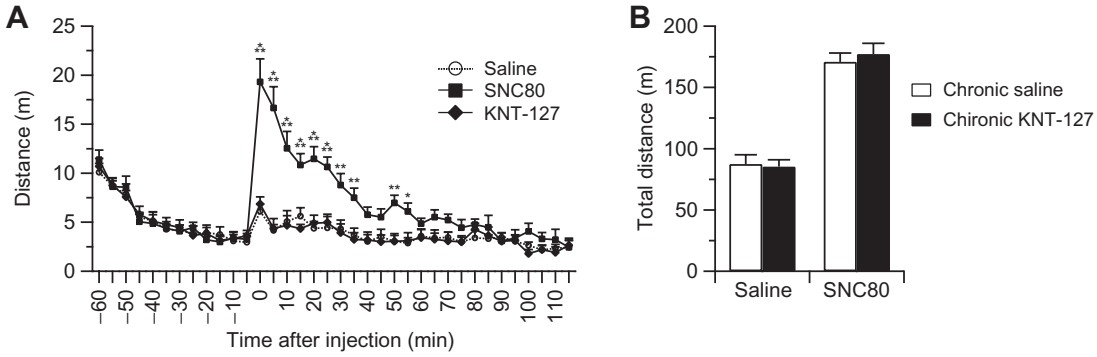

Figure 6

Chronic KNT-127 did not induce tolerance to SNC80-evoked locomotor stimulation. Locomotion (total distance of the movement) was measured automatically by the automatic tracking system immediately after the drug administration. (A) Acute effect of KNT-127 (1 mg·kg^{-1}, s.c.) was compared with that of SNC80 on naïve C57BL/6J mice. KNT-127 produced no effect on locomotor activity, whereas SNC80 induced a significant hyperlocomotion. Data are expressed as means ± SEM of seven to eight mice per group. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from vehicle-treated group for individual time points; repeated-measures two-way ANOVA with Bonferroni *post hoc* test. (B) SNC80 (3 mg·kg^{-1}, i.p.) stimulated locomotor activity in both chronic KNT-127 and chronic saline animals, indicating that chronic KNT-127 treatment did not lead to the development of tolerance to SNC80-induced hyperlocomotion. No significant effect was observed by comparing saline chronic group versus KNT-127 chronic group, Student's *t*-test.

produced no tolerance to the antidepressant actions of DOP receptor agonists, but rather a shift in SNC80 activity by promoting climbing instead of swimming behaviour.

In contrast to SNC80, KNT-127 does not induce hyperlocomotion and does not affect SNC80-induced locomotor stimulation

A number of reports have shown that SNC80 induces locomotor stimulation, whereas other recently described DOP receptor agonists do not affect activity in rodents (Chu Sin Chung and Kieffer, 2013). Hence, we first compared the effects of acute SNC80 and KNT-127 on activity of naïve C57BL/6J mice. As consistently reported, acute SNC80 (10 mg·kg^{-1}) induced drastic hyperlocomotion, whereas KNT-127 (5 mg·kg^{-1}) had no effect (Figure 6A). We then treated two groups of WT C57BL/6J mice with either saline or KNT-127 repeatedly for 5 days as in previous tolerance experiments. On day 6, mice in each group were challenged with either saline or SNC80 and locomotion was analysed (Figure 6B). SNC80-evoked locomotor stimulation was observed similarly in chronic KNT-127 and chronic saline control animals. This indicates that chronic treatment with KNT-127 did not induce tolerance to the hyperlocomotor effect of SNC80.

In contrast to SNC80, KNT-127 does not induce DOP internalization in vivo

We previously reported that DOP receptor agonists display differential receptor internalization properties *in vivo*, as SNC80 induced internalization whereas AR-M1000390, ADL5747 and ADL5859 did not (Pradhan *et al.*, 2009; 2010; Nozaki *et al.*, 2012). We therefore compared KNT-127 and SNC80 for triggering DOP receptor internalization *in vivo* using DOP–eGFP mice as in previous studies. As expected, SNC80 (10 mg·kg^{-1}) induced receptor internalization in the striatum, hippocampus, spinal cord and DRGs of DOP–eGFP mice (Figure 7). However, KNT-127 at 10 mg·kg^{-1} did not alter receptor distribution, as a strong fluorescent signal was detected at the cell surface in all tissues, similarly to saline controls (Figure 7).

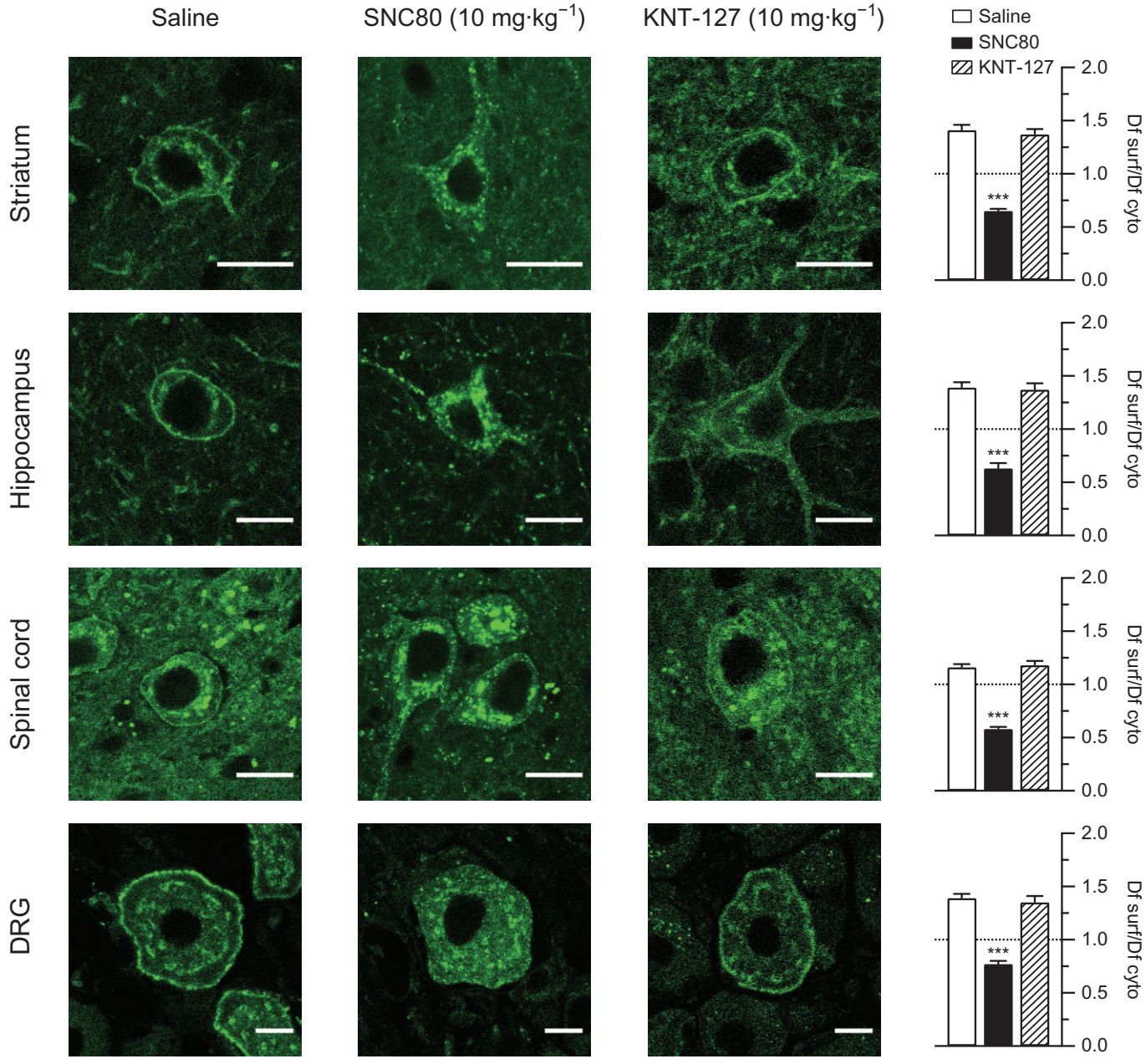

Figure 7

Confocal imaging of SNC80 and KNT-127 induced δ receptor redistribution in DOP–eGFP mice. All mice were perfused 30 min after the drug administration (SNC80: 10 mg·kg^{-1}, i.p.; KNT-127: 10 mg·kg^{-1}, s.c.). Confocal images were taken at striatum, hippocampus, spinal cord and DRG. Bar on each image indicates a length of 10 μm. Ratio of mean fluorescence density on cell surface (Df surf) and cytoplasm (Df cyto) was defined by total fluorescence intensity of cell surface or cytoplasm. Data are expressed as means ± SEM of four mice per group for each region or tissue. Data were first averaged for each brain region or tissue for each mouse, and statistical comparison was performed between the four experimental groups. ***$P < 0.001$, significantly different from vehicle-treated group; one-way ANOVA followed by Bonferroni *post hoc* test.

Discussion

In the present study, we have investigated some unexplored *in vivo* properties of the novel DOP receptor agonist KNT-127. This compound has been shown to display a high affinity for DOP receptors *in vitro* (Nagase *et al.*, 2010), analgesic properties in chemical pain tests and antidepressant effects in the forced swim test in mice (Nagase *et al.*, 2010; Saitoh *et al.*, 2011). We show here for the first time that KNT-127 inhibited inflammatory hyperalgesia in the chronic paw and tail CFA pain models at doses of 0.3–10.0 mg·kg^{-1} that are similar to the analgesic doses of other DOP receptor agonists (Gaveriaux-Ruff and Kieffer, 2011; Gaveriaux-Ruff *et al.*, 2011; Nozaki *et al.*, 2012), indicating a similar potency of KNT-127 and these other agonists. We found that only 10 mg·kg^{-1} KNT-127 could induce antinociception in the tail immersion assay but did not modify mechanical sensitivity in naïve mice as well as at the contralateral paw, in the CFA inflammatory paw model. Taken collectively, this indicates a modest KNT-127-induced thermal antinociception at 10 mg·kg^{-1}, whereas a very significant antihyperalgesia was obtained at 1–5 mg·kg^{-1} KNT-127 in CFA inflammatory pain animals. Previously, systemic SNC80 has been shown to have no or little effect on acute pain in most publications including our previous work. The pioneering paper on SNC80 (Bilsky *et al.*, 1995) showed that SNC80 injected i.p.-induced antinociception in the tail flick test with an A_{50} value of 57 mg·kg^{-1}, indicating that SNC80 produced antinociception although at doses much higher than MOP receptor agonists. We and others have found in previous studies that the DOP receptor agonists AR-M1000390, ADL5747 and ADL5859 could reverse hyperalgesia in animal models of inflammatory or neuropathic pain, whereas antihyperalgesic doses of these drugs had no effect in naïve animals (Le Bourdonnec *et al.*, 2008; 2009; Pradhan *et al.*, 2009; Nozaki *et al.*, 2012).

In addition, analgesia induced by KNT-127 was abolished in DOP receptor KO mice. In these KO mice, interruption of *Oprd1* exon-1 has led to gene inactivation and to the absence of both ligands and antibody binding sites (Filliol *et al.*, 2000; Gaveriaux-Ruff *et al.*, 2008; Wang *et al.*, 2010; Nozaki *et al.*, 2012). The recently solved three-dimensional structure of DOP receptors (Granier *et al.*, 2012) together with previous mutagenesis studies (Pradhan *et al.*, 2011) have shown that the ligand-binding sites comprises residues in transmembrane domains 3–7. The abolition of KNT-127-induced analgesia in KO animals confirms its high *in vivo* selectivity towards DOP receptors, as demonstrated pharmacologically (Saitoh *et al.*, 2011). The present findings of KNT-127-induced analgesia in an inflammatory pain model strengthens previous reports of an important role for DOP receptors in the control of chronic pain (Gaveriaux-Ruff and Kieffer, 2011).

Furthermore, our results indicate that, under identical experimental condition, both KNT-127 and SNC80 showed antidepressant activity in the forced swim test. This result confirms previous findings (Saitoh *et al.*, 2011) and extends general evidence on mood-improving properties of DOP receptor agonists (Chu Sin Chung and Kieffer, 2013; Saitoh and Yamada, 2013). Although the mechanisms for the antidepressant effects of KNT-127 have not yet been fully characterized, this compound evokes the release of dopamine and glutamate in rat striatum (Tanahashi *et al.*, 2012) that may participate in the increase of swimming behaviour rather than climbing behaviour (Cryan *et al.*, 2005) induced by KNT-127. As previously showed, either climbing or swimming behaviour in the forced swim test reflects the activation of either noradrenergic or serotoninergic systems, respectively (Cryan *et al.*, 2005; Nguyen *et al.*, 2013), which suggests that KNT-127 and SNC80 antidepressant effects may be mediated by these different neurotransmitter systems. Whether KNT-127 acts on noradrenergic, serotonergic or other systems to induce its antidepressant effect may be explored in the future.

Acute KNT-127 administration did not induce any hyperlocomotion (present study) nor convulsion (Saitoh *et al.*, 2011) or motor coordination deficit (Saitoh and Yamada, 2013). KNT-127 profile is hence similar to that of the other agonists AR-M1000390, ADL5747 and ADL5857 (Le Bourdonnec *et al.*, 2008; 2009; Nozaki *et al.*, 2012) and in contrast to AZD2327 or SNC80 that produce hyperlocomotion and convulsions depending on animal species and experimental conditions (Jutkiewicz *et al.*, 2005; Jutkiewicz, 2006; Hudzik *et al.*, 2011; Chu Sin Chung and Kieffer, 2013). Using DOP–eGFP mice, we showed that KNT-127 induced no receptor sequestration at four different sites in the CNS (striatum, hippocampus, spinal cord and DRGs), similarly to AR-M1000390, ADL5747 and ADL5857, and in contrast to SNC80 (present study and Pradhan *et al.*, 2010; Nozaki *et al.*, 2012). In transfected cells, SNC80 and DPDPE induced similar internalization but biased DOP receptor coupling to G proteins and recruitment of β-arrestin, leading to receptor recycling or sequestration respectively (Audet and Bouvier, 2012; Audet *et al.*, 2012). In the same study, intrathecal SNC80 induced acute tolerance to analgesia whereas DPDPE produced no acute tolerance, providing an *in vivo* correlate to *in vitro* ligand-biased cellular findings. Moreover, ligand-biased signalling at DOP receptors was shown to depend on the cellular background, with distinct implications of β-arrestin and kinase in HEK-transfected cells or cultured neurons (Charfi *et al.*, 2014). Along the same lines, *in vivo* administration of KNT-127 and SNC80 may induce differential signalling in distinct regions of the nervous system, and therefore distinct behavioural consequences. Also, KNT-127 and other DOP receptor agonists may display different affinities for potential DOP-MOP or DOP-KOP receptor heterodimers (van Rijn *et al.*, 2013) that might explain the differences between their actions and those of SNC80.

Repeated KNT-127 administration induced tolerance to analgesia but not to SNC80-induced hyperlocomotion, similarly to chronically administered AR-M1000390 (Pradhan *et al.*, 2011). The biased effects of long-term administered agonists on analgesia and other behaviours may be explained by differences in receptor internalization (present study and Pradhan *et al.*, 2011), recycling or sequestration (Nagi and Pineyro, 2011; Audet and Bouvier, 2012), which are considered as key mechanisms underlying these chronic responses. However, as analgesic tolerance was obtained after repeated treatment of all SNC80, AR-M1000390 and KNT-127, DOP receptor agonists-induced tolerance may be based on sequestration-independent mechanisms including alterations of receptor coupling to calcium channels in sensory neurons, as shown previously for AR-M1000390 (Pradhan *et al.*, 2010). Repeated KNT-127 induced no tolerance to KNT-127-induced

antidepressant effects and a slight decrease of SNC80 antidepressant effect together with a shift from climbing to swimming. This differential effect of chronic KNT-127 on the acute antidepressant properties of SNC80 and KNT-127 reveals a novel ligand-biased agonism for the DOP receptor-mediated antidepression. It is known that activation of DOP receptors regulates brain-derived neurotrophic factor (BDNF) expression (Zhang *et al.*, 2006; Tian *et al.*, 2013) and exerts neuroprotective and neurogenesis effects that may participate in mood improvement, similarly to other antidepressants (Gardier, 2009). The comparison of the DOP receptor agonists discussed here or of novel DOP receptor compounds for their capacity to regulate BDNF or other molecules such as biogenic amines known to play major roles on mood control may be the subject of future studies.

In summary, this paper presents novel results for KNT-127, compared with previous reports. We show here that KNT-127 (i) induced antinociception at the 10 $mg \cdot kg^{-1}$ dose in the tail immersion test; (ii) induced antihyperalgesia in the CFA paw inflammatory pain model at lower dose than the antinociceptive dose; (iii) induced an antidepressant effect in a biased manner, compared with SNC80; (iv) when administered repeatedly produces tolerance to analgesia but not to the antidepressant actions of SNC80 or KNT-127 itself; and (v) did not induce DOP receptor sequestration. In summary, KNT-127 shows similar pharmacological and ligand-biased effects to other recently synthesized non-peptidic DOP receptor agonists, such as ADL5747, ADL5859 or AR-M1000390 (Pradhan *et al.*, 2010; Nozaki *et al.*, 2012). Thus, while overall these DOP receptor agonists produce beneficial analgesia and mood-promoting effects, only SNC80 and AZD2327 evoke convulsions, hyperlocomotion and receptor sequestration. Furthermore, repeated administration of all agonists produces analgesic tolerance, with repeated SNC80 also inducing tolerance to hyperlocomotor, anxiolytic and antidepressant effects. These results generate new questions about DOP receptor agonist-induced ligand-biased agonism that possibly regulate distinct or selective intracellular signalling, neurotransmission or long-term adaptations. Activation of DOP receptors also produces neuroprotective effects and several clinical trials target this receptor (Chu Sin Chung and Kieffer, 2013). Overall, the broadened repertoire of compounds acting at the DOP receptor will allow us to understand better how this receptor is finely tuned, with improved therapeutic applications in several diseases.

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Author contributions

C. N., C. G.-R. and B. L. K. participated in research design. C. N. conducted the experiments. A. M., H. N. and T. N. contributed new reagents or analytic tools. C. N. performed data analysis. C. N. and C. G.-R. wrote or contributed to the writing of the manuscript.

Conflict of interest

All authors declare that there is no conflict of interest around the present research.

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