Delta opioid mechanisms for ADL5747 and ADL5859 effects in mice: analgesia, locomotion and receptor internalization

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Abbreviations
ADL: Adolor
ADL5859: N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl)benzamide
ADL5747: N,N-diethyl-3-hydroxy-4-(spiro[chromene-2,4'-piperidine]-4-yl)benzamide
SNC80:
4-[(R)-[(2S,5R)-4-allyl-2,5-dimethyl-piperazin-1-yl]-(3-methoxyphenyl)methyl]-N,N-diethyl-benzamide

AR-M100390: N,N-diethyl-4-(phenylpiperidin-4-ylidene-methyl)-benzamide

CFA: Complete Freund’s Adjuvant

DRG: dorsal root ganglia

Nav: voltage-gated sodium channel

DOR: delta opioid receptor

eGFP: enhanced green fluorescence protein

KO: knockout

SNL: sciatic nerve ligation

WT: wild-type

NS: not significant

SEM: standard error of the mean

ANOVA: analysis of variance

Recommended section: Behavioral Pharmacology
Abstract

ADL5859 and ADL5747 are novel delta opioid agonists that show good oral bioavailability, analgesic and antidepressive effects in the rat, and represent potential drugs for chronic pain treatment. Here we used genetic approaches to investigate molecular mechanisms underlying their analgesic effects in the mouse. We tested analgesic effects of ADL5859 and ADL5747 in mice using mechanical sensitivity measures in both Complete Freund’s Adjuvant and sciatic nerve ligation pain models. We examined their analgesic effects in delta opioid receptor constitutive knockout (KO) mice as well as in mice with a conditional deletion of delta receptor in peripheral Nav1.8-expressing neurons (cKO mice). Both ADL5859 and ADL5747 as well as the prototypal delta agonist SNC80 as a control, significantly reduced inflammatory and neuropathic pain. The antiallodynic effects of all three delta opioid agonists were abolished in constitutive delta receptor KO mice, and strongly diminished in delta receptor cKO mice. We also measured two other well-described effects of delta agonists, increase in locomotor activity, and agonist-induced receptor internalization using knock-in mice expressing eGFP-tagged delta receptors. In contrast to SNC80, ADL5859 and ADL5747 induced neither hyperlocomotion nor receptor internalization in vivo. In conclusion, both ADL5859 and ADL5747 show efficient pain-reducing properties in the two models of chronic pain. Their effects are mediated by delta opioid receptors, with a main contribution of receptors expressed on peripheral Nav1.8 positive neurons. The lack of in vivo receptor internalization and locomotor activation, typically induced by SNC80, suggests agonist-biased activity at the receptor for the two drugs.
Introduction

Opiates produce their strong analgesic and addictive properties by acting at opioid receptors, classified as mu, delta and kappa receptors. Mu opioid agonists, such as morphine, are most powerful analgesics widely used in the clinic since more than a century. These compounds, however, produce side effects including constipation, respiratory depression and sedation (McNicol et al., 2003), and are often ineffective under chronic pain conditions like neuropathic pain (Glajchen, 2001). Furthermore, 15%-25% adults suffer from chronic pain (Brennan et al., 2007), and the treatment of persistent pain remains a true challenge. In this context, delta opioid receptors have emerged as a promising therapeutic target, in particular since delta opioid agonists exhibit analgesic activity in models of chronic pain, and lack classical morphine-like side effects (Gaveriaux-Ruff and Kieffer, 2011). No delta opioid agonist has reached the clinic, as yet, and progress is made towards the development of novel useful compounds.

In the past decade, preclinical pharmacological tools and genetic approaches have provided a comprehensive view of delta receptor function in rodent models (Pradhan et al., 2011). The development of several selective non-peptidic delta opioid agonists with small molecular weights and the analysis of novel mouse lines with selected mutations targeting the delta opioid receptor have clarified the role of delta receptors in pain control and mood disorders (Gaveriaux-Ruff and Kieffer, 2011; Pradhan et al., 2011). In brief, pharmacological data in rodents have demonstrated that delta opioid agonists weakly influence acute pain perception (Gallantine and Meert, 2005), but efficiently decrease persistent pain including inflammatory (Fraser et al., 2000; Brandt et al., 2001; Dondio et al., 2001), cancer (Brainin-Mattos et al., 2006; Otis et al., 2011) and neuropathic pain (Dondio et al., 2001; Holdridge and Cahill, 2007; Kabli and Cahill, 2007). The analysis of conventional knockout mice showed the existence of an endogenous delta opioid receptor tone, which reduces
chronic pain (Nadal et al., 2006; Gaveriaux-Ruff et al., 2008) and improves mood (Filliol et al., 2000; Gaveriaux-Ruff and Kieffer, 2002). Further analysis of pain responses and delta opioid analgesia in conditional knockout animals has highlighted a critical role of peripheral receptors in chronic pain control (Gaveriaux-Ruff et al., 2011).

The two novel non-peptidic compounds ADL5859 (Le Bourdonnec et al., 2008) and ADL5747 (Le Bourdonnec et al., 2009) were recently developed with structures distinct from other delta agonists classes to obtain molecules with higher selectivity for delta receptors (Fig. 1). They showed delta opioid selectivity based on nanomolar affinities and signaling potencies for the receptor in vitro, with no detectable binding at over a hundred non-opioid receptors, channels, or enzymes (Le Bourdonnec et al., 2008; Le Bourdonnec et al., 2009). In rats, the two drugs display good oral bioavailability, and produce antidepressant (ADL5859) as well as antihyperalgesic effects in a model of inflammatory pain (Le Bourdonnec et al., 2008; Le Bourdonnec et al., 2009). Here we took advantage of the several existing mutant mouse lines targeting delta opioid receptor gene to investigate molecular bases of ADL5747 and ADL5859 effects in vivo. We first tested analgesic activities of the two compounds in models of inflammatory and neuropathic pain in wild-type mice. We then tested whether these drugs produce analgesic effects in mice lacking delta receptors, either throughout the body (Filliol et al., 2000) or specifically in peripheral Nav1.8-positive nociceptive neurons (Gaveriaux-Ruff et al., 2011). Nav1.8 is a voltage-gated sodium channel expressed specifically in more than 85% of primary nociceptive neurons (Stirling et al., 2005; Liu and Wood, 2011) that include both unmyelinated C and thinly myelinated Adelta fibers (Foulkes and Wood, 2008). Nav1.8 expressing neurons are involved in sensing cold and mechanical pressure, inflammatory and visceral hyperalgesia (Liu and Wood, 2011). Our data show that, as SNC80, both ADL5747 and ADL5859 efficiently reduce inflammatory and neuropathic
pain mainly by recruiting delta opioid receptors expressed by peripheral Nav1.8-expressing neurons.

Finally, our previous work has revealed the existence of biased agonism at the delta opioid receptor in vivo. We showed that the two delta agonists SNC80 and ARM390 display comparable analgesic properties, but distinct locomotor and trafficking effects (Pradhan et al., 2009), and that these differential agonist properties lead to highly distinct forms of tolerance (Pradhan et al., 2010). Because biased agonism has important implications for receptor biology and drug design (Galandrin et al., 2007), we further characterized ADL effects in vivo and examined here locomotor and internalizing properties of ADL5747 and ADL5859 compounds. We show that, as for the ARM390 compound and in contrast to SNC80, the ADL compounds are ineffective on these two in vivo integrated and cellular responses, respectively.

2. Materials & Methods

2.1. Animals

Mice were 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 h and 20:00 h). Food and water were available ad libitum except during behavioural observations. For behavioral studies, all mice were habituated to their new experimental environment and handled for one week before starting the experiments. Researchers were blind to genotype and treatment during behavioral experiment. All data are presented as means ± SEM.

All experimental procedures and animal husbandry were conducted according to standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609EEC) and approved by the local ethical committee (Comité d'éthique pour l'expérimentation animale IGBMC-ICS).
Conventional knockout mice: Delta opioid receptor knockout (KO) mice and their wild type (WT) counterparts with a mixed genetic background (C57BL6/J x SV129Pas) aged 7-10 weeks were used (Filliol et al., 2000). 8-10 mice were used in each group.

Nav1.8-delta receptor conditional KO mice: Floxed mice, Nav1.8-delta receptor conditional KO (Nav1.8-cKO) mice, and total KO (CMV-KO) mice with a mixed genetic background (C57BL6/J x SV129Pas) were used. The delta receptor floxed mice have been generated by our laboratory (Gaveriaux-Ruff et al., 2011). These floxed mice were interbred with Nav1.8-Cre mice from John Wood laboratory in London (Stirling et al., 2005) or CMV-Cre mice to generate the Nav1.8-cKO or CMV-KO mice, respectively (Gaveriaux-Ruff et al., 2011). 6-8 mice aged 7-10 weeks were used in each group.

DOR-eGFP mice: Knock-in mice with delta receptor in fusion to delta opioid receptor (Scherrer et al., 2006) (4 mice per drug, 2 male and 2 female) with a mixed genetic background (C57BL6/J x SV129Pas) aged 10-13 weeks were used for imaging experiments.

Induction of inflammatory pain: Complete Freund's Adjuvant (CFA) was used to induce the inflammatory pain on the hindpaw or tail of mice. Hindpaw CFA model was used to evaluate the analgesic properties of ADL compounds. Tail CFA mice were used to examine the effect of inflammatory pain on delta agonists-induced locomotor activity. After the measurement of baseline pain threshold, 8 μL or 20 μL of CFA (F-5881, Sigma-Aldrich, Saint-Quentin Fallavier, France) were injected subcutaneously into the plantar surface of the left hindpaw or 3 cm from the tip of the tail, under inhalation anaesthetic (Flothane 2%, ref.5573901, CSP Centre de Spécialité Pharmaceutique, Cournon-d'Auvergne, France), respectively. Pain testing was conducted 2 or 3 days after CFA injection.
Induction of neuropathic pain: Sciatic nerve ligation (SNL) induced neuropathic pain was produced by a tight ligation of about half diameter of left common sciatic nerve by 7-0 braid silk suture under deep ketamine/xylazine anesthesia (100/10 mg/kg mixture, Ketamine, Virbac, Carros, France; Xylazine, Rompun, Bayer Healthcare, Puteaux, France) according to the surgical method described by Malmberg and Basbaum (Malmberg and Basbaum, 1998). Sham mice followed the same surgical procedure except for the nerve ligation. Baseline pain threshold was measured before the surgery. Pain measurement was performed on week 2 post-surgery.

2.2. Behavioral assessment

Assessment of mechanical allodynia: Von-Frey test (up-down method (Chaplan et al., 1994)) was used to determine the mechanical sensitivity (50% withdrawal threshold) on CFA-treated mice. Briefly, 8 von Frey filaments were chosen (0.008, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, and 2.0 g force, Bioseb, Valbonne, France), and the test was initiated with the 0.4 g hair. Clear paw withdrawal, shaking or licking of the paw indicated a positive response and promoted the use of the next weaker filament. Absence of a paw withdrawal response prompted the use of the next stronger filament. This procedure was stopped four measures after the first change in animal responding. The threshold of response was calculated by using the up-down Excel program generously provided by the Basbaum's laboratory (UCSF, San Francisco, USA). Kinetics for analgesia was performed 45 min and 2h after SNC80 injection, and 30 min, 1h, 2h and 4h after ADL5747 or ADL5859 injection. Systemic analgesia was measured 45 min and 60 min following SNC80 and ADL compounds administration, respectively. Local ADL5747-induced analgesia was tested 30 min after treatment.

Assessment of thermal hyperalgesia: On tail CFA mice, thermal sensitivity was measured using the tail immersion test by immersing the tail (5 cm from the tip) into a water bath at
46°C. Each individual mouse was lightly restrained in 50 mL cylinder. Tail withdrawal latencies were determined and a cut-off of 30 s was established.

Assessment of locomotor activity: Locomotor activity during exploratory behavior was determined in acrylic cages (21 cm×11 cm×17 cm) on an actimetry platform. Assessment of locomotor activity was carried out for 30 min preinjection (habituation to the cage) and 90 min postinjection, and locomotion (total distance of the movement) was measured in 5-min windows automatically by the video camera with the automatic tracking system (Viewpoint, Lyon, France) during that time.

2.3. Perfusion and Microscopy

Mice were anaesthesized with a ketamine/xylasine mixture (100/10 mg/kg, same suppliers as above) and intracardially perfused with 9.25% sucrose/ddH2O followed by 4% paraformaldehyde/0.1M phosphate buffer (PB; pH 7.4). Perfusion was conducted 45 min (SNC80) or 60 min (ADL compounds) after drug administration. Brain, spinal cord and dorsal root ganglia (DRG) were dissected, postfixed for 2 h at 4°C in 4% paraformaldehyde/0.1M PB, and cryoprotected at 4°C in 30% sucrose/0.1 M PB solution until the tissue sank. Tissues were then frozen in isopentane and cut to 30μm thick sections in a cryostat, with using freely floating sections for hippocampus and striatum. Sections were mounted on glass slides, and DOR-eGFP receptor distribution in striatum, hippocampus, spinal cord and DRG was observed under a Leica confocal microscope (SP2UV; 63× objective and numerical aperture of 1.32) and the LCS (Leica) software was used for image acquisition.

Quantification of cell surface mean fluorescence intensity was determined using ImageJ software. Nuclear fluorescence defined the background level. Fluorescence intensity of cell membrane and cytoplasm is used to calculate the ratios of surface (Df surf) versus
cytoplasmic (Df cyto) fluorescence densities (for further details, see (Scherrer et al., 2006)).

Df surf/Df cyto value of 1.0 results from equal densities of DOR-eFGP at the cell surface and in the cytoplasm. In total, 2–3 neurons/region/mouse were analyzed, and there were 4 mice/group.

2.4. Drugs

SNC80 (10 mg/kg, Tocris, Bristol, UK) was dissolved in saline and injected intraperitoneally (i.p.). Control groups for SNC80 received i.p. saline. ADL 5747 and ADL5859 (10 - 300 mg/kg, Adolor Corp., Exton, USA) were dissolved in distilled water with 0.5% Hydroxypropyl Methylcellulose/0.1% Tween80 and administered by gavage per os (p.o.) as described for rats (Le Bourdonnec et al., 2008; Le Bourdonnec et al., 2009), for systemic administration. Indeed in a clinical phase I study with ADL5859, this route was well tolerated and suitable for daily dosing. Control groups received distilled water with 0.5% Hydroxypropyl Methylcellulose/0.1% Tween80 (p.o.). For local administration, ADL5747 in saline or saline control were injected 2 days after CFA intraplantarly into the inflamed hindpaw in a 5 μl volume.

2.5. 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 test to determine statistically significant differences.
3. Results

**ADL5747 and ADL5859 produce dose-dependent analgesia in both inflammatory pain and neuropathic pain mouse models**

We first examined the analgesic effects of ADL5747- and ADL5859 in C57BL6/J x SV129Pas wild-type mice, matching our delta opioid receptor knockout lines. SNC80, the prototypic non-peptidic delta agonist, was used as a reference compound. In the classical CFA-induced inflammatory pain model, CFA induced strong mechanical allodynia, which was fully reversed by SNC80 (P value = 0.692 vs. Baseline threshold, NS) at the 5 mg/kg dose (Fig. 2A). Administration of either ADL5747 or ADL 5859 also produced significant antiallodynia, with best efficacy within the 30-100 mg/kg dose range for the two compounds (Fig. 2A). All three agonists produced a bell-shape-type dose response for anti-hyperalgesia. We then tested the kinetics of delta agonist effects using optimal doses (SNC80 5 & 10 mg/kg, ADL compounds 30 & 100 mg/kg). The SNC80 effect disappeared within 2 hours, whereas the two ADL compound were still active after 120 min and analgesia terminated 4 hours following drug administration (Fig. 2B).

We also tested ADL5747- and ADL5859-induced analgesia in the SNL neuropathic pain model in these mice. SNL decreased the mechanical threshold, as classically described, and this effect was significantly attenuated by SNC80 (Fig. 2C). Administration of either ADL5747 or ADL 5859, at a dose optimal to reduce inflammatory pain, also efficiently reversed mechanical allodynia in SNL mice (Fig. 2C). Finally, there was no allodynia in contralateral paws animals for both CFA and SNL animals, and no ADL compound effects in sham-operated SNL controls (data not shown). Altogether, the data indicate that ADL5747 and ADL5859 reduced mechanical hypersensitivity in both inflammatory and neuropathic pain models, with their action terminated at 4 hours after administration.
**ADL5747 and ADL5859 are ineffective in delta opioid receptor knockout mice**

We examined the *in vivo* selectivity of ADL compounds for the delta opioid receptor by comparing analgesic effects in delta opioid receptor KO mice and their WT controls. In WT mice, and in both CFA ([Fig. 3A](#)) and SNL ([Fig. 3B](#)) models, administration of ADL5747 and ADL5859 potently reversed mechanical allodynia 60 min post-injection. In particular, SNL-induced mechanical allodynia was fully reversed by the two ADL compounds at the analgesic dose of 30 mg/kg (ADL5747: \( P = 0.167 \), ADL5859: \( P = 0.078 \), vs. baseline threshold, NS). These effects were comparable to those obtained with SNC80 45 min post-injection ([Fig. 2](#)). At the same optimal analgesic dose of 30 mg/kg, ADL5747 and ADL5859 did not modify pain thresholds in KO mice. As for SNC80 therefore, the delta opioid receptor is necessary for ADL compounds-induced analgesia.

This result also indicates their effect is selective at this 30 mg/kg dose ([Fig. 3](#)). We further tested ADL compounds at the 100 mg/kg dose in the inflammatory pain model, and found a slight analgesic effect in KO mice ([Fig. 3A](#)). At this high dose, therefore, ADL compounds may show off-target effects *in vivo*, and this dose was not further used in our experiments. Finally, pain thresholds were unchanged and ADL compounds showed no activity at contralateral paws of both CFA and SNL animals, or in sham-operated animals (data not shown).

**ADL5747 and ADL5859 show strongly reduced analgesic effects in peripheral Nav1.8-delta receptor conditional KO mice**

To examine whether peripheral opioid delta receptors may be involved in ADL compounds-induced analgesia, we administered ADL5747 locally into the hindpaw of CFA inflamed mice. Local ADL5747 induced a dose-dependent decrease of mechanical allodynia in control mice ([Fig. 4](#)), with no effect at the contralateral paws (not shown). This effect was...
delta receptor-selective, as local ADL5747-induced analgesia was abolished in KO mice. These results suggest that the ADL compounds-induced analgesia may be mediated by peripheral delta receptors. We then analyzed the effects of ADL5747 in conditional knockout mice lacking delta receptors in Nav1.8 primary nociceptive neurons (Nav1.8-cKO, see (Gaveriaux-Ruff et al., 2011)). The intraplantar injection of 30 nmoles ADL4757 did not produce any analgesia in the Nav1.8-cKO mice.

To further determine the implication of peripheral delta receptors in both ADL5747- and ADL5859-induced analgesia, we administered both compounds systemically and examined their effects in both inflammatory and neuropathic pain models, using Nav1.8-cKO mice. In control floxed mice, as for WT mice in the previous experiment (Fig. 3), SNC80 (10 mg/kg) significantly suppressed mechanical allodynia using both CFA (Fig. 5A) and SNL (Fig. 5B) models. Administration of either ADL5747 or ADL5859 (10-30 mg/kg) also produced strong antiallodynia in floxed mice, as for control WT mice (Fig. 3). In Nav1.8-cKO mice, ADL compounds were ineffective at the 10 mg/kg dose, indicating that peripheral delta receptors expressed on Nav1.8 neurons are mandatory for mediating analgesia at this dose. At the 30 mg/kg dose, however, there was a significant analgesic (10-20%) effect for both compounds, suggesting that other delta receptor populations may also contribute to ADL compounds analgesia. Finally, pain scores on contralateral paws of either CFA and SNL animals, or sham-operated animals were also unchanged and ADL compounds showed no activity (data not shown).

**ADL5747 and ADL5859 show no locomotor activating effect**

SNC80 is well-known to increase locomotor activity in rats and mice (Jutkiewicz et al., 2005; Scherrer et al., 2006; Ito et al., 2008; Pradhan et al., 2010) whereas ADL compounds did not induce hyperlocomotion in rats (Le Bourdonnec et al., 2008; Le Bourdonnec et al., 2009).
Therefore, we investigated locomotor effects of ADL compounds in naïve WT mice. As previously described, SNC80 at 5 & 10 mg/kg induced a strong hyperlocomotor effect from 5 to 90 min post-injection (Fig. 6A, D). However, ADL5859 and ADL5747 had no effect when tested at 30 and 100 mg/kg (Fig. 6B-D) except for a marginal effect 5 min after administration. In addition, we examined whether inflammatory pain may influence the locomotor properties of delta agonists. Tail-CFA model was used, and administration of CFA into tail produced a significant heat hyperalgesia (Fig. 7A). SNC80 at 5 mg/kg and both ADL compounds at 30 mg/kg significantly alleviated thermal hypersensitivity. Finally, in these CFA-pain mice, analgesic dose of SNC80 induced hyperlocomotion whereas ADL5859 and ADL5747 did not stimulate locomotor activity (Fig. 7B, C), as previously shown in naïve mice.

**ADL5747 and ADL5859 do not induce DOR-eGFP internalization in vivo**

Recently we reported that SNC80 induces strong delta receptor internalization in vivo whereas another delta opioid agonist with similar analgesic potency, AR-M100390, produced no detectable receptor internalization in vivo (Pradhan et al., 2009). Interestingly the latter compound did not desensitize the receptor acutely and produced an analgesia-specific tolerance, thereby contrasting with SNC80 that desensitized the receptor and triggered a tolerance to delta opioid agonist-induced analgesia, hyperlocomotion and anxiolytic effect (Pradhan et al., 2010). Because internalization properties of the ligand may be predictive of in vivo behavioral outcomes, we tested whether ADL compounds produce receptor sequestration in naïve mice. As done earlier, we took advantage of a knockin mouse line expressing a functional delta receptor in fusion with the green fluorescent protein (DOR-eGFP). As expected, SNC80 (10 mg/kg) induced obvious receptor internalization in hippocampus, striatum, spinal cord and DRG (Fig. 8) of DOR-eGFP mice. However, neither ADL5747 nor
ADL5859 tested at the optimal analgesic dose (30 mg/kg) had any visible effect on receptor distribution at cellular level, since a strong fluorescent signal was detected at the cell surface in all tissue sections similar to vehicle controls (Fig. 8). Therefore, ADL5747 and ADL5859 display activity profiles similar to AR-M100390, as the two compounds produce remarkable antiallodynic effects without increasing locomotor activity or triggering receptor internalization.
4. Discussion

In the present study, we show that ADL5747 and ADL5859 dose-dependently suppress CFA or SNL-induced mechanical pain in WT mice after oral administration. The ability of ADL compounds to increase paw withdrawal thresholds represents true antiallodynic activity, as no effect was observed in contralateral paws or sham-operated animal. These results support and extend previous findings with these compounds in a rat model of inflammatory pain (Le Bourdonnec et al., 2008; Le Bourdonnec et al., 2009). Analgesia produced by ADL5747 and ADL5859 in the mouse differed somehow from analgesia obtained in the rat study (Le Bourdonnec et al., 2009). First, the reduction of inflammatory pain in CFA mice seemed weaker as compared to antiallodynia observed in the rat (3-10 fold higher doses required for an analgesic effect). Additionally, the two ADL compounds showed similar dose-dependent effects in mice, whereas ADL5747 was less effective than ADL5859 in the rat. These discrepancies may be explained by distinct intensities of CFA-induced inflammatory pain between the two studies, by differences in mechanical sensitivities of paw pressure (Le Bourdonnec et al., 2009) and Von Frey filament (this study) tests, as well as potentially distinct bioavailability of ADL compounds across the two species. Also, differences in the ADL analgesic effects between mice and rats may be explained by distinct expression patterns of delta opioid receptor between the two species (Mennicken et al., 2003). Interestingly, neuropathic pain was not examined in the rat studies. Here we show almost complete reversal of mechanical pain in SNL-animals mice, indicating that the two compounds are at least as efficient to reduce chronic pain induced by nerve injury than tissue inflammation. Interestingly also, both ADL compounds had a longer duration of action as compared to SNC80 in the two chronic pain models. This is consistent with the pharmacokinetic properties of the compounds evaluated in previous studies (rat and dog), indicating a long half-life for each compound (5.1h for ADL5859 and 12.2h for ADL5747 (Le
We found that analgesic effects of ADL5747 and ADL5859 were abolished in conventional delta receptor KO mice at doses up to 30 mg/kg, and this applied to both CFA and SNL pain models. These results provide a genetic demonstration for the in vivo selectivity of ADL compounds up to this dose. We also observed a slight but significant effect of both compounds at the dose of 100 mg/kg in KO mice suggesting that, at this high dose, the two drugs may also induce analgesia partly via other mechanisms. In the CFA-induced inflammatory pain model, the three agonists appeared to produce a bell-shape-type anti-hyperalgesia dose response. This may be the result of the agonists action on other receptors at the highest doses (20 mg/kg SNC80, 300 mg/kg ADL5747 and ADL5859), or of the combination of relative activation of several signalling pathways following receptor activation depending on the dose. Because the ADL 30mg/kg dose showed optimal effect, the data indicate that specific delta analgesia may be obtained with the two compounds without off-target effects.

Our further analysis using local and systemic injections in Nav1.8-KO mice showed the implication of peripheral delta receptors for ADL5747- and ADL5859-induced analgesia. ADL compounds-induced analgesic effects are abolished following local injection or at the low systemic dose (10 mg/kg), and strongly reduced at the higher delta-receptor selective dose (30 mg/kg). These findings indicate that delta receptors on Nav1.8 neurons represent important targets for ADL compounds in the control of both inflammatory and neuropathic pain. Further, as for ADL compounds, SNC80 analgesia was also abolished or almost abolished in Nav1.8 conditional KO, and in the two chronic pain paradigms (present results and (Gaveriaux-Ruff et al., 2011)). Therefore, the importance of delta opioid receptors on of Nav1.8 neurons applies to all the agonists tested so far, indicating that delta opioid receptor-mediated blockade of primary nociceptive inputs is a main mechanism for delta
opioid analgesia. This does not exclude the participation of other receptor populations located in the peripheral or central nervous system. One implication of these findings is that delta drugs that potentially poorly cross the blood brain barrier may retain significant analgesic properties.

On the other hand, there was a small but significant remaining analgesic effect in delta receptor peripheral conditional knockout (Nav1.8-KO) mice, for both ADL5747 and ADL5859 at the delta receptor selective dose of 30 mg/kg. This indicates that delta opioid receptors at other sites of nociceptive pathways also partly contribute to ADL agonist activities at this dose. Several pharmacological studies indicate that the local application of delta agonists in central regions such as the spinal cord (Bilsky et al., 1995; Kawaraguchi et al., 2004; Scherrer et al., 2009) or cerebral ventricle (Bilsky et al., 1995; Fraser et al., 2000; Cao et al., 2001) produces analgesia. The activation of peripheral receptors by ADL compounds is therefore required but may not be necessarily sufficient to reduce chronic pain (Gaveriaux-Ruff and Kieffer, 2011).

Even though SNC80 and the two ADL compounds show similar in vitro receptor binding and signaling properties, as well as in vivo analgesic effects (this work and (Le Bourdonnec et al., 2008; Le Bourdonnec et al., 2009)), we found profound differences in their ability to trigger receptor internalization at the cellular level. As reported earlier (Scherrer et al., 2006; Pradhan et al., 2009; Pradhan et al., 2010) and in this study, analgesic doses of SNC80 triggered massive receptor internalization at all delta receptor-expressing sites that were examined (hippocampus, striatum, spinal cord and DRG), whereas analgesic doses of ADL compounds had no effect on receptor distribution in neurons. This observation parallels our previous set of studies (Pradhan et al., 2009; Pradhan et al., 2010), which compared behavioral and in vivo cellular effects of SNC80 with those of another delta receptor agonist, ARM-100390 (ARM390). As for ADL compounds, ARM390 showed binding affinities and
signaling potencies similar to SNC80, but did not produce any detectable receptor internalization in vivo. Remarkably, the differential internalization potencies of SNC80 and ARM390 correlated with distinct adaptive responses of delta receptors to repeated drug injections (Pradhan et al., 2010). Chronic ARM390 produced analgesic tolerance without altering locomotor or anxiolytic receptor-mediated responses (Pradhan et al., 2010), whereas chronic SNC80 produced receptor down-regulation accompanied with tolerance to the analgesic, hyperlocomotor and anxiolytic responses to delta drugs, although no tolerance was obtained for chronic SNC80-induced antidepressant effect in rats (Jutkiewicz et al., 2005; Saitoh et al., 2008). Noticeably, SNC80 produced delta receptor internalization in both naïve (this work and (Scherrer 2006)) and CFA-pain animals (Pradhan et al., 2009; Pradhan et al., 2010), and ARM390 induced no internalization neither in naïve animals (Pradhan et al., 2009; Pradhan et al., 2010) nor in CFA-pain animals (Pradhan et al., 2009; Pradhan et al., 2010), indicating that inflammatory pain did not change the internalizing properties of delta agonists.

Altogether, our study demonstrates the existence of two additional delta agonists with high analgesic efficacy and low internalizing properties, extending the repertoire of biased agonists at the delta receptor (Pradhan et al., 2009; Pradhan et al., 2010).

There was another main difference between SNC80 and ADL compound activities in vivo. SNC80 significantly increased locomotor activity, as reported in many previous studies, whereas ADL5859 and ADL5747 did not affect on locomotion neither in naïve animals nor in CFA-pain animals. This shows that the locomotor properties of SNC80 and ADL agonists are not affected by inflammatory pain. Whether inability of the two ADL compounds to stimulate animal activity is related to inability to internalize the receptor is presently unknown. We may speculate that locomotor activating effects of delta agonists require receptor internalization, in which case the behavioral activation would result from the specific activation of an internalization-dependent signaling pathway in vivo. The detailed mechanistic basis of this
particular behavioral response, and whether beta-arrestins are involved for example (Qiu et al., 2007; Urs et al., 2011), will be the subject of future studies.

In conclusion, our study shows that ADL5859 and ADL5747 efficiently reduce inflammatory and neuropathic pain in mice, show delta opioid receptor selectivity in vivo at doses up to 30 mg/kg and implicate delta receptors on peripheral Nav1.8 neurons to produce their analgesic effects. Furthermore, and in line with our previous study (Pradhan et al., 2010), our results suggest that ADL compounds show biased activity at the delta receptor and may activate signaling pathways distinct from SNC80 in vivo. It is likely that the number of delta drugs with biased activity will increase in the future as more compounds are synthesized and characterized for a broad set of signaling and biological activities (Galandrin et al., 2007; Zheng et al., 2010). Altogether, data obtained from ADL5859 and ADL5747 in this study increase our knowledge on delta agonist properties in vivo at cellular and behavioral levels, and strengthen the potential utility of these two molecules within the repertoire of novel delta opioid agonists (Pradhan et al., 2011).
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Authorship contributions

Participated in research design: Nozaki, Windh, Little, Gaveriaux-Ruff and Kieffer.

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Footnotes

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Figure Legends

**Fig. 1.** Chemical structures of SNC80, ADL5747 and ADL5859.

**Fig. 2.** ADL5747-, ADL5859- and SNC80-induced analgesia in CFA-induced inflammatory (A, B) or SNL-induced neuropathic (C) pain models in wild type mice. Nociceptive thresholds were determined by the von-Frey test. (A) Dose-dependent analgesia of SNC80 and ADL compounds. Broken lines indicate basal mechanical thresholds. SNC80 analgesia was measured 45 min post-injection, while both ADL5747 and ADL5859 were tested 60 min post-administration. Significant drug effects are indicated by *P<0.05, **P<0.01 and ***P<0.001, one-way ANOVA followed by Bonferroni post-hoc test, n = 8-10 mice/group.

(B, C) Time-course of SNC80- or ADL compounds-induced analgesia in CFA (B) and SNL (C) mice. ADL compounds and SNC80 showed significant antiallodynic effects in both CFA and SNL pain models. Significant drug effects are indicated by *P<0.05, **P<0.01 and ***P<0.001, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test, n = 8-10 mice/group.

**Fig. 3.** Effect of total receptor knockout on antinociceptive effect of SNC80 and ADL compounds. Conventional delta receptor knockout (KO) mice and their wild type (WT) counterparts were used for testing. Chronic pain was induced by CFA (A) or SNL (B). The nociceptive threshold was determined before induction of pain (BL), 45 min after SNC80 (10 mg/kg) or vehicle injection and 60 min after the ADL compound by von-Frey filaments. Both SNC80 and ADL compounds induced analgesic effect in WT mice, whereas these antinociception were abolished in KO mice, except for ADL compounds at dose of 100 mg/kg. Data are expressed as means ± SEM of 8-10 mice/group. Significance is indicated by...
***P<0.001, drug-treated group vs vehicle-treated group (two-way ANOVA followed by Bonferroni post-hoc test).

**Fig. 4.** Analgesic effect of intraplantar ADL5747 under CFA-induced mechanical allodynia.
Injection of 10 and 30 nmoles ADL5747 in the hindpaw of floxed mice induced a dose-dependent antiallodynic effect. The analgesic effect of local ADL5747 was abolished in delta receptor total knockout (CMV-KO) and conditional knockout (Nav1.8-KO) animals. (n=7-8/group, ** P <0.01 ADL5747 vs saline; two-way ANOVA followed by Bonferroni post-hoc test).

**Fig. 5.** Involvement of peripheral delta receptor on antinociceptive effect induced by either SNC80 or ADL compounds. Conditional delta receptor knockout (Nav1.8-KO) mice and floxed mice were used for testing. Chronic pain was induced by CFA (A) or SNL (B). The nociceptive threshold was determined before induction of pain (BL), 45 min after SNC80 (10 mg/kg) or vehicle injection and 60 min after the ADL compound (10-30 mg/kg) injection by von-frey filament. Both SNC80 and ADL compounds induced analgesic effect in floxed mice, whereas these antiallodynic effects were completely abolished in Nav1.8-KO mice, except ADL compounds at dose of 30 mg/kg. Data are expressed as means ± SEM of 8-10 mice/group. Significance is indicated by *P<0.05 and ***P<0.001, drug-treated group vs vehicle-treated group (two-way ANOVA followed by Bonferroni post-hoc test).

**Fig. 6.** Locomotor activity after administration of SNC80 and ADL compounds in naïve mice.
Locomotion (total distance of the movement) was measured automatically by the automatic tracking system immediately after the drug administration. Although SNC80 (A) induced significant hyperlocomotion, ADL 5747 (B) and ADL5859 (C) had no effect on locomotor
activity. Total activity (D) also shows the hyperlocomotor effect of SNC80, and the absence of effect of the ADL compounds. Data are expressed as means ± SEM of 8-10 mice/group. Significance is indicated by *P<0.05, **P<0.01 and ***P<0.001, drug-treated group vs vehicle-treated group for individual time points, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test (A-C) or Student's t-test (D).

**Fig. 7.** Locomotor activity after administration of SNC80 and ADL compounds in tail-CFA mice. (A) Analgesic effect of SNC80 (5 mg/kg) and ADL compounds (30 mg/kg) in tail-CFA mice. Pain threshold was determined by tail immersion test before the CFA injection (BL), at the time point of pre- and post-injection of drugs 2 days after tail-CFA. Post-injection period for SNC80 was 45 min, while that for both ADL5747 and ADL5859 were 60 min. Heat sensitivity was increased by CFA injection to tail, and all the delta agonists induced anti-hyperalgesic effect under these conditions. Significant drug effects are indicated by ***P<0.001, one-way ANOVA followed by Bonferroni post-hoc test, n = 8-10 mice/group. (B) Locomotion was measured automatically by the automatic tracking system immediately after drug administration. SNC80 (5 mg/kg) produced significant hyperlocomotion whereas ADL 5747 and ADL5859 (30 mg/kg, respectively) had no effect on locomotor activity. Total activity (C) also indicates SNC80 hyperlocomotor effect, and the absence of effect of the ADL agonists. Data are expressed as means ± SEM of 7-9 mice/group. Significance is indicated by *P<0.05, **P<0.01 and ***P<0.001, drug-treated group vs vehicle-treated group for individual time points, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test (B) or Student's t-test (C).

**Fig. 8.** Confocal imaging of SNC80, ADL5747 and ADL5859 induced delta receptor redistribution in DOR-eGFP mice. All mice were perfused 45 min after the drug
administration. Confocal images were taken at striatum, hippocampus, spinal cord and dorsal root ganglia. Bar on each image indicates the 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 4 mice/group for each region or tissue. White bars, control group; black bars, SNC80 group; left stripped bar, ADL5747; right stripped bars, ADL5859. Data were first averaged for each brain region or tissue for each mouse, and statistical comparison was performed between the four experimental groups. A significant effect of delta agonist vs vehicle control is indicated by ***P<0.001, drug-treated group vs vehicle-treated group (one-way ANOVA followed by Bonferroni post-hoc test).
SNC80

ADL5747

ADL5859

Figure 1
Figure 2
Figure 7
Figure 8