



RESEARCH PAPER

Tolerance to high-internalizing δ opioid receptor agonist is critically mediated by arrestin 2

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BACKGROUND AND PURPOSE

Opioid δ receptor agonists are potent antihyperalgesics in chronic pain models, but tolerance develops after prolonged use. Previous evidence indicates that distinct forms of tolerance occur depending on the internalization properties of δ receptor agonists. As arrestins are important in receptor internalization, we investigated the role of arrestin 2 (β -arrestin 1) in mediating the development of tolerance induced by high- and low-internalizing δ receptor agonists.

EXPERIMENTAL APPROACH

We evaluated the effect of two δ receptor agonists with similar analgesic potencies, but either high-(SNC80) or low-(ARM390) internalization properties in wild-type (WT) and arrestin 2 knockout (KO) mice. We compared tolerance to the antihyperalgesic effects of these compounds in a model of inflammatory pain. We also examined tolerance to the convulsant effect of SNC80. Furthermore, effect of chronic treatment with SNC80 on δ agonist-stimulated [³⁵S]-GTP γ S binding was determined in WT and KO mice.

KEY RESULTS

Arrestin 2 KO resulted in increased drug potency, duration of action and decreased acute tolerance to the antihyperalgesic effects of SNC80. In contrast, ARM390 produced similar effects in both WT and KO animals. Following chronic treatment, we found a marked decrease in the extent of tolerance to SNC80-induced antihyperalgesia and convulsions in arrestin 2 KO mice. Accordingly, δ receptors remained functionally coupled to G proteins in arrestin 2 KO mice chronically treated with SNC80.

CONCLUSIONS AND IMPLICATIONS

Overall, these results suggest that δ receptor agonists interact with arrestins in a ligand-specific manner, and tolerance to high- but not low-internalizing agonists are preferentially regulated by arrestin 2.

Abbreviations

ARM390, N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide; CFA, complete Freund's adjuvant; KO, knockout; SNC80, (+)-4-[(α R)- α -((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethyl benzamide; WT, wild-type

Introduction

The opioid **δ receptors**, also known as δ OR, are GPCRs that play an important role in the regulation of pain processing and emotional responses (Gaveriaux-Ruff *et al.*, 2011; Pradhan *et al.*, 2011). Recently, δ receptor agonists have attracted increasing research interest as a potential alternative to **μ receptor** agonists (Gendron *et al.*, 2016). Compared to μ receptor agonists, δ receptor agonists are poor analgesics for acute pain (Gallantine and Meert, 2005), but they are highly effective in models of chronic pain. This difference in antinociceptive efficacy is likely due to increased functionality of δ receptors during chronic pain states (Fraser *et al.*, 2000; Hurley and Hammond, 2000; Cahill *et al.*, 2003; Kabli and Cahill, 2007; Gaveriaux-Ruff *et al.*, 2008; Pradhan *et al.*, 2013). Unlike μ receptor agonists, δ receptor agonists do not show significant abuse liability (Negus *et al.*, 1998; Brandt *et al.*, 2001; Stevenson *et al.*, 2005) and lack several side effects associated with μ receptor activation (constipation, respiratory depression, etc.) (Gallantine and Meert, 2005). However, some δ receptor agonists can cause convulsions, which has limited their clinical development (Comer *et al.*, 1993; Broom *et al.*, 2002a). In addition, prolonged exposure to δ or μ receptor agonists leads to the development of tolerance, which hinders their therapeutic use.

The extent and duration of GPCR signalling are governed by multiple factors, including receptor desensitization, internalization, down-regulation and recycling. Arrestins are key regulators of GPCR desensitization and internalization (Luttrell and Lefkowitz, 2002; Reiter *et al.*, 2012). In addition, arrestins act as multifunctional scaffolding proteins and signalling intermediates with effects on cytoskeletal remodeling (Mittal *et al.*, 2013), protein ubiquitination (Shenoy, 2014), trafficking of ion channels (Nagi *et al.*, 2015) and gene transcription (Ma and Pei, 2007). Similar to many GPCRs, ligand-activated δ receptors are also modulated by arrestins (Lowe *et al.*, 2002; Zhang *et al.*, 2005; Qiu *et al.*, 2007; Raehal and Bohn, 2011). In cellular models, both arrestin 2 (β -arrestin 1) and arrestin 3 (β -arrestin 2) can mediate δ receptor desensitization and internalization after receptor phosphorylation (Qiu *et al.*, 2007; Hong *et al.*, 2009). Interestingly, emerging evidence suggests that arrestins 2 and 3 play distinct roles in downstream functional outcomes upon GPCR activation (Taylor *et al.*, 2016). In line with this functional specialization of arrestin isoforms, δ receptor export from the Golgi to the cell surface is selectively regulated by arrestin 2 through the RhoA/ROCK/LIMK pathway (Mittal *et al.*, 2013). To add one more layer of complexity, several studies have demonstrated the existence of agonist-specific recruitment of arrestin isoforms at many GPCRs, including the δ receptor. In mouse embryonic fibroblasts, DPDPE preferentially recruited arrestin 3 to induce phosphorylation-dependent δ receptor internalization (Qiu *et al.*, 2007). In contrast, arrestin 2 mediated etorphine-activated δ receptor internalization in SK-N-BE cells, while facilitating receptor desensitization, but not internalization, in response to DPDPE or deltorphin I (Bowman *et al.*, 2015). Recently, we reported that *in vivo*, the binding of the high-internalizing agonist **SNC80** produces preferential interaction between δ receptors and arrestin 2, and low-internalizing agonists like **ARM390** or JNJ20788560 preferentially promote arrestin 3-

receptor interactions (Pradhan *et al.*, 2016). An increased interaction between the δ receptor and arrestin 3 facilitated receptor resensitization and protected against the development of behavioural tolerance to low-internalizing agonists (Pradhan *et al.*, 2016).

In the present study, we explored the long-term behavioural consequences of the ligand-specific interaction between arrestin 2 and δ receptors using SNC80 and ARM390. We found that arrestin 2 mediates the development of tolerance to the antihyperalgesic and convulsive effects of SNC80, but not tolerance to the antihyperalgesic effects of ARM390. Furthermore, assays monitoring receptor function *ex vivo* after the establishment of tolerance to SNC80 revealed that δ receptors remain functionally coupled to G proteins in the absence of arrestin 2. These studies demonstrate that the ligand-selective recruitment of arrestins by δ receptor agonists can have profound effects on tolerance to chronic drug administration.

Methods

Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015) and the US National Research Council's Guide for the Care and Use of Laboratory Animals. Mice were group-housed with a maximum of five animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. Arrestin 2 knockout (KO) mice were generously provided by R. Lefkowitz (Duke University). Both male and female mice were used. Mice, aged 8–20 weeks, were housed in a temperature- and humidity-controlled animal colony on a 12 h light/dark cycle. Mice had free access to food and water at all times. All animal experiments were performed according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines as administered by the University of Illinois at Chicago and University of Michigan Animal Care Committees. A total of 242 mice were used in these experiments – 118 wild-type (WT) and 124 arrestin 2 KO.

Drugs

All drugs were administered in a volume of 10 mL·kg⁻¹. SNC80 (Tocris Bioscience, Pittsburgh, PA, USA) was dissolved in acidic 0.9% saline, pH 5.5, and injected i.p. ARM390 (AstraZeneca, R&D, Wilmington, DE, USA) was dissolved in dH₂O and administered by oral gavage. Unless otherwise stated, mechanical sensitivity was tested 45 min after treatment with SNC80 or ARM390. All other drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless noted otherwise.

Inflammatory pain model

The complete Freund's adjuvant (CFA)-induced inflammatory pain model was performed as described previously (Pradhan *et al.*, 2016; Vicente-Sanchez *et al.*, 2016). Briefly, inflammation was induced by injecting 13 μ L of CFA (Sigma-Aldrich) into the plantar surface of the hindpaw, and animals were tested 72 h after injection. Mice were habituated to the testing area for 20 min daily for 2 days before

baseline testing. The threshold for mechanical responses of the hindpaw to punctate mechanical stimuli (mechanical hyperalgesia) was assessed according to the up-and-down method (Chaplan *et al.*, 1994). A series of eight von Frey filaments with bending force ranging from 0.01 to 2 g was used. A response was defined as a lifting or shaking of the paw upon stimulation. Mechanical responses were determined prior to CFA injection to establish baseline sensitivity. Animals were randomly assigned to treatment groups, and the experimenter was blinded to the drug treatment and/or genotype when testing.

Observation of SNC80-induced convulsions

Mice were randomized to receive a s.c. injection of SNC80 (3.2, 10, 32 mg·kg⁻¹) every 24 ± 1 h for 5 days. Following SNC80 administration, mice were observed continuously in individual cages for 30 min for convulsions. Convulsions were comprised of a tonic phase characterized by sudden tensing of the musculature and extension of the forepaws followed by clonic contractions that extended the length of the body. The severity of each convulsion was quantified using the following modified Racine scale (Racine, 1972) adapted from Jutkiewicz *et al.* (2006): scale 1 – teeth chattering or face twitching; 2 – head bobbing or twitching; 3 – tonic extension or clonic convulsion lasting less than 3 s; 4 – tonic extension or clonic convulsion lasting longer than 3 s; 5 – tonic extension or clonic convulsion lasting more than 3 s with loss of balance. Post-convulsion catalepsy-like behaviour was assessed by a placing response in which a horizontal rod was placed under the forearms of the mouse, and a positive catalepsy score was assigned if the mouse did not move its forepaws onto the rod or cage floor within 30 s.

GTPγS assay

Brain membrane preparations were carried out as described previously (Befort *et al.*, 2001). Animals that were treated and tested chronically with vehicle or SNC80 for 5 days were anaesthetised with isoflurane gas and killed by decapitation 24 h after the final treatment day. Whole brains from WT and KO mice chronically treated and tested with agonist or vehicle were removed, immediately frozen in isopentane on dry ice and stored at –80°C prior to use. Whole brain membranes were prepared by homogenizing the brain in ice-cold 0.25 M sucrose solution 10 vol (mL·g⁻¹ wet weight of tissue). Samples were then centrifuged at 1100× g for 10 min. Supernatants were collected and diluted five times in buffer containing 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA, following which they were centrifuged at 25000× g for 30 min. The pellets were homogenized in 2 mL ice-cold sucrose solution (0.32 M), aliquoted and kept at –80°C until further use.

For the [³⁵S]-GTPγS binding assay, 5 µg of protein was used per well. Samples were incubated with varying concentrations of SNC80 (10⁻⁵ to 10⁻¹² M) for 1 h at 25°C in assay buffer containing 50 mM Tris HCl (pH 7.4), 3 mM MgCl₂, 100 mM NaCl, 0.2 mM EGTA, 30 µM GDP and 0.1 nM [³⁵S]-GTPγS (Perkin Elmer USA). Incubation was terminated by rapid filtration and washing in ice-cold buffer (50 mM Tris HCl, 5 mM MgCl₂, 50 mM NaCl, pH 7.4). Bound radioactivity was quantified using a liquid scintillation counter. Non-

specific binding was defined as binding in the presence of 10 µM GTPγS, and basal binding indicates binding in the absence of any agonist. Samples were run in triplicate. Plates were counterbalanced so that different combinations of groups were represented on a plate, and the experimenter was blinded to the groups.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). All data are reported as mean ± SEM. All graphs were produced in GraphPad Prism version 6 or 7, and all statistical analysis was performed in SigmaStat software. Experiments were designed to have an equal *n* per group. However, animals were excluded from analysis if there was an error in drug injection, genotyping or if they were outliers. Outliers were defined using the Grubb's test, and based on this analysis, one data point was removed from the dataset shown in Figure 3A. In addition, in the convulsion experiments, arrestin 2 KO mice died or were killed following 32 mg·kg⁻¹ SNC80; and one arrestin 2 KO mouse died due to a misplaced injection that damaged the liver for the 10 day SNC80 experiment outlined in Figure 3C. For pain and convulsion experiments, two-way repeated-measures ANOVAs were performed or where cited two-way ANOVA was used. For all tests, the level of significance α was set to 0.05. *Post hoc* analysis was conducted using a Holm–Sidak *post hoc* analysis. *Post hoc* analysis was only performed when *F* values achieved *P* < 0.05. For convulsion data, a Tukey's *post hoc* analysis was performed. For the GTPγS assays, curve fitting was performed using GraphPad Prism. SNC80 was fit with a nonlinear fit, one-site model. *R*² values were used to assess goodness of fit. EC₅₀ values were determined from pooled, fitted data (*n* = 3–4 mice per group). Each data point for each mouse was the average of a triplicate, and this average was considered as *n* = 1.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Effect of arrestin 2 deletion on the antihyperalgesic response to SNC80 and ARM390

To evaluate the role of arrestin 2 in δ receptor-mediated antihyperalgesia, CFA was injected into the hindpaw, and 72 h later, mice were injected with different doses of SNC80 or ARM390. To assess potential differences in the duration of drug action, mice were tested 45 min (Figure 1A, C) and 3 h (Figure 1B, D) following injection. In WT mice, SNC80 and ARM390 produced dose-dependent increases in mechanical threshold 45 min after administration (Figure 1A, C). After 3 h, SNC80 was no longer effective in WT animals potentially

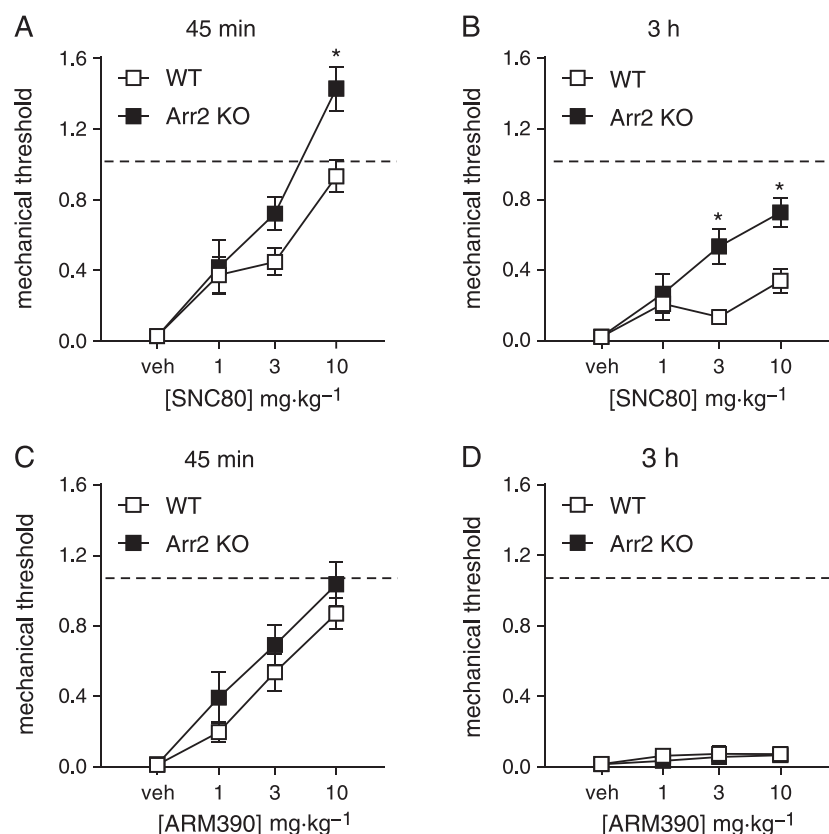


Figure 1

Potency and duration of SNC80- or ARM390-induced antihyperalgesia in WT and arrestin 2 KO mice. In a CFA model of inflammatory pain, we performed a dose- and time-response to SNC80 (i.p.) and ARM390 (p.o.). (A) The antihyperalgesic effect of SNC80 was significantly enhanced in arrestin 2 KO mice, and the effect of SNC80 was longer lasting in these animals (B), $n = 8$ per group. Two-way ANOVA with Holm-Sidak *post hoc* analysis at both time points, $P < 0.05$ genotype, dose and interaction; * $P < 0.05$. (C) ARM390 showed similar effects in arrestin 2 KO and WT mice (two-way ANOVA, $P < 0.05$ effect of dose), and regardless of genotype, this antihyperalgesic effect was lost 3 h post-administration (D), $n = 7$; vehicle and 10 mg·kg⁻¹; $n = 9$; 1 and 3 mg·kg⁻¹. Dashed lines represent baseline mechanical responses assessed pre-CFA injection.

due to drug clearance. However, in arrestin 2 KO mice SNC80, but not ARM390, was enhanced at this time point; and SNC80 continued to produce significant antihyperalgesic effects (Figure 1B). ARM390 failed to produce antihyperalgesic effects in either genotype 3 h post-injection (Figure 1D). These data show that SNC80, but not ARM390, has increased efficacy and duration of action in arrestin 2 KO mice.

Effect of arrestin 2 deletion on acute analgesic tolerance to SNC80 and ARM390

We have previously shown that a single injection of the high-internalizing δ receptor agonist SNC80 produced acute analgesic tolerance while the low-internalizing agonist ARM390 did not (Pradhan *et al.*, 2009). In those experiments, we tested Emax doses of drug (10 mg·kg⁻¹). Because SNC80 is more effective in arrestin 2 KO mice than WT mice, we tested equipotent doses of the drug between the two genotypes. Thus, KOs received a 3 mg·kg⁻¹ dose of SNC80 and WTs a 10 mg·kg⁻¹ dose. For ARM390, both genotypes were administered a 10 mg·kg⁻¹ dose. CFA was injected into the mouse paw, and severe mechanical hypersensitivity was observed 72 h later (Figure 2, dashed line vs. vehicle controls).

In WT and arrestin 2 KO mice, an initial injection of SNC80 and ARM390 attenuated CFA-induced hyperalgesia (Figure 2, Injection 1). Consistent with previous findings, a second injection of SNC80 given 4 h later was ineffective in WT animals (Figure 2A, Injection 2). However, this acute behavioural desensitization was not observed in arrestin 2 KO mice (Figure 2B, Injection 2). A second injection of ARM390 was equally effective in both WT and KO mice. These results imply that acute tolerance to SNC80, but not ARM390, is preferentially mediated by arrestin 2.

Effect of arrestin 2 deletion on the development of chronic analgesic tolerance to SNC80 and ARM390

To confirm the role of arrestin 2 in SNC80-induced behavioural tolerance, we examined the consequences of repeated SNC80 or ARM390 treatment in arrestin 2 WT and KO animals. WT mice treated daily with SNC80 showed full tolerance to the antihyperalgesic effects of SNC80 by the third day of treatment (Figure 3A). The development of tolerance to SNC80 was significantly delayed in arrestin 2 KO mice (Figure 3A). To rule out the possibility that these effects were

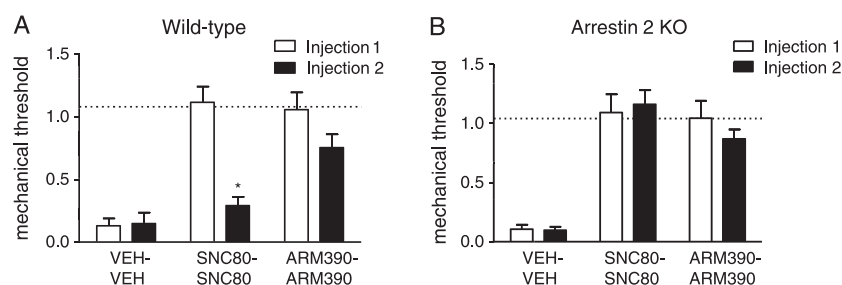


Figure 2

Acute behavioural tolerance to SNC80 is not observed in arrestin 2 knockout mice. Mechanical responses were determined in the CFA model of inflammatory pain. Equipotent doses of SNC80 and ARM390 were compared. Injection 1, mechanical responses in (A) WT and (B) arrestin 2 KO mice treated with vehicle (control, $n = 10$ per genotype), SNC80 ($3 \text{ mg}\cdot\text{kg}^{-1}$ for KO mice, 13 per group, and $10 \text{ mg}\cdot\text{kg}^{-1}$ for WT mice, 12 per group i.p.), or ARM390 ($10 \text{ mg}\cdot\text{kg}^{-1}$, p.o., 12 per genotype). Injection 2, animals re-challenged with the same drug and dose 4 h following Injection 1. Dashed lines represent baseline mechanical responses pre-CFA injection. For consistency, all animals were injected i.p. and p.o. Two-way RM ANOVA for each genotype, $P < 0.05$ drug, time and interaction for WT; $P < 0.05$ for drug in KO. * $P < 0.05$ as compared to Injection 1, Holm–Sidak *post hoc* analysis. In addition, $P < 0.05$ when Injection 2 of SNC80 was compared across genotypes; *t*-test.

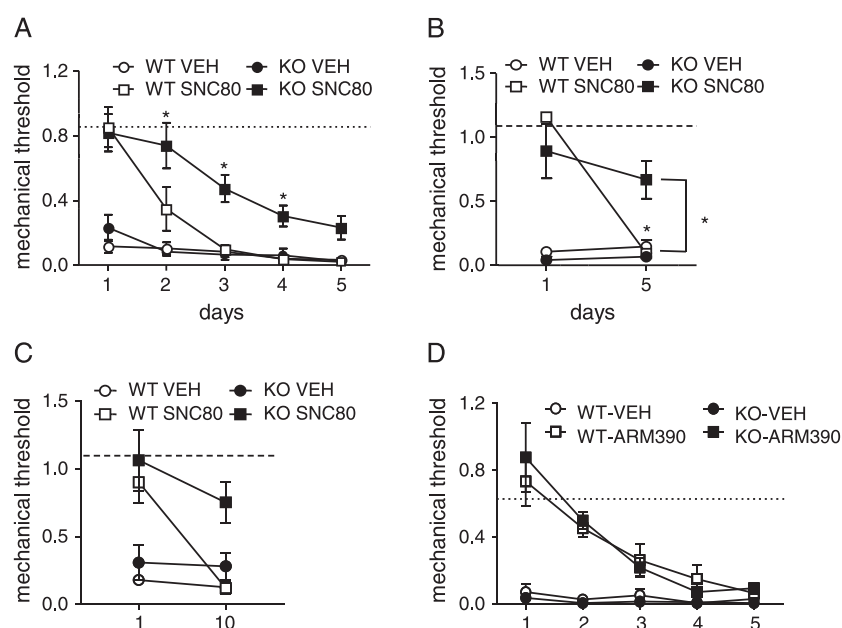


Figure 3

The extent of chronic analgesic tolerance after repeated exposure to SNC80 is attenuated in arrestin 2 KO mice. Arrestin 2 WT and KO mice were injected with equipotent doses of SNC80 ($10 \text{ mg}\cdot\text{kg}^{-1}$ for WT, and $3 \text{ mg}\cdot\text{kg}^{-1}$ for KO) daily for 5 days, and tested daily, 45 min following each injection (A, $n = 10$ per WT group, $n = 12$ per KO group; two-way RM ANOVA $P < 0.05$ time, genotype and interaction; * $P < 0.05$ as compared to WT-SNC80 group). We also treated mice daily but only tested on the first and the fifth day of treatment (B, $n = 8$ per group; two-way RM ANOVA, $P < 0.05$ genotype \times time interaction, * $P < 0.05$ as compared to day 1, and as compared to WT-SNC80 on day 5); or on the first and tenth day of treatment (C, $n = 5$ –6 per group; two-way RM ANOVA, $P < 0.05$ genotype and time). Unlike SNC80, tolerance to repeated ARM390 treatment was unaltered in arrestin 2 KO mice (D). WT and KO mice were injected with ARM390 ($10 \text{ mg}\cdot\text{kg}^{-1}$, p.o.) daily for 5 days, and tested 45 min following each injection. $n = 6$ per group, two-way RM ANOVA $P < 0.05$ for time only.

due to WT and KO mice developing behavioural/associative tolerance to the assay at different rates, we repeated the same experiment but tested only on the first and fifth days of treatment (Figure 3B). Again, SNC80 failed to produce antihyperalgesic effects in WT animals after repeated treatment, indicating pharmacological tolerance. However, in arrestin 2 KO mice, the last injection of SNC80 still produced

significant antihyperalgesic effects (Figure 3B). In order to determine if an even longer term treatment would induce pharmacological tolerance in arrestin 2 KOs, we treated animals with vehicle or SNC80 daily for 10 days. Even after this prolonged treatment, SNC80 continued to be effective in arrestin 2 KO mice as compared to WT controls (Figure 3C). In contrast to SNC80, WT and arrestin 2 KO mice developed

chronic tolerance to the antihyperalgesic effects of ARM390 at similar rates (Figure 3D). Arrestin 2 appears to be necessary for the development of tolerance to SNC80, but not to the low-internalizing agonist, ARM390.

The effect of arrestin 2 deletion on tolerance to SNC80-induced convulsions

To explore the role of arrestin 2 in the development of tolerance to other δ receptor-mediated behaviours, tolerance to SNC80-induced convulsions was evaluated in WT and arrestin 2 KO mice (Figure 4). ARM390 does not produce convulsions, even at high doses, and therefore was not included to study the development of tolerance to this phenomenon. Mice were treated with SNC80 daily for 5 days, and the severity of convulsions was evaluated using a modified Racine scale. In WT mice, the dose of 32 mg·kg⁻¹ SNC80 was tested as 10 mg·kg⁻¹ does not reliably induce convulsions. SNC80 produced severe convulsive effects on day 1 of treatment, but failed to produce significant convulsive effects on subsequent days in WTs. In contrast, arrestin 2 KO mice exhibited significant convulsive effects in response to lower doses of SNC80 (3.2 or 10 mg·kg⁻¹), and this effect was observed on all 5 days. A group of four arrestin 2 KO mice was also tested with 32 mg·kg⁻¹ SNC80. However, on day 1, this dose of SNC80 produced fatal convulsions in two mice and a sustained convulsion in a third mouse that prompted the experimenters to kill it immediately by pentobarbital overdose. Therefore, this dose of SNC80 was not further evaluated in arrestin 2 KO animals. Overall, mice lacking arrestin 2

showed increased sensitivity and attenuated tolerance to SNC80-induced convulsive effects.

The effect of arrestin 2 deletion on G protein-coupling to δ receptors after chronic exposure to SNC80

We then investigated receptor functionality of δ receptors after the establishment of tolerance to SNC80. Arrestin 2 WT and KO animals were injected and tested with vehicle or equipotent doses of SNC80 (10 mg·kg⁻¹ WT, 3 mg·kg⁻¹ KO) once daily for 5 days, and tissue was collected 24 h after the final treatment day. SNC80-induced G protein-coupling to δ receptors was examined in brain membrane preparations. [³⁵S]GTP γ S binding was severely attenuated in the brains of WT animals treated repeatedly with SNC80, in line with the observed chronic tolerance observed in these animals. In contrast, SNC80-treated arrestin 2 KO mice showed [³⁵S]GTP γ S binding that was comparable to SNC80-naïve controls (Figure 5A, B). These results indicate that in arrestin 2 KO mice δ receptor-G protein coupling is preserved, even after chronic treatment with SNC80.

Discussion

In this study, we examined the role of arrestin 2 in the development of tolerance to the behavioural effects of a high- (SNC80) or a low- (ARM390) internalizing δ receptor agonist. We found that KO of arrestin 2 results in increased efficacy and duration of action of the antihyperalgesic effects of SNC80 and increased the potency of SNC80 to produce convulsions. Loss of arrestin 2 also attenuated the development of tolerance to both the antihyperalgesic and convulsive effects of chronic SNC80 treatment. Although some chronic tolerance to the antihyperalgesic effects of SNC80 was observed, it was considerably diminished and was significantly dependent on repeated testing (behavioural/associative tolerance). In accordance with these findings, δ receptors in KO mice treated chronically with SNC80 remained functionally coupled to G proteins, an effect not observed in the WTs. In contrast, the low-internalizing agonist ARM390 produced similar analgesic effects and tolerance in both WT and KO arrestin 2 animals. Overall, these results suggest a ligand-selective recruitment of arrestins by different δ receptor agonists and reveal that SNC80 may preferentially recruit arrestin 2 to promote tolerance whereas ARM390 does not.

We have previously shown that loss of arrestin 2 enhances the antihyperalgesic effects of SNC80 and inhibits the development of acute tolerance (Pradhan *et al.*, 2016). This study reproduces those results in another group of animals and also demonstrates a potential role for arrestin 2 in regulating the development of tolerance to chronic daily administration of SNC80. After repeated administration, a marked reduction in tolerance to the antihyperalgesic effects of SNC80 was clear in arrestin 2 KO animals tested only twice, at the beginning and end of a 5- or 10-day treatment, but not in animals tested every day. A previous study showed that repeated testing of mice treated with vehicle for 5 days resulted in a 50% decrease of the analgesic effects of a subsequent challenge with SNC80 or ARM390 (Pradhan *et al.*, 2010). It is known that environmental cues and processes related

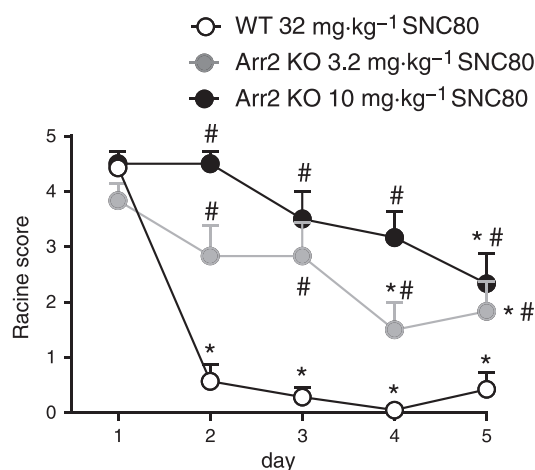


Figure 4

Development of tolerance to the convulsive effects of SNC80 is attenuated in arrestin 2 KO mice. The severity of convulsions produced by acute administration of SNC80 in WT mice (32 mg·kg⁻¹) or arrestin 2 KO mice (3.2 or 10 mg·kg⁻¹) did not differ significantly. In WT mice, SNC80 failed to produce significant convulsive effects after the first day of treatment. Repeated daily administration of SNC80 produced significant convulsive effects in arrestin 2 KO mice on all test days. For all groups $n = 6$, two-way RM ANOVA, $P < 0.05$ time, group and interaction. * $P < 0.05$ compared to same treatment group on day 1, # $P < 0.05$ compared to the WT group on the same day.

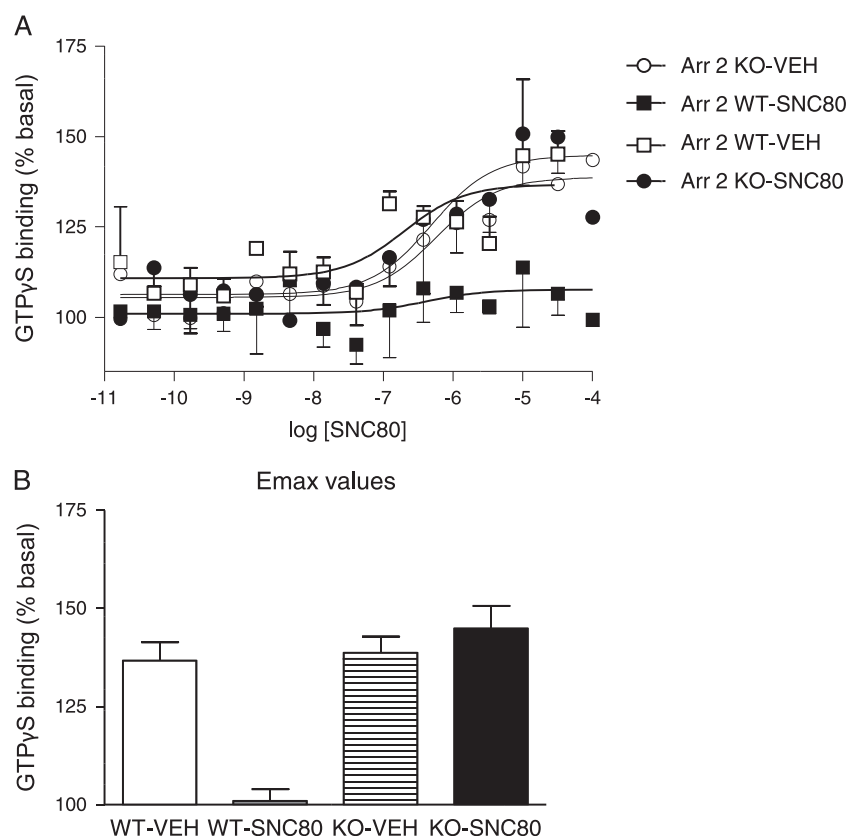


Figure 5

Arrestin 2 KO mice showed intact G protein- δ receptor coupling after chronic SNC80 treatment. Graphs show (A) concentration–response curves for SNC80-induced [35 S]-GTP γ S binding and (B) Emax values calculated from these responses. The y-axis shows mean \pm SEM specific [35 S]-GTP γ S binding expressed as percentage basal binding; $n = 3$ mice per group.

to memory and learning can facilitate tolerance development during repeated exposure to opioids (Gamble and Milne, 1989; Mitchell *et al.*, 2000). Thus, our data demonstrate that the tolerance that develops to SNC80-induced antihyperalgesia in arrestin 2 KO is significantly dependent on associative learning. Our data also show that the development of tolerance to the convulsive effects of SNC80 was attenuated in arrestin 2 KO mice, suggesting that arrestin 2 is also an important regulator of this behaviour. Although seizure activity can have profound effects on behaviour, it is unlikely to have affected antihyperalgesic responses to SNC80, as convulsive effects are lost within 10–15 min post-administration (Broom *et al.*, 2002a). In addition, the arrestin 2 KO mice developed behavioural/associative tolerance similar to WT mice, suggesting that their learning and memory was not affected. Loss of δ receptor in GABAergic forebrain neurons was sufficient to block all SNC80-induced seizurogenic activity (Chung *et al.*, 2015). It remains to be seen whether arrestin 2 acts as a negative regulator of δ receptor-mediated convulsions within these neurons. Furthermore, the role these neurons play in the development of tolerance to SNC80-induced convulsions should be examined. Taken together, these behavioural results also imply that both central (convulsions) and peripheral (antihyperalgesia) effects of SNC80 are regulated by arrestin 2.

Tolerance to the convulsive effects of SNC80 developed rapidly relative to antihyperalgesic tolerance, and almost no convulsions were observed in WT mice in the second day of SNC80 treatment. These results are in keeping with a previous report that showed that tolerance to the convulsive effects of SNC80 developed faster than tolerance to its antidepressant-like effects and that this rapid tolerance corresponded with differential tolerance rates in δ receptor signalling (Jutkiewicz *et al.*, 2005). One hypothesis is that δ receptor agonists have a low efficacy requirement to produce convulsive effects, as compared to their pain-relieving properties (Broom *et al.*, 2002b).

Consistent with the *in vivo* data, our GTP γ S assays revealed that δ receptors remain functionally coupled to G proteins in arrestin 2 KO mice repeatedly treated with SNC80. SNC80 has been shown to induce robust δ receptor internalization *in vitro* and *in vivo* (Lecoq *et al.*, 2004; Scherrer *et al.*, 2006; Pradhan *et al.*, 2009, 2010; Charfi *et al.*, 2014). This loss of surface δ receptor expression likely accounts for the decreased δ receptor function observed after repeated administration of SNC80 to WT mice. Following prolonged agonist exposure, internalized δ receptors are targeted to lysosomes and degraded (Ko *et al.*, 1999; Tsao and von Zastrow, 2000; Whistler *et al.*, 2002), a process that is also induced after chronic treatment with SNC80 (Lecoq *et al.*, 2004; Pradhan *et al.*, 2010). This receptor

down-regulation results in generalized tolerance to all the behavioural effects triggered by SNC80 (Pradhan *et al.*, 2010). Because arrestin 2 is involved in δ receptor internalization (Zhang *et al.*, 2005, 2008; Qiu *et al.*, 2007; Mittal *et al.*, 2013), the increased analgesic effects of SNC80 and decreased tolerance observed in the absence of arrestin 2 could be due to diminished receptor internalization. Therefore, more δ receptors would be accessible to SNC80 resulting in increased receptor activation. Mittal *et al.* (2013) found that loss of arrestin 2 increased the export of δ receptors to the cell membrane in response to SNC80 via a deregulation of the ROCK-LIMK pathway. This enhancement of agonist-induced externalization could also account for the differences observed in the behavioural effects of SNC80 in arrestin 2 KO mice. We hypothesize that our findings could result from a combination of both phenomena, a decrease in receptor internalization along with an increase in δ receptors externalization, thus resulting in a net enhancement of SNC80 effects.

Unlike SNC80, the antihyperalgesic effects of ARM390 were not altered in arrestin 2 KO mice. Previous BRET studies demonstrated that the low-internalizing agonist ARM390 recruits arrestin 2 at the δ receptor; however, no changes in the antihyperalgesic effects of ARM390 were observed in KOs of arrestin 2 (Pradhan *et al.*, 2016). Moreover, no significant differences in the development of acute and chronic tolerance to ARM390 were detected between arrestin 2 KO mice and WT animals. Importantly, the mechanisms involved in the development of tolerance to SNC80 and ARM390 are different. ARM390 does not promote robust receptor endocytosis, and animals treated with ARM390 do not develop acute analgesic tolerance (Pradhan *et al.*, 2010; Pradhan *et al.*, 2016). In addition, chronic tolerance to ARM390 is independent of receptor internalization and relies on cellular adaptations occurring down-stream of the receptor (Pradhan *et al.*, 2010). Thus, contrary to SNC80, recruitment of arrestin 2 at ARM390-activated δ receptors may serve a different function to the traditional role of arrestins as attenuators of GPCR signalling.

Numerous lines of evidence *in vitro* indicate that SNC80-activated δ receptors recruit both arrestin 2 and arrestin 3 (Cen *et al.*, 2001; Audet *et al.*, 2012; Mittal *et al.*, 2013; Chiang *et al.*, 2016; Pradhan *et al.*, 2016). However, our results suggest functional specialization *in vivo* for arrestin 2 in modulating the antihyperalgesic effects of SNC80 in the CFA model of chronic inflammatory pain. Furthermore, KO of arrestin 3 had no effect on SNC80-induced analgesia and acute tolerance (Pradhan *et al.*, 2016). Notwithstanding, a recent study has shown a direct correlation between the efficacy of SNC80 and other δ receptor agonists to induce recruitment of arrestin 3 and increased alcohol consumption (Chiang *et al.*, 2016). Thus, it is a possibility that our results are specific to regions controlling pain processing and convulsions and that arrestin 3 may play an important role in regulation of SNC80-activated δ receptor in other CNS areas. Further work will be needed to explain potential regional differences in agonist-specific receptor-arrestin interactions.

These results suggest that arrestin 2 differentially regulates δ receptor effects in an agonist-dependent manner. The ligand-specific recruitment of arrestins observed in our studies is likely due to distinct conformational changes induced

by the binding of different δ receptor agonists. The δ receptor agonists induce specific receptor conformations that possess different affinities for arrestins, resulting in distinct receptor desensitization, internalization and signalling profiles that ultimately dictate physiological outcomes (Aguila *et al.*, 2012; Audet *et al.*, 2012). In BRET studies, it has been shown that agonists like SNC80 stabilize a δ receptor conformation in which its C-terminal tail becomes closer to G $\beta\gamma$ subunits. This spatial rearrangement of the receptor increased the stability of its interaction with arrestin 3 and caused poor recycling and marked receptor desensitization (Audet *et al.*, 2012). Differentially, the δ receptor agonist DPDPE moved the C-terminal tail of the δ receptor away from G $\beta\gamma$ subunits and promoted a transient interaction with arrestin 3 that resulted in receptor recycling and sustained analgesia (Audet *et al.*, 2012). These studies support the notion that agonists for the same receptor can promote distinct receptor-arrestin complexes.

Our study indicates that arrestin 2 may be selectively recruited *in vivo* by high-internalizing δ agonists to modulate chronic pain. The specific deletion of arrestin 2 produced enhanced δ receptor function to the high-internalizing agonist SNC80 even after chronic treatment but did not modify the behavioural effects to the low-internalizing agonist ARM390. Collectively, our results demonstrate the behavioural significance that ligand-specific interactions between arrestin 2 and the δ receptor may have and reveal a potential role of arrestin 2 as a mediator of the analgesic effects and tolerance to high-internalizing δ receptor agonist.

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Author contributions

A.V.S., I.J.D., A.F.T., H.A. and A.A. performed the experiments. A.V.S., I.J.D., E.M.J. and A.A.P. analysed the data. A.V.S., I.J.D., E.M.J. and A.A.P. planned the experiments, wrote and edited the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies,

publishers and other organisations engaged with supporting research.

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