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

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Running Title: Internalizing DOR agonist regulated by arrestin 2

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ABSTRACT

Background and purpose: Delta opioid receptor (δOR) 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 δOR agonists. Due to the importance of arrestins 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 δOR agonists.

Experimental approach: We evaluated the effect of two δOR agonists with similar analgesic potencies, but either high-(SNC80) or low-(ARM390) internalization properties in wild-type and arrestin 2 knockout 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. Further, we determined the effect of WT and KO on chronic treatment with SNC80 in δOR agonist-stimulated [^{35}S]GTPγS binding.

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 the arrestin 2 knockout mice. Accordingly, δORs remained functionally coupled to G proteins in arrestin 2 KO mice chronically treated with SNC80.

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Conclusion and implications: Overall, these results suggest that δOR 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: delta opioid receptor (δOR), nitroglycerin (NTG), (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl benzamide (SNC80), N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide (ARM390), guanosine 5`-(γ-thio)triphosphate (GTPγS), Complete Freunds' Adjuvant (CFA), knockout (KO), wild-type (WT)

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INTRODUCTION

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

The extent and duration of GPCR signaling is governed by multiple factors, including receptor desensitization, internalization, down-regulation, and recycling. Arrestins are key regulators of GPCR desensitization and internalization (Luttrell *et al.*, 2002; Reiter *et al.*, 2012).

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In addition, arrestins act as multifunctional scaffolding proteins and signaling 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 *et al.*, 2007). Similar to many GPCRs, ligand-activated δORs are also modulated by arrestins (Lowe *et al.*, 2002; Qiu *et al.*, 2007; Raehal *et al.*, 2011; Zhang *et al.*, 2005a). In cellular models, both arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin 2) can mediate δOR desensitization and internalization after receptor phosphorylation (Hong *et al.*, 2009; Qiu *et al.*, 2007). Interestingly, emerging evidence suggests that arrestin 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, δOR 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 δOR. In mouse embryonic fibroblasts, DPDPE preferentially recruited arrestin 3 to induce phosphorylation-dependent δOR internalization (Qiu *et al.*, 2007). In contrast, arrestin 2 mediated etorphine-activated δOR 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, binding of the high-internalizing agonist SNC80 produces preferential interaction between δOR and arrestin 2, and low-internalizing agonists like ARM390 or JNJ20788560 preferentially promote arrestin 3-receptor interactions (Pradhan *et al.*, 2016). Increased interaction between the δOR and arrestin 3

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facilitated receptor resensitization and protected against the development of behavioral tolerance to low internalizing agonists (Pradhan *et al.*, 2016).

In the present study, we explored the long-term behavioral consequences of the ligand-specific interaction between arrestin 2 and δOR 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 δORs remain functionally coupled to G proteins in the absence of arrestin 2. These studies demonstrate that the ligand-selective recruitment of arrestins by δOR agonists can have profound effects on tolerance to chronic drug administration.

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METHODS

ANIMALS

All animal use procedures complied with the US National Research Council's Guide for the Care and Use of Laboratory Animals (Council, 2011) and the ARRIVE guidelines (Kilkenny *et al.,* 2010). Mice were group-housed with a maximum of 5 animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. Arrestin 2 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 WT and 124 arrestin 2 KO.

DRUGS

All drugs were administered in a volume of 10 ml kg^{-1}. SNC80 (Tocris Bioscience, Pittsburgh, PA) was dissolved in acidic 0.9% saline, pH 5.5, and injected intraperitoneally. ARM390 (AstraZeneca) was dissolved in dH2O and administered by oral gavage. Unless otherwise noted,

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mechanical sensitivity was tested 45 min after treatment with SNC80 or ARM390. All other drugs were purchased from Sigma Aldrich, St Louis, USA unless otherwise noted.

INFLAMMATORY PAIN MODEL

The 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 µl of CFA (Sigma-Aldrich, St. Louis, MO) 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 d 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 8 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 subcutaneous injection of SNC80 (3.2, 10, 32 mg kg^{-1}) every 24±1 hrs for 5 days. Following SNC80 administration, mice were observed continuously in

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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. (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 sec; 4- tonic extension or clonic convulsion lasting longer than 3 sec; 5- tonic extension or clonic convulsion lasting more than 3 sec with loss of balance. Post-convulsion catalepsy-like behavior 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 sec.

GTPyS ASSAY

Brain membrane preparations were carried out as described previously (Befort *et al.*, 2001). Animals treated and tested chronically with vehicle or SNC80 for 5 days were anesthetized with isoflurane gas and euthanized by decapitation 24h after the final treatment day. Whole brain from WT and KO mice chronically treated and tested with agonist or vehicle was 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.

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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 [^{35}S]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 TrisHCl (pH 7.4), 3 mM MgCl2, 100 mM NaCl, 0.2 mM EGTA, 30 µM GDP and 0.1 nM [^{35}S]GTPγS (Perkin Elmer USA). Incubation was terminated by rapid filtration and washing in ice-cold buffer (50 mM TrisHCl, 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/group. However, animals were excluded from analysis if there was an error in drug injection, genotyping, or if they were

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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, for convulsion experiments arrestin 2 KO mice died or were with euthanized following 32 mg/kg SNC80; and 1 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 2-way ANOVA was used. For all tests, 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 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. EC_{50} 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).

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RESULTS

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

To evaluate the role of arrestin 2 in δOR-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 minutes (Figure 1A and C) and 3 hours (Figure 1B and D) following injection. In wild-type mice, SNC80 and ARM390 produced dose-dependent increases in mechanical threshold 45 minutes after administration (Figure 1A and 1C). After 3 hours, SNC80 was no longer effective in wild-type animals likely due to drug clearance. However, in arrestin 2 knockout mice SNC80, but not ARM390, was enhanced at this timepoint; and SNC80 continued to produce significant antihyperalgesic effects (Figure 1B). ARM390 failed to produce antihyperalgesic effects in either genotype 3 hours post-injection (Figure 1D). These data show that SNC80, but not ARM390, has increased efficacy and duration of action in arrestin 2 knockout 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 δOR 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). 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 dose of SNC80 and WTs a 10

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mg/kg dose. For ARM390 both genotypes were administered a 10 mg/kg dose. CFA was injected into the mouse paw, and severe mechanical hypersensitivity was observed 72h 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 hours later was ineffective in WT animals (Figure 2A, Injection 2). However, this acute behavioral 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 behavioral tolerance, we examined the consequences of repeated SNC80 or ARM390 treatment in arrestin 2 WT and KO animals. Wild-type 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 knockout mice (Figure 3A). To rule out the possibility that these effects were due to wild-type and knockout mice developing behavioral/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

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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, wild-type 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 δOR-mediated behaviors, 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 five 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 one 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

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(3.2 or 10 mg kg^{-1}) and this effect was observed on all five days. A group of four arrestin 2 knockout mice was also tested with 32 mg kg^{-1} SNC80. However, on day 1 this dose of SNC80 produced fatal convulsions in 2 mice and a sustained convulsion in a third mouse that prompted the experimenters to euthanize 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 δORs after chronic exposure to SNC80

We then investigated receptor functionality of δORs 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 five days, and tissue was collected 24h after the final treatment day. SNC80-induced G protein-coupling to δORs was examined in brain membrane preparations. [^{35}S]GTPγS 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 [^{35}S]GTPγS binding that was comparable to SNC80-naïve controls (Fig. 5A, B). These results indicate that in arrestin 2 knockouts δOR-G protein coupling is preserved, even after chronic treatment with SNC80.

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DISCUSSION

In this study, we examined the role of arrestin 2 in the development of tolerance to the behavioral effects of a high- (SNC80) or a low- (ARM390) internalizing δOR agonist. We found that knockout 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 (behavioral/associative tolerance). In accordance with these findings, δORs in knockout 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 wild-type and knockout arrestin 2 animals. Overall, these results suggest a ligand-selective recruitment of arrestins by different δOR 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 reproduced 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

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SNC80 was clear in arrestin 2 knockout animals tested only twice, at the beginning and end of a five or ten day treatment; but not in animals tested every day. A previous study showed that repeated testing of mice treated with vehicle for five 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 to memory and learning can facilitate tolerance development during repeated exposure to opioids (Gamble *et al.*, 1989; Mitchell *et al.*, 2000). Thus, our data demonstrates that the tolerance that develops to SNC80-induced antihyperalgesia in arrestin 2 KOs 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 knockout mice, suggesting that arrestin 2 is also an important regulator of this behavior. Although seizure activity can have profound effects on behavior, it is unlikely to have affected antihyperalgesic responses to SNC80, as convulsive effects are lost within 10-15 minutes post-administration (Broom *et al.*, 2002a). In addition, the arrestin 2 KOs developed behavioral/associative tolerance similar to WTs, suggesting that their learning and memory was not affected. Loss of δOR 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 δOR-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 behavioral results also imply that both central (convulsions) and peripheral (antihyperalgesia) effects of SNC80 are regulated by arrestin 2.

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Tolerance to the convulsive effects of SNC80 developed rapidly relative to antihyperalgesic tolerance, and almost no convulsions were observed in wildtype mice 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 δOR signaling (Jutkiewicz *et al.*, 2005). One hypothesis is that δOR 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 GTPγS assays revealed that δORs remain functionally coupled to G proteins in arrestin 2 knockouts repeatedly treated with SNC80. SNC80 has been shown to induce robust δOR internalization *in vitro* and *in vivo* (Charfi *et al.*, 2014; Lecoq *et al.*, 2004; Pradhan *et al.*, 2009; Pradhan *et al.*, 2010; Scherrer *et al.*, 2006). This loss of surface δOR expression likely accounts for the decreased δOR function observed after repeated administration of SNC80 to WT mice. Following prolonged agonist exposure, internalized δORs are targeted to lysosomes and degraded (Ko *et al.*, 1999; Tsao *et al.*, 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 downregulation results in generalized tolerance to all the behavioral effects triggered by SNC80 (Pradhan *et al.*, 2010). Because arrestin 2 is involved in δOR internalization (Mittal *et al.*, 2013; Qiu *et al.*, 2007; Zhang *et al.*, 2008; Zhang *et al.*, 2005b), the increased analgesic effects of SNC80 and decreased tolerance observed in the

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absence of arrestin 2 could be due to diminished receptor internalization. Therefore, more δORs would be accessible to SNC80 resulting in increased receptor activation. Mittal et al. (2013) found that loss of arrestin 2 increased the export of δORs 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 behavioral effects of SNC80 in arrestin 2 KOs. We hypothesize that our findings could result from a combination of both phenomena, a decrease in receptor internalization along with an increase in δORs 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 δOR, however no changes in the antihyperalgesic effects of ARM390 were observed in knockouts 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 knockouts and wild-type 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.*, 2016; Pradhan *et al.*, 2010). 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

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ARM390-activated δORs may serve a different function to the traditional role of arrestins as attenuators of GPCR signaling.

Numerous lines of evidence *in vitro* indicate that SNC80-activated δORs recruit both arrestin 2 and arrestin 3 (Cen et al., 2001; Pradhan et al., 2016;Van Rijn et al., 2015; Audet et al., 2012; Mittal et al., 2013). 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, knockout 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 δOR agonists to induce recruitment of arrestin 3 and increased alcohol consumption (Chiang *et al.*, 2015). 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 δOR 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 δOR 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 δOR agonists. δOR agonists induce specific receptor conformations that possess different affinities for arrestins, resulting in distinct receptor desensitization, internalization, and signaling profiles which ultimately dictate physiological outcomes (Aguila *et al.*, 2011; Audet *et al.*, 2012). In BRET studies, it has been shown that agonists like SNC80 stabilize a δOR conformation in which its C-

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terminal tail becomes closer to Gβγ 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 δOR agonist DPDPE moved the C-terminal tail of the δOR away from Gβγ 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 δOR function to the high-internalizing agonist SNC80 even after chronic treatment; but did not modify the behavioral effects to the low-internalizing agonist ARM390. Collectively, our results demonstrate the behavioral significance that ligand-specific interactions between arrestin 2 and the delta opioid receptor may have, and reveal a potential role of arrestin 2 as a mediator of the analgesic effects and tolerance to high internalizing δOR agonist.

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Conflicts of Interest

AAP has previously received funding from Trevena Inc.

CONTRIBUTIONS

AVS, IJD, AFT, HA, AA performed experiments. AVS, IJD, EMJ, AAP analyzed data. AVS, IJD, EMJ, AAP planned the experiments, and wrote and edited the manuscript.

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FIGURE LEGENDS

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 (IP) and ARM390 (PO). (A) The anti-hyperalgesic 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/group. 2-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 (2-way ANOVA, $p<0.05$ effect of dose), and regardless of genotype this antihyperalgesic effect was lost 3 hours post-administration (D), n=7; vehicle and 10 mg/kg; n=9; 1 and 3 mg/kg. Dashed lines represent baseline mechanical responses assessed pre-CFA injection.

Figure 2. Acute behavioral 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) wildtype and (B) arrestin 2 KO mice treated with vehicle (control, n=10/genotype), SNC80 (3 mg/kg for KOs, 13/group, and 10 mg/kg for WTs, 12/group IP), or ARM390 (10 mg/kg, PO, 12/genotype). Injection 2, animals re-challenged with the same drug and dose 4h following Injection 1. Dashed lines represent baseline mechanical responses pre-CFA injection. For consistency, all animals were injected IP and PO. 2-way RM ANOVA for each genotype, $p<0.01$

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drug, time, and interaction for WT; $p<0.01$ for drug in KO. **$p<0.01$ as compared to Injection 1, Holm-Sidak post-hoc analysis. In addition, $p<0.01$ 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 mg/kg for WTs, and 3 mg/kg for KOs) daily for 5 days, and tested daily, 45 minutes following each injection (A, n=10/WT group, n=12/KO group; 2 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/group; 2-way RM ANOVA, $p<0.05$ genotype X 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/group; 2-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 mg/kg, PO) daily for 5 days, and tested 45 minutes following each injection. n=6/group, 2-way RM ANOVA $p<0.05$ for time only.

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Figure 4. Development of tolerance to the convulsive effects of SNC80 is attenuated in arrestin 2 KOs. The severity of convulsions produced by acute administration of SNC80 in WT mice (32 mg/kg) or arrestin 2 KO mice (3.2 or 10 mg/kg) did not differ significantly. In wild-type 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 KOs on all test days. $n = 6$ for all groups, 2-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 KOs showed intact G protein-δOR coupling after chronic SNC80 treatment. Graphs show the (A) concentration-response curves for SNC80-induced $[^{35}S]GTP\gamma S$ binding, and the (B) calculated Emax values from these responses. The y-axis shows mean ± SEM specific $[^{35}S]GTP\gamma S$ binding expressed as percentage basal binding. n=3mice/group. Chronic SNC80 treatment impaired agonist stimulated $[^{35}S]GTP\gamma S$ binding in wildtype but not arrestin 2 KO mice.

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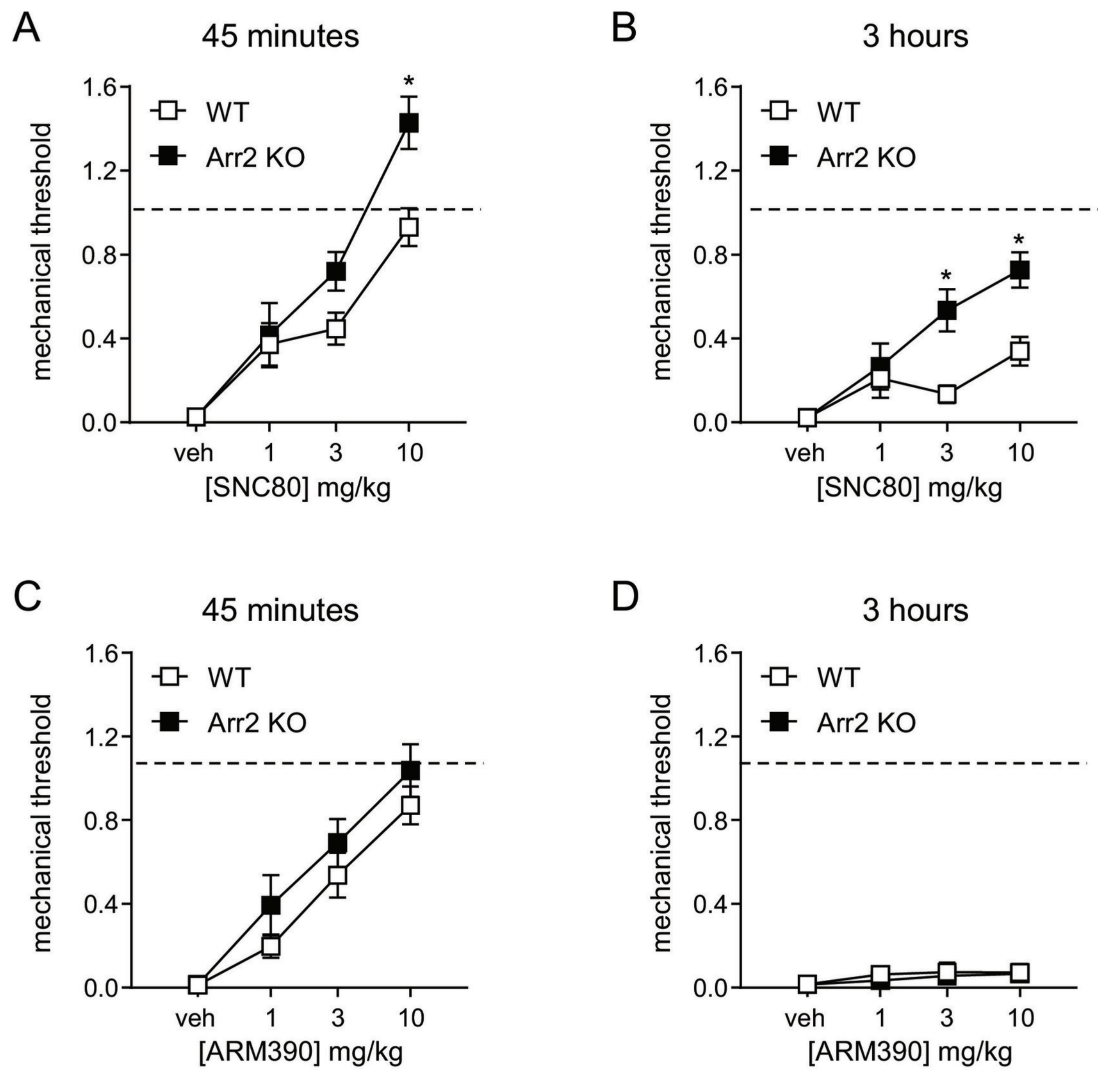

Figure 1

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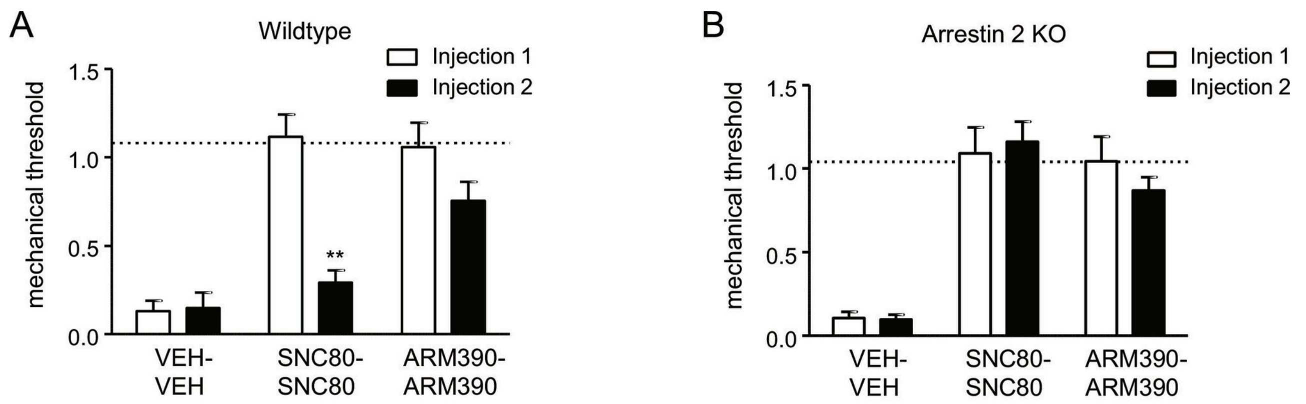

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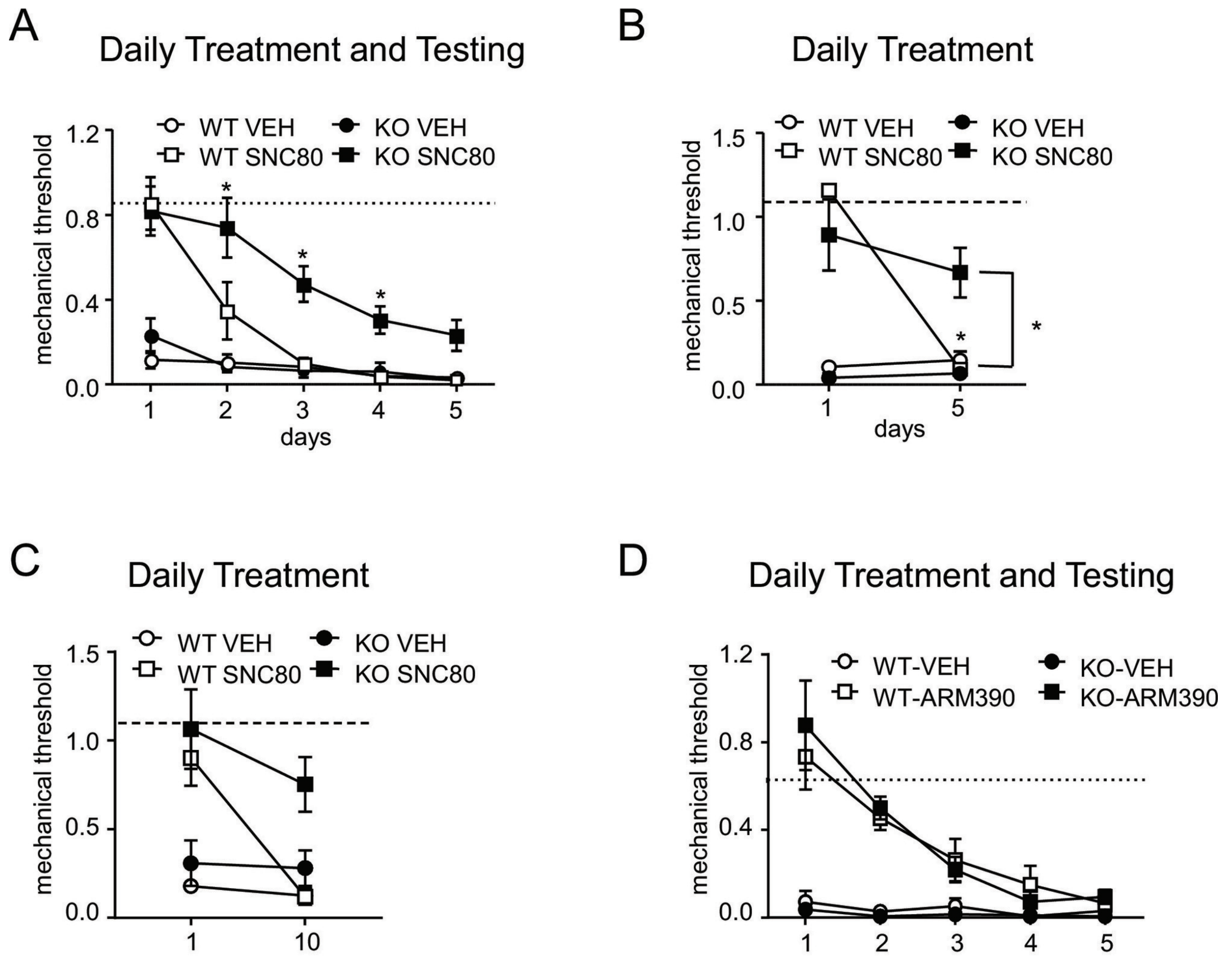

Figure 3

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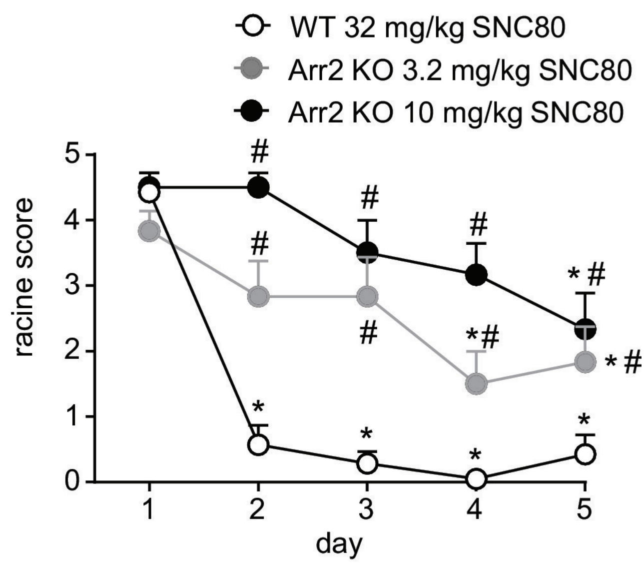

Figure 4

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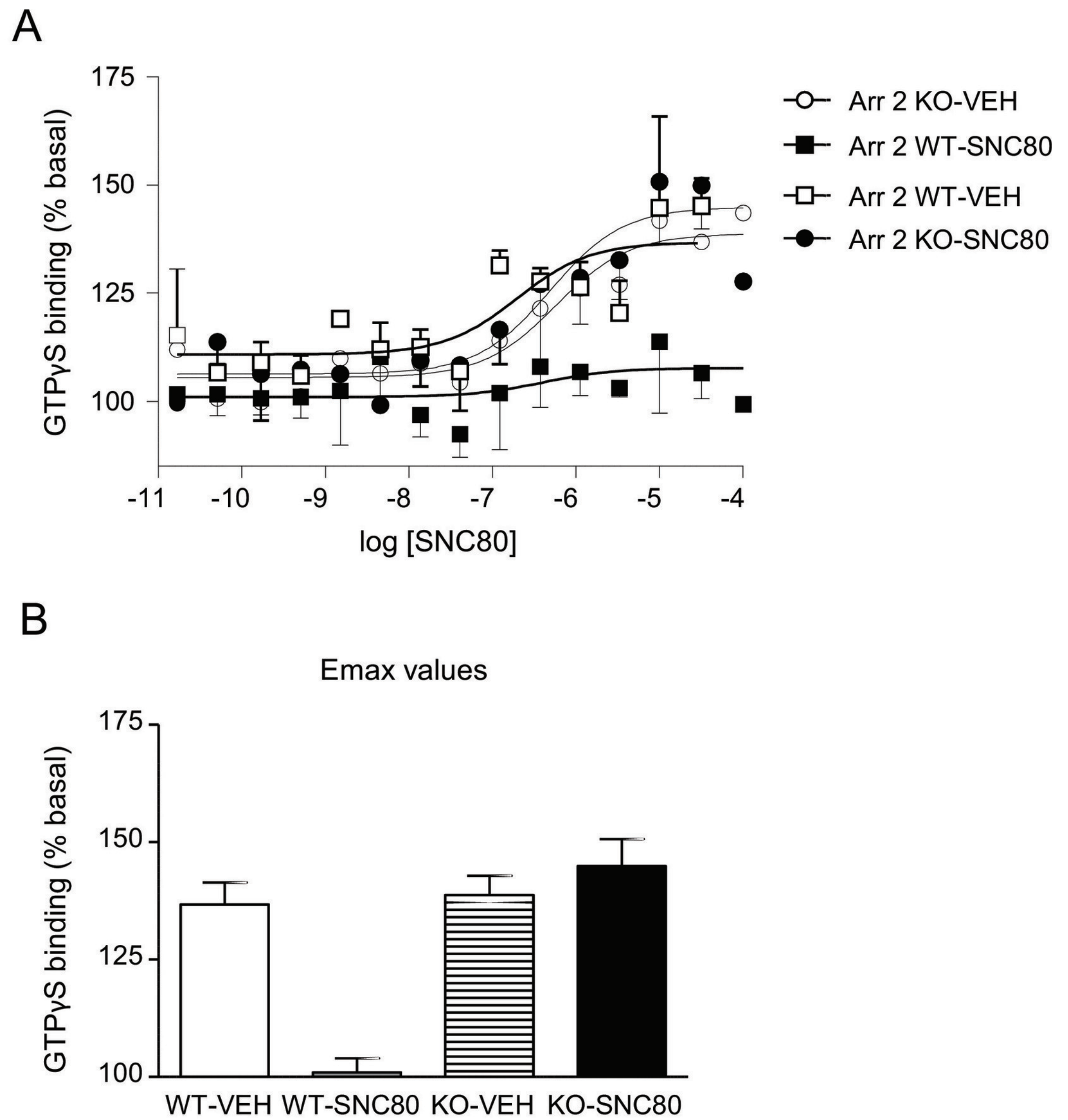

Figure 5

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