Traynor John (Orcid ID: 0000-0002-1849-8316)
Jutkiewicz Emily (Orcid ID: 0000-0002-1154-1684)

Role of signaling molecules in behaviors mediated by the δ-receptor agonist SNC80

Running Title: Signaling bias and δ-receptor activity in vivo

Isaac J. Dripps[1], Brett T. Boyer[1], Richard R. Neubig MD, PhD[2], Kenner C. Rice PhD[3], John R Traynor PhD[1], Emily M. Jutkiewicz PhD[1]

[1]- Department of Pharmacology, University of Michigan Medical School [2]- Department of Pharmacology and Toxicology, Michigan State University [3]- Drug Design and Synthesis Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse

Corresponding Author:

Emily M. Jutkiewicz
A220A MSRB III
1150 W. Medical Center Dr.
Ann Arbor, MI 48109
Phone: 734-764-8612
Fax: 734-763-4450
Email: ejutkiew@umich.edu

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List of Abbreviations

δ-receptor - delta opioid receptor
CFA- complete Freund's adjuvant
RGS- regulator of G protein signaling
GPCR- G protein-coupled receptor
GRK- G protein-coupled receptor kinase
FST- forced swim test
IP- intraperitoneal
NTG- nitroglycerin
SC- subcutaneous

Abstract

Background and Purpose: G protein-coupled receptors exist in multiple conformations that can engage distinct signaling mechanisms which in turn may lead to diverse behavioral outputs. In rodent models, activation of the delta opioid receptor (δ-receptor) has been shown to elicit antihyperalgesia, antidepressant-like effects, and convulsions. We recently showed that these δ-receptor-mediated behaviors are differentially regulated by the GTPase-activating protein regulator of G protein signaling 4 (RGS4), which facilitates termination of G protein signaling. To further evaluate the signaling mechanisms underlying δ-receptor-mediated antihyperalgesia, antidepressant-like effects, and convulsions, we observed how changes in $G\alpha_o$ or arrestin proteins in vivo affected behaviors elicited by the δ-receptor agonist SNC80 in mice.

Experimental Approach: Transgenic mice with altered expression of various signaling molecules were used in the current studies. Antihyperalgesia was measured in a nitroglycerin-induced thermal hyperalgesia assay. Antidepressant-like effects were evaluated in the forced swim test. Mice were also observed for convulsive activity following SNC80 treatment.

Key Results: In $G\alpha_o$ RGS-insensitive heterozygous knock-in mice, the potency of SNC80 to produce antihyperalgesia and antidepressant-like effects was enhanced with no change in SNC80-induced convulsions. Conversely, in $G\alpha_o$ heterozygous knockout mice, SNC80-induced antihyperalgesia was abolished while antidepressant-like effects and convulsions were unaltered. No changes in SNC80-induced behaviors were observed in arrestin 3 knockout mice. SNC80-induced convulsions were potentiated in arrestin 2 knockout mice.

Conclusions and Implications: Taken together, these findings suggest that different signaling molecules may underlie the convulsive effects of the δ-receptor relative to the antihyperalgesic and antidepressant-like effects.

Introduction

G protein-coupled receptors (GPCRs) are a diverse family of membrane bound receptors that regulate a wide array of biological functions. Canonically, GPCRs regulate these processes through activation of G proteins which subsequently interact with a variety of downstream effectors. Following agonist activation, a GPCR is phosphorylated by G protein-coupled receptor kinases (GRKs) and internalized following recruitment of arrestins. In recent years, it has become apparent that GPCRs can signal through G protein-independent mechanisms (Galandrin et al. 2007) by directly recruiting arrestins that can also promote signaling from GPCRs (Reiter et al. 2012). Furthermore, ligands that act at the same orthosteric site on a receptor can stabilize distinct active conformations that preferentially signal through distinct G protein or arrestin subtypes. This phenomenon, known as functional selectivity or biased agonism, has been observed with multiple GPCRs including the β2 adrenergic receptor (Drake et al. 2008), the CB1 cannabinoid receptor (Hudson et al. 2010), as well as μ-, κ-, and δ-opioid receptors (Pradhan et al. 2012).

The δ-receptor is a class A GPCR and interacts with $G\alpha_{i/o}$ proteins. Activation of the δ-receptor in rodents has been shown to produce antinociception, antihyperalgesia, anxiolytic effects, and antidepressant-like effects without the constipation, respiratory depression, and abuse liability observed with μ-receptor agonists (for review see Chu Sin Chung and Kieffer 2013). In addition, some δ-receptor agonists cause convulsions, which has limited their clinical utility (Comer et al. 1993; Hong et al. 1998).

The signaling pathways that bring about δ-receptor-mediated behaviors are only beginning to be understood. Targeted knockdown of specific G protein subunits using antisense nucleotides inhibited δ-receptor-mediated spinal and supraspinal antinociception in mice, implicating multiple $G\alpha_{i/o}$ subtypes in the regulation of these effects (Standifer et al. 1996; Sánchez-Blázquez and Gárzon 1998). Loss of regulator of G protein signaling 4 (RGS4) potentiated the antinociceptive, antihyperalgesic, and antidepressant-like effects of the δ-receptor agonist SNC80 suggesting that these behaviors are generated through G protein signaling

(Dripps et al. 2017). However, this study also found that the frequency of SNC80-induced convulsions was not altered in RGS4 knockout mice suggesting that δ-receptor-mediated convulsions may signal through a G protein-independent mechanism. Loss of arrestin 2 (β-arrestin 1) increased the potency of SNC80 to induce mechanical antihyperalgesia, whereas loss of arrestin 3 (β-arrestin 2) produced acute tolerance to the antihyperalgesic effects of the δ-receptor agonists ARM390 and JNJ20788560 (Pradhan et al. 2016).

Use of a δ-receptor agonist that is biased towards producing the analgesic and antidepressant-like effects could be an effective strategy for improving the safety and clinical utility of δ-receptor ligands. A detailed understanding of the intracellular signaling pathways that give rise to δ-receptor-mediated behaviors, and δ-receptor-mediated convulsions in particular, is critical for the development of such drugs. Therefore, to gain a better understanding of the downstream signaling mechanisms that give rise to δ-receptor-mediated behaviors, we evaluated how altering $G\alpha_o$ and arrestin molecules affected these behaviors. Specifically, we examined the potency of SNC80 to produce antihyperalgesia, antidepressant-like effects, and convulsions in $G\alpha_o$ heterozygous knockout mice, $G\alpha_o$ RGS-insensitive heterozygous knock-in mice, as well as arrestin 2 and arrestin 3 knockout mice.

Materials and 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. Mice had free access to food and water at all times. Animals were housed in pathogen free rooms maintained between 68-79°F and humidity between 30-70% humidity with a 12h light/dark cycle with lights on at 7:00 AM. Experiments were conducted in the housing room during the light cycle. All mice were used between 8 and 15 weeks of age at time of experiment and weighed 16-32 g. Mice were

tested only once, and all analyses are between-subject with the exception of the hot plate test (within-subject analysis). For *in vivo* experiments, 6 mice per experimental condition (e.g., per drug and per genotype) were used with a total of 904 mice used for the entire study. Treatment conditions were randomized across cages of mice and across at least 3 independent experiments. For in vivo studies, power analysis (α=0.05; 1-β=0.9) revealed that for a calculated effect size of 1-3 (Cohen's d), depending on the experiment, that a sample size of 4-6 mice per experimental condition would be needed (G*Power 3.1.9.2, Faul *et al.*, 2007).

The arrestin 3 knockout mouse strain ($Arrb2^{tm1Rjl/J}$) was obtained from The Jackson Laboratory (Bar Harbor, Maine, https://www.jax.org/strain/011130). Arrestin 2 knockout mice ($Arrb1^{tm1jse}$, https://www.jax.org/strain/011131) were gifted by Dr. Amynah A. Pradhan (University of Illinois at Chicago). $G\alpha_o$ RGS-insensitive heterozygous knock-in mice (Goldstein *et al.*, 2009) were obtained from Dr. Richard Neubig and $G\alpha_o$ knockout mice were obtained from Dr. Richard Mortensen (Duan *et al.*, 2007). Mice were backcrossed at least ten generations into a C57BL/6 background and maintained in-house as heterozygote harem (1 male, 2 female) breeding groups except for arrestin 2 knockout mice which were maintained as homozygote harem breeding groups. C57BL/6 mice are the background strain for all the genetic knockout strains used in this study. C57BL/6 mice were used for all studies as this species is commonly used in pharmacological and behavioral research and is consistent with our previous studies (Dripps et al 2017). Wild-type littermates (+/+) were used as controls for all strains except arrestin 2 knockout mice in which case arrestin 3 wild-type littermates were used. For studies in which transgenic mice were not required, C57BL/6N mice (17-30g) were obtained from Envigo (formerly Harlan, Indianapolis, IN). Breeder mice in the $G\alpha_o$ RGS-insensitive knock-in, $G\alpha_o$ knockout, and arrestin 3 knockout mouse colonies were supplemented with gamma-irradiated peanuts in the shell (S6711, Bio-Serv, Flemington, NJ) to enhance litter size and production of transgenic mice.

Drugs

All drugs were injected at a volume of 10 ml·kg^{-1}unless otherwise noted. SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was dissolved in 1 M HCl and diluted in sterile water to a concentration of 3% HCl. Nitroglycerin (NTG) was provided by Dr. Adam Lauver (Department of Pharmacology and Toxicology, Michigan State University) at a concentration of 5 mg/ml and was diluted in saline. Desipramine hydrochloride (Sigma-Aldrich, St. Louis, MO), sumatriptan succinate (Sigma-Aldrich, St. Louis, MO) and morphine sulfate were dissolved in saline. All drugs were given subcutaneously (sc) except for NTG which was administered via intraperitoneal (ip) injection.

Forced Swim Test

The forced swim test (FST) is an assay that is widely used to evaluate the antidepressant-like effects of drugs in rodents (Barkus, 2013). Our experiments were adapted from Porsolt *et al.* (1977) and performed as previously described (Dripps *et al.*, 2016). Briefly, sixty min after SNC80 (0.1, 0.32, 1, 3.2, 10, or 32 mg·kg^{-1}) or vehicle injection, each mouse was placed in a 4L beaker filled with 15 cm of 25±1°C water and its behavior was recorded for 6 min using a Sony HDR-CX220 digital camcorder. Videos were analyzed by individuals blind to the experimental conditions and the amount of time the animals spent immobile was quantified. Immobility was defined as the mouse not actively traveling through the water and making only movements necessary to stay afloat. The time the mouse spends immobile after the first 30 sec of the assay was recorded.

Nitroglycerin-Induced Hyperalgesia

The NTG-induced hyperalgesia assay was adapted from Bates *et al.* (2010) using modifications described in Pradhan *et al.* (2014) and performed as previously described (Dripps *et al.*, 2017). In brief, male and female mice were used to evaluate NTG-induced hyperalgesia. Hyperalgesia was assessed by immersing the tail (~5cm from the tip) in a 46°C water bath and determining the latency for the animal to withdraw its tail with a cut-off time of 60 sec. After

determining baseline withdrawal latencies, 10 mg·kg^{-1} NTG (ip) was administered to each animal. Tail withdrawal latency was assessed again 1 hr after NTG administration. At 90 min post-NTG, animals received an injection of SNC80 (0.32, 1, 3.2, 10, or 32 mg·kg^{-1}) or vehicle, and mice were observed continuously in individual cages for 30 min to observe for convulsions (see section below). Tail withdrawal latencies were assessed again 30 min after SNC80 administration.

SNC80-Induced Convulsions

Mice were observed continuously in individual cages for convulsions. Unless otherwise noted, NTG treatment had no significant effect on the frequency or nature of SNC80-induced convulsions (see supplemental information). Convulsions were typically comprised of a single 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. Mice would frequently lose balance and fall on their side, although so called barrel rolling was rarely observed. Convulsions were followed by a period of catalepsy that lasted 2-5 min after which the animals were hyperlocomotive but otherwise indistinguishable from untreated controls. The severity of each convulsion was quantified using the following modified Racine (1972) scale adapted from Jutkiewicz *et al.* (2006): 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 placing a horizontal rod under the forepaws of the mouse and a positive catalepsy score was assigned if the mouse did not remove its forepaws after 30 sec. Two arrestin 2 knockout mice that received 32 mg/kg SNC80 exhibited sustained convulsions after the observation period and were euthanized by pentobarbital overdose.

Hot Plate Test

The hot plate test was adapted from Lamberts et al. (2011) and was chosen because it has previously been used to evaluate the antinociceptive effects of opioids in arrestin 3 knockout mice (Bohn *et al.*, 1999). Briefly, mice were placed on a 52°C hot plate and the latency to lick forepaw(s) or jump was measured with a cutoff time of 60s in order to prevent tissue damage. To determine baseline latency, mice were placed on the hot plate 30 min after each of two injections of saline. Following an injection of 32 $mg \cdot kg^{-1}$ morphine, latency was assessed every 30 min.

δ-Receptor Saturation Binding

Mice were decapitated following cervical dislocation, the forebrain was removed immediately, and membranes were freshly prepared as previously described (Broom *et al.*, 2002a). Tissue collection without anesthesia was used to limit modification to δ-receptor number, conformation, and/or localization and is conditionally acceptable with justification under the American Veterinary Medical Association Guidelines for the Euthanasia of Animals. Protein concentrations were determined with a BCA assay kit (Thermo Scientific, Rockford, IL). Specific binding of the δ-receptor agonist [^{3}H]DPDPE was determined as described using 10μM of the opioid antagonist naloxone to define non-specific binding as described (Broom *et al.*, 2002a). Reactions were incubated for 60 min at 26ºC and stopped by rapid filtration through GF/C filter mats soaked in 0.1% PEI using a MLR-24 harvester (Brandel, Gaithersburg, MD). Bound [^{3}H]DPDPE was determined by scintillation counting and B_{max} and K_d values calculated using nonlinear regression analysis with GraphPad Prism version 6.02 (GraphPad, San Diego, CA). To ensure the reliability of single values, membranes from each mouse (n = 5 per group) were assayed in triplicate.

Data Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). All data analysis was performed using GraphPad Prism version 6.02 (GraphPad, San Diego, CA). For all tests, level of significance (α)

was set to 0.05. Post hoc analysis was conducted using the Sidak's post hoc test to correct for multiple comparisons. Post hoc analysis was only performed when F values achieved $p < 0.05$. All values in the text are reported as mean ± SEM. ED_{50} values were calculated using GraphPad Prism version 6.02 by extrapolating the 50% maximum effect from the straight line analysis of the averaged treatment group data used to generate each dose effect function.

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 (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2015; Alexander et al., 2017).

Results

δ-receptor-mediated behaviors in $G\alpha_o$ RGS-insensitive mice

It has previously been demonstrated that loss of RGS4 potentiates δ-receptor-mediated antihyperalgesia and antidepressant-like effects, but not δ-receptor-mediated convulsions (Dripps *et al.*, 2017). To further investigate the signaling mechanisms involved in these behaviors, we characterized these behaviors in $G\alpha_o$ RGS-insensitive heterozygous mice. The $G\alpha_o$ RGS-insensitive heterozygous mice have one copy of *GNAO1* with a G184S point mutation that prevents binding of all RGS proteins to $G\alpha_o$ and should enhance signaling from those G proteins (Goldstein *et al.*, 2009; Lamberts *et al.*, 2013). First, the potency of SNC80 to reverse NTG-evoked thermal hyperalgesia was evaluated in $G\alpha_o$ RGS-insensitive heterozygous mice (+/GS) and their wild-type littermates (+/+; Figure 1A). The +/GS mice did not differ significantly from wild-type littermates in their baseline tail withdrawal latencies prior to NTG treatment (+/+: 42.4 ± 2.5 s, +/GS: 41.4 ± 1.4 s). Administration of 10 $mg \cdot kg^{-1}$ NTG significantly decreased tail withdrawal latency to a similar degree in both genotypes (+/+: 6.1 ± 1.3 s, +/GS: 5.4 ± 0.3 s). Two-way ANOVA revealed a significant interaction (SNC80 dose X genotype, $F(5,60) = 7.61$),

as well as significant main effects of SNC80 dose ($F(5,60) = 56.15$) and genotype ($F(1,60) = 53.07$). There was an approximately 3.5-fold leftward shift in the SNC80 dose effect curve (ED_{50} values: +/+: 4.8 $mg \cdot kg^{-1}$; +/GS: 16.6 $mg \cdot kg^{-1}$) and a significant increase in the maximum effect observed in the +/GS mice compared with their wild-type littermates. Overall, the potency and efficacy of SNC80 to increase tail withdrawal latency was enhanced in $G\alpha_o$ RGS-insensitive heterozygous mice.

The potency of SNC80-induced antidepressant-like effects in $G\alpha_o$ RGS-insensitive heterozygous mice was evaluated in the FST (Figure 1B). In the absence of drug treatment, +/GS mice had lower immobility scores than wild-type littermates. SNC80 significantly decreased immobility scores to a greater extent in +/GS mice compared to wild-type littermates. Due to the basal differences in immobility scores, scores were normalized to a percentage relative to vehicle treated mice of the appropriate genotype (Figure 1 C). Two-way ANOVA of the transformed data revealed significant main effects of SNC80 dose ([vehicle and 0.32-10 $mg \cdot kg^{-1}$ only] $F(4,50) = 17.1$) and genotype ($F(1,50) = 4.80$), as well as a significant interaction (SNC80 dose X genotype, $F(4,50) = 6.23$). To investigate whether $G\alpha_o$ RGS-insensitive heterozygous mice were hyper-responsive to a wider array of antidepressive drugs, the effects of the tricyclic antidepressant desipramine were evaluated in the FST (Figure 1D). Desipramine produced decreases in immobility (main effect of desipramine dose: $F(2,30) = 12.43$), but there was no effect of genotype and no significant interaction.

Although loss of RGS4 did not alter SNC80-induced convulsions, other RGS proteins may play a role in regulating this behavior. Therefore, we evaluated SNC80-induced convulsions in $G\alpha_o$ RGS-insensitive heterozygous mice and wild-type littermates and scored convulsion severity using a modified Racine scale (Figure 1E). SNC80 produced similar dose-dependent increases in convulsion severity in both genotypes. There were no significant differences in the frequency of convulsions or time of onset and duration of SNC80-induced convulsions (see supplemental information).

It is possible that the enhanced behavioral effects of SNC80 in $G\alpha_o$ RGS-insensitive heterozygous mice are due to a change in receptor density or agonist affinity for the δ-receptor relative to their wild-type littermates. To evaluate potential changes in these parameters, saturation binding with the radiolabeled δ-receptor agonist [^{3}H]DPDPE was performed using brain tissue from $G\alpha_o$ RGS-insensitive +/+ and +/GS mice. There were no significant differences in total receptor number of the +/GS mice compared to wild-type littermates (Table 1; Figure 1F). In addition, there were no changes in the affinity of [^{3}H]DPDPE for the δ-receptor in the $G\alpha_o$ RGS-insensitive heterozygous mice.

δ-receptor-mediated behaviors in $G\alpha_o$ heterozygous knockout mice

To further evaluate the role of $G\alpha_o$ in δ-receptor-mediated behaviors, we characterized δ-receptor-mediated antihyperalgesia, antidepressant-like effects, and convulsions in $G\alpha_o$ heterozygous knockout mice. $G\alpha_o$ null mice rarely survived to weaning (Lamberts *et al.*, 2011). Therefore we chose to only evaluate $G\alpha_o$ wild-type and heterozygous knockout mice.

Prior to NTG administration, there were no significant differences in tail withdrawal latency in $G\alpha_o$ wild-type and heterozygous knockout mice (+/+: 41.2 ± 1.8 s, +/-: 40.3 ± 2.0 s). Administration of 10 mg·kg^{-1} NTG produced similar decreases in tail withdrawal latency in both genotypes (+/+: 4.9 ± 0.5 s, +/-: 4.1 ± 0.3 s). In $G\alpha_o$ wild-type mice, SNC80 produced dose-dependent increases in tail withdrawal latency following NTG administration (Figure 2A). This effect was abrogated in $G\alpha_o$ heterozygous knockout mice. Two-way ANOVA revealed significant main effects of SNC80 dose [vehicle and 10-100 mg·kg^{-1} only] ($F(4,50) = 30.85$) and genotype ($F(1,50) = 256.1$), as well as a significant interaction (SNC80 dose X genotype, $F(4,50) = 19.04$). To investigate whether the antihyperalgesic effects of non-δ-receptor drugs were altered in $G\alpha_o$ heterozygous knockout mice, the effects of the 5-$HT_{1B/1D}$ agonist sumatriptan on NTG-induced thermal hyperalgesia were examined (Figure 2B). Sumatriptan produced similar robust increases in tail withdrawal latency in wild-type and $G\alpha_o$ heterozygous knockout mice

(two-way ANOVA main effect of sumatriptan dose: $F(2,30) = 91.28$, but no main effect of genotype and no interaction).

In the FST, SNC80 produced significant decreases in immobility in both the $G\alpha_o$ wild-type and heterozygous knockout mice (Figure 2C; Two-way ANOVA main effect of SNC80 dose: $F(4,50) = 22.05$)). However, there were no significant differences between genotypes in the immobility scores produced in response to a given dose of SNC80. SNC80 also produced similar dose-dependent increases in convulsion severity in $G\alpha_o$ wild-type and heterozygous knockout mice (Figure 2D). There were no significant differences in frequency of convulsions or the time of onset and duration of SNC80-induced convulsions (see supplemental information).

The diminished effect of SNC80 on NTG-induced hyperalgesia in $G\alpha_o$ heterozygous knockout mice could be due to decreased receptor density of or agonist affinity at the δ-receptor relative to wild-type littermates. To evaluate potential changes in density or agonist affinity, saturation binding with the radiolabeled δ-receptor agonist [^{3}H]DPDPE was performed using brain tissue from $G\alpha_o$ wild-type and heterozygous knockout mice. There were no significant differences in total receptor number or affinity of [^{3}H]DPDPE for the δ-receptor in the $G\alpha_o$ heterozygous knockout mice relative to wild-type littermates (Table 1; Figure 2E).

δ-receptor-mediated behaviors in arrestin 2 and arrestin 3 knockout mice

To evaluate the potential role of arrestin-mediated mechanisms, we evaluated SNC80-induced antihyperalgesia, antidepressant-like effects, and convulsions in arrestin 2 and arrestin 3 knockout mice. There were no significant differences in SNC80-induced antihyperalgesia, antidepressant-like effects, or convulsions in the arrestin 3 knockout mice compared to wild-type and heterozygote knockout littermates (Figures 3A-C; supplemental information). However, the increase in hot-plate latency produced by a single bolus dose of 32 $mg \cdot kg^{-1}$ morphine in the 52 ºC hot plate test was potentiated in arrestin 3 knockout mice (Figure 3D) consistent with previously published data (Bohn *et al.*, 1999; Two-way repeated measures ANOVA: main

effects of time ($F(5,75) = 61.04$), genotype ($F(2,15) = 13.37$), and a significant interaction ($F(10,75) = 6.77$).

In arrestin 2 knockout mice, SNC80-induced increases in tail withdrawal latency following NTG administration were similar to wild-type controls (Figure 4A). Arrestin 2 knockout mice had no significant differences in SNC80-induced decreases in immobility in the forced swim test relative to wild-type mice (Figure 4B). In contrast, SNC80-induced convulsions were profoundly altered in arrestin 2 knockout mice such that the potency of SNC80 to induce convulsions was significantly increased in arrestin 2 knockout mice as evidenced by a leftward shift in the dose response curve (Figure 4C). Two-way ANOVA (vehicle and 1-32 $mg \cdot kg^{-1}$ only) revealed significant effects of genotype ($F(1,50) = 106$), SNC80 dose ($F(4,50) = 147$), and a significant interaction ($F(4,50) = 27.75$). There were no significant differences in the time of onset and duration of these convulsions (see supplemental information). However, arrestin 2 knockout mice exhibited significantly more convulsions in response to a single dose of SNC80 relative with wild-type controls (Figure 4D). Two-way ANOVA (vehicle and 1-32 $mg \cdot kg^{-1}$ only) revealed significant effects of genotype ($F(1,50) = 26.9$), SNC80 dose ($F(4,50) = 37.32$), and a significant interaction ($F(4,50) = 5.84$). These subsequent convulsions were similar in nature to the initial SNC80-induced convulsions, consisting of both tonic and clonic phases followed by a brief (1-2 min) period of catalepsy. In order to reduce the number of animals used, SNC80-induced convulsions were typically evaluated prior to antihyperalgesia measurements (see Methods). However, these mice received NTG prior to SNC80, which may have influenced convulsion frequency. Therefore, SNC80-induced convulsions were also evaluated in drug-naïve mice to confirm the altered convulsive effects of SNC80 in arrestin 2 knockout mice. NTG administration did not alter convulsion severity but produced a non-significant increase in the number of convulsions elicited by 3.2 $mg \cdot kg^{-1}$ SNC80 (Figure 4C and 4D). Despite the significant changes to SNC80-induced convulsions observed in arrestin 2 knockout mice, loss of arrestin 2 did not alter the potency of the chemical convulsant pentylenetetrazol (see supplemental information).

Discussion

In this report, we sought to further elucidate the downstream signaling molecules that give rise to δ-receptor-mediated behaviors. We found that $G\alpha_o$ and arrestins differentially regulate the antihyperalgesia, antidepressant-like effects, and convulsions produced by the δ-receptor agonist SNC80. In the NTG-induced thermal hyperalgesia assay, SNC80 produced antihyperalgesia in wild-type mice, consistent with previous studies (Pradhan *et al.*, 2014; Dripps *et al.*, 2017). SNC80 also decreased immobility in the forced swim test, consistent with the well-established antidepressant-like effects of δ-receptor agonists (Broom *et al.*, 2002b; Naidu *et al.*, 2007; Saitoh *et al.*, 2011). RGS proteins negatively regulate G protein signaling by accelerating Gα-mediated GTP hydrolysis which returns Gα to an inactive state. This function reduces the lifetime of active Gα and diminishes downstream signaling (Traynor and Neubig, 2005). The potency of SNC80 to produce antihyperalgesia and antidepressant-like effects was significantly increased in the $G\alpha_o$ RGS-insensitive heterozygous mice. These data indicate that these δ-receptor-mediated behaviors signal through $G\alpha_o$ and are negatively regulated by RGS proteins, consistent with our previous finding that RGS4 negatively regulates these behaviors (Dripps *et al.*, 2017). Furthermore, these enhanced effects of SNC80 were observed in mice with only one mutant copy of $G\alpha_o$, demonstrating that δ-receptor-mediated signaling *in vivo* is highly sensitive to the effects of RGS proteins. Interestingly, the magnitude of these behavioral changes are consistent with those seen in RGS4 knockout mice, suggesting that other RGS proteins likely do not play a significant role in regulating the antihyperalgesic and antidepressant-like effects of the δ-receptor. Overall, these findings suggest that the enhanced δ-receptor-mediated antihyperalgesia and antidepressant-like effects observed in the +/GS mice are likely due to prolongation of δ-receptor-mediated G protein signaling and amplification of downstream effectors.

To confirm the role of $G\alpha_o$ in δ-receptor-mediated behaviors, we examined the behavioral effects of SNC80 in $G\alpha_o$ heterozygous knockout mice. $G\alpha_o$ is highly expressed in the dorsal root ganglion of the spinal cord and in the brain, comprising 0.5-1 % of total brain membrane proteins

(Yoo et al., 2002; for review Jiang and Bajpayee, 2009). SNC80-induced antihyperalgesia was abolished in $G\alpha_o$ heterozygous knockout mice, suggesting that $G\alpha_o$ is required for the antihyperalgesic effects of δ-receptor agonists. Furthermore, this profound effect was produced by only a 50% reduction in $G\alpha_o$, indicating that δ-receptor-mediated antihyperalgesia likely requires robust amplification of downstream signaling and/or is a high efficacy-requiring behavior (i.e., has a small receptor reserve). It is possible that larger doses of SNC80 could produce antihyperalgesia in $G\alpha_o$ heterozygous knockout mice, however such doses are likely to be nonselective. Taken together, our findings indicate that $G\alpha_o$ plays a critical role in mediating signaling required for δ-receptor-mediated antihyperalgesia.

In contrast, decreased expression of $G\alpha_o$ did not affect δ-receptor-mediated antidepressant-like effects in the forced swim test. The δ-receptor could be capable of signaling through other G proteins in order to produce antidepressant-like effects and/or compensate for the reduction in $G\alpha_o$ expression. Alternatively, it is possible that the efficacy requirement for δ-receptor-mediated antidepressant-like effects is relatively low (i.e., has large receptor reserve) compared to that for δ-receptor-mediated antihyperalgesia in which case one functional copy of *GNAO1* and approximately 50% of $G\alpha_o$ protein subunits (Lamberts *et al.,* 2011) could be sufficient to produce a full response in the forced swim test. Broom *et al.* (2002a) proposed that the efficacy requirement for δ-receptor-mediated antinociception was higher than that required for convulsions. The relative efficacy requirement for antidepressant-like effects has not been evaluated and will be compared with efficacy requirements for convulsions and antihyperalgesic effects in future studies.

In the present study, δ-receptor-mediated convulsions were not altered in $G\alpha_o$ RGSi and $G\alpha_o$ knockout mice. In addition, we previously observed that SNC80-induced convulsions were unaltered in RGS4 knockout mice (Dripps *et al.,* 2017). Overall, these data may suggest signaling mechanisms mediating δ-receptor agonist-induced convulsions are distinct from those mediating antihyperalgesia and antidepressant-like effects. These behavioral measures could be

regulated differentially by specific G protein subunits, G protein-independent signaling, and/or the selective expression of signaling molecules within specific brain circuits or regions.

To address this question, we explored the hypothesis that SNC80-induced convulsions are produced by a G protein-independent, arrestin-mediated mechanism. As first shown by Bohn *et al.* (1999), we observed potentiation of morphine-induced antinociception in arrestin 3 knockout mice. Although class A GPCRs are thought to preferentially interact with arrestin 3 (Oakley *et al.,* 2000), no significant changes in δ-receptor-mediated behaviors, including convulsions, were observed in arrestin 3 knockout mice. It should be noted that these data are the result of acute administration of SNC80 and it is possible that arrestin 3 could play a role in regulating the effects of repeated doses of SNC80 or other δ-receptor agonists. This observation with SNC80 is consistent with previous reports that found that loss of arrestin 3 in mice did not alter the analgesic profile of δ-receptor agonists and had no effect on the enhanced coupling of δ-receptors to voltage-dependent calcium channels observed in the Complete Freund's Adjuvant (CFA) model of chronic inflammatory pain (Pradhan *et al.,* 2013; Pradhan *et al.,* 2016). Overall, our findings indicate that arrestin 3 is not required for δ-receptor-mediated antihyperalgesia, antidepressant-like effects, or convulsions.

In arrestin 2 knockout mice, we observed no changes in the effects of SNC80 in response to NTG-induced thermal hyperalgesia. However, it was previously demonstrated that the effects of SNC80 on CFA-induced mechanical hyperalgesia were potentiated in arrestin 2 knockout mice (Pradhan *et al.,* 2016). It is possible that the δ-receptor-mediated responses to these distinct pain modalities (CFA vs NTG; mechanical vs thermal) are differentially regulated by arrestin 2. Future studies should investigate differences in the signaling molecules and pathways mediating different types of δ-receptor-mediated antihyperalgesia. The convulsive effects of SNC80 were strongly enhanced in arrestin 2 knockout mice. The potency of SNC80 to induce convulsions was enhanced in arrestin 2 knockout mice, suggesting that arrestin 2 acts as a negative regulator of δ-receptor-mediated convulsions. Secondly, arrestin 2 knockout mice convulsed multiple times in response to a single dose of SNC80.

Tolerance to δ-receptor-mediated convulsions is typically acute and long lasting (Comer *et al.,* 1993; Hong *et al.,* 1998). In addition, the changes in the electroencephalographic waveform produced by SNC80 return to normal baseline activity following the end of catalepsy (Jutkiewicz *et al.,* 2006). To our knowledge, this is the first report of multiple convulsive events in response to a δ-receptor agonist in rodents. One possible explanation for this observation is that loss of arrestin 2 produces these behavioral changes by preventing δ-receptor desensitization and/or upregulating δ-receptor trafficking to the cell membrane resulting in enhanced δ-receptor signaling (Mittal *et al.,* 2013). However, in the current study, δ-receptor-mediated antidepressant-like effects and thermal antihyperalgesia were not significantly altered in arrestin 2 knockout mice. Therefore, it is possible that the behavioral effects of SNC80 are differentially regulated by arrestin 2 due to differences in regional expression, behavioral mechanisms, and/or signaling downregulation, and/or tolerance to the convulsive effects of SNC80. Thus, loss of arrestin 2 could allow signaling pathways that would normally be terminated to persist and produce multiple convulsive events. Future work will examine whether arrestin 2 also regulates tolerance to other behavioral effects of δ-receptor agonists.

Overall, our data demonstrate an important role for $G\alpha_o$, but not arrestins, in regulating the acute antihyperalgesic and antidepressant-like effects of the δ-receptor. However, δ-receptor-mediated convulsions appear to be negatively regulated by arrestin 2 and were not altered by manipulations to $G\alpha_o$ function (see Figure 5). Taken together, these findings suggest that different signaling pathways underlie the convulsive effects of the δ-receptor relative to the antihyperalgesic and antidepressant-like effects. Perhaps due in part to this phenomenon, it has been shown that some δ-receptor agonists do not produce convulsions at doses far exceeding those needed to produce antinociception and antidepressant-like effects (Le Bourdonnec *et al.,* 2008; Saitoh *et al.,* 2011; Chung *et al.,* 2015). However, the properties of δ-receptor agonists that determine their convulsive nature remain unclear. Ongoing work will continue to investigate the signaling mechanisms responsible for the behavioral effects of different δ-receptor agonists.

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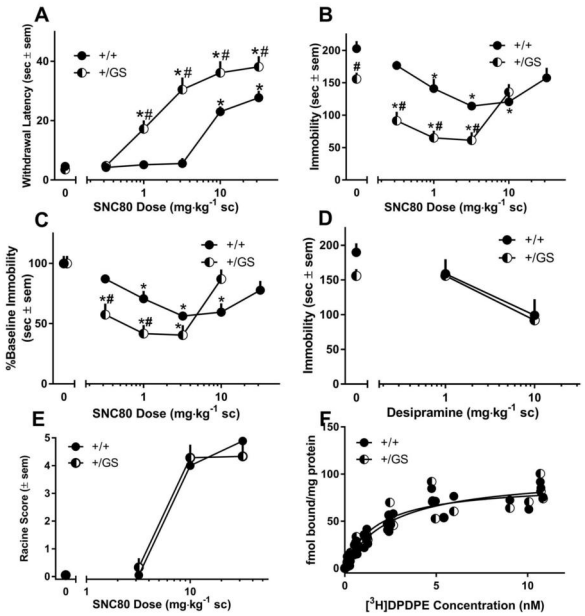

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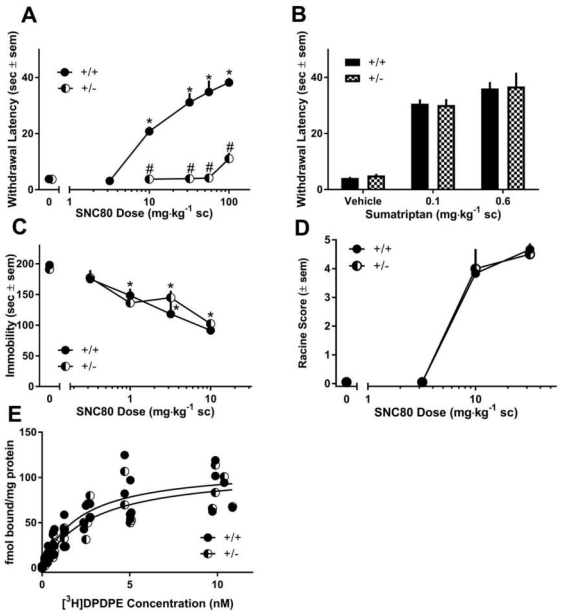

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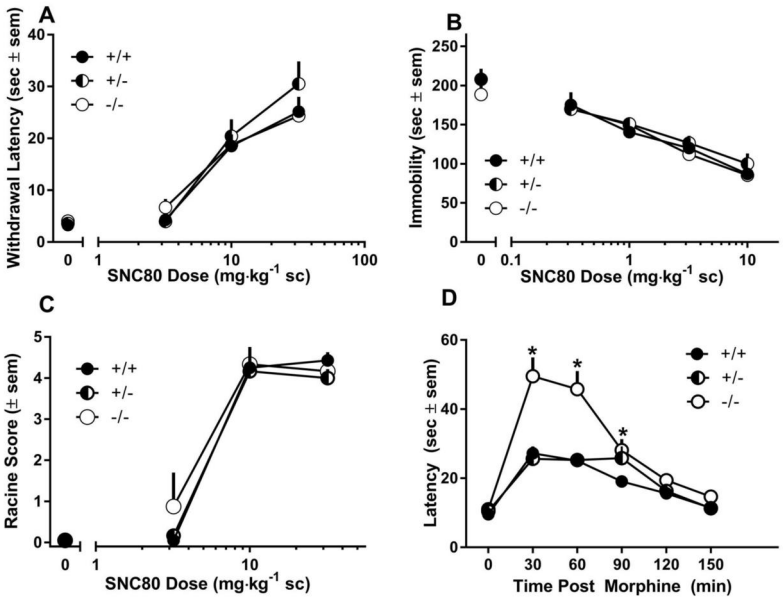

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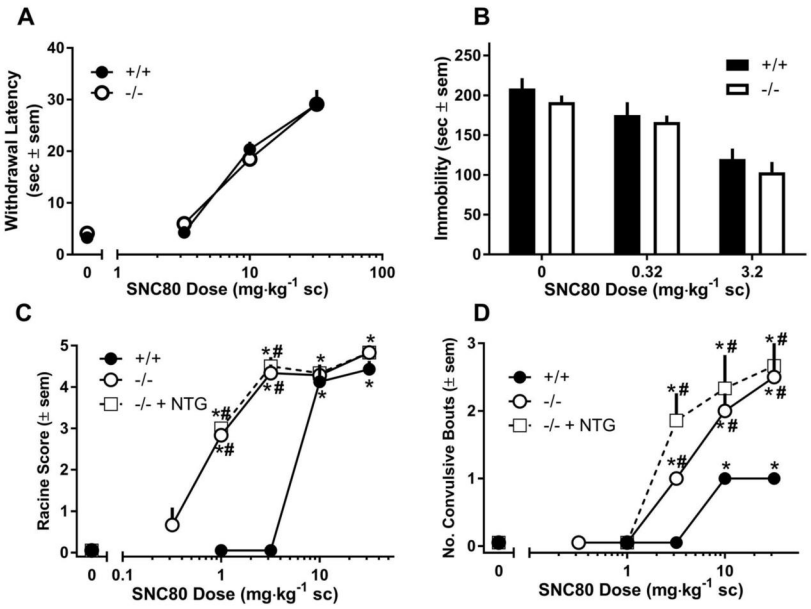

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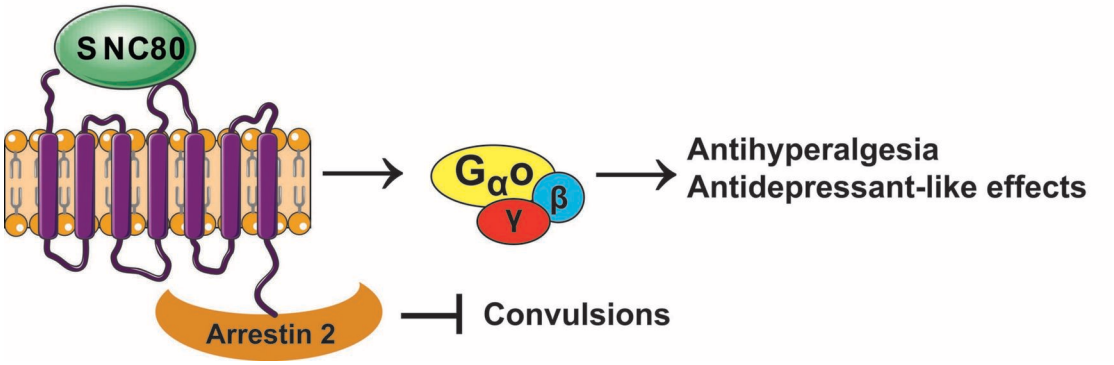

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