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Pharmacological Properties of δ-Opioid Receptor–Mediated Behaviors: Agonist Efficacy and Receptor Reserve[S]

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ABSTRACT

δ-Opioid receptor (δ-receptor) agonists produce antihyperalgesia, antidepressant-like effects, and convulsions in animals. However, the role of agonist efficacy in generating different δ-receptor–mediated behaviors has not been thoroughly investigated. To this end, efficacy requirements for δ-receptor–mediated antihyperalgesia, antidepressant-like effects, and convulsions were evaluated by comparing the effects of the partial agonist BU48 and the full agonist SNC80 and changes in the potency of SNC80 after δ-receptor elimination. Antihyperalgesia was measured in a nitroglycerin-induced thermal hyperalgesia assay. An antidepressant-like effect was evaluated in the forced swim test. Mice were observed for convulsions after treatment with SNC80 or the δ-opioid receptor partial agonist BU48. Ligand-induced G protein activation was measured by [^{35}S]guanosine 5′-O-[γ-thio] triphosphate binding in mouse forebrain tissue, and δ-receptor number was measured by [^{3}H]D-Pen^{2,5}-enkephalin saturation binding. BU48 produced antidepressant-like effects and convulsions but antagonized SNC80-induced antihyperalgesia and G protein activation. The potency of SNC80 was shifted to the right in δ-receptor heterozygous knockout mice and naltrindole-5′-isothiocyanate–treated mice, and the magnitude of potency shift differed across assays, with the largest shift occurring in the thermal hyperalgesia assay, followed by the forced swim test and then convulsion observation. Naltrindole antagonized these SNC80-induced behaviors with similar potencies, suggesting that these effects are mediated by the same type of δ-receptor. These data suggest that δ-receptor–mediated behaviors display a rank order of efficacy requirement, with antihyperalgesia having the highest requirement, followed by antidepressant-like effects and then convulsions. These findings further our understanding of the pharmacological mechanisms mediating the in vivo effects of δ-opioid receptor agonists.

SIGNIFICANCE STATEMENT

δ-Opioid receptor (δ-receptor) agonists produce antihyperalgesia, antidepressant-like effects, and convulsions in animal models. This study evaluates pharmacological properties, specifically the role of agonist efficacy and receptor reserve, underlying these δ-receptor–mediated behaviors. These data suggest that δ-receptor–mediated behaviors display a rank order of efficacy requirement, with antihyperalgesia having the highest requirement, followed by antidepressant-like effects and then convulsions.

Introduction

The δ-opioid receptor (δ-receptor) is a class A G protein–coupled receptor that couples to inhibitory $G\alpha_{i/o}$ proteins. Activation of δ-receptors has been shown to elicit a number of behavioral effects, including antinociception and antihyperalgesia in mice (Hong et al., 1998; Pradhan et al., 2014), rats (Fraser et al., 2000; Gallantine and Meert, 2005), and monkeys (Negus et al., 1998; Brandt et al., 2001). δ-Opioid receptor agonists have also been shown to produce antidepressant-like effects in a number of rodent models [for review, see Lutz and Kieffer (2013)]. Some (Comer et al., 1993; Hong et al., 1998), but not all (Saitoh et al., 2018), δ-receptor agonists also produce convulsions and seizure activity.

To better understand the pharmacological properties mediating behavioral effects of δ-receptor agonists, we sought to directly compare two structurally distinct δ-receptor ligands that differ in intrinsic efficacy: the piperazinyl benzamide SNC80 and the morphinan derivative BU48. SNC80 is the

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ABBREVIATIONS: BU48, *N*-cyclopropylmethyl-[7α,8α,2′,3′]-cyclohexano-1′[S]-hydroxy-6,14-endo-ethenotetrahydronororipavine; FST, forced swim test; [^{3}H]DPDPE, [^{3}H]D-Pen^{2,5}-enkephalin; NIH, National Institutes of Health; NTG, nitroglycerin; NTI, naltrindole; 5′-NTII, naltrindole-5′-isothiocyanate; OD, optical density; δ-receptor, δ-opioid receptor; RGS4, regulator of G protein signaling 4; [^{35}S]GTPγS, [^{35}S]guanosine 5′-O-[γ-thio]triphosphate; SNC80, (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide.

prototypical nonpeptidic δ-receptor agonist and is highly efficacious at stimulating G protein activation in vitro in C6 glioma cells (Clark et al., 1997) and ex vivo (Jutkiewicz et al., 2004). In vivo, SNC80 has been shown to produce antihyperalgesia (Pradhan et al., 2014), antidepressant-like effects (Saitoh et al., 2004), and convulsions (Hong et al., 1998) in mice. BU48 is less efficacious than SNC80 in vitro, producing approximately 40% stimulation of [^{35}S]GTPγS binding relative to SNC80 in C6 glioma cells expressing only δ-receptors (Broom et al., 2000). This report also found that BU48 produces δ-receptor–mediated convulsions but did not produce δ-receptor–mediated antinociception in NIH Swiss mice (Broom et al., 2000), suggesting that the efficacy requirement for δ-receptor–mediated convulsions is low relative to that for antinociception. Consistent with this hypothesis, small doses (3 and 10 mg/kg s.c.) of the δ-receptor irreversible antagonist naltrindole-5′-isothiocyanate (5′-NTII) antagonized the antinociceptive effects of the δ-receptor agonist BW373U86 but did not decrease the frequency of BW373U86-induced convulsions in NIH Swiss mice (Broom et al., 2002a). Also compatible with these findings was a study that demonstrated SNC80 and its analog SNC162 were equipotent at producing convulsions, but of the two compounds, SNC80 was significantly more potent, and potentially more efficacious, at decreasing immobility in the rat forced swim test (Jutkiewicz et al., 2004). Nevertheless, the relationship of agonist efficacy to the different behavioral effects of δ-receptor agonists has not been thoroughly evaluated.

Therefore, the present study evaluated the role of agonist efficacy and receptor reserve involved in δ-receptor–mediated behaviors. To do this, we compared the behavioral effects of the δ-receptor full agonist SNC80 and the partial agonist BU48 and the ability of a competitive δ-receptor antagonist naltrindole (NTI) to attenuate these behaviors. Effects of receptor reserve were assessed by comparing the shifts in the dose-response curves for SNC80-induced antihyperalgesia, antidepressant-like effects, and convulsions in heterozygous δ-opioid knockout mice and in mice treated with the irreversible δ-receptor antagonist 5′-NTII as compared with controls.

Materials and Methods

Subjects. All procedures complied with the regulations and policies set forth in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the Institutional Committee on the Use and Care of Animals at the University of Michigan. Mice were housed in groups with a maximum of five 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 specific pathogen–free rooms maintained between 68°F and 79°F and between 30% and 70% humidity with a 12-hour 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 with a single dose of drug, and all analyses are between-subject. For studies in which transgenic mice were not required, male C57BL/6N mice were obtained from Envigo (formerly Harlan, Indianapolis, IN).

The *Oprd1*^{tm1Kff}/J mouse strain was obtained from The Jackson Laboratory [Bar Harbor, ME, https://www.jax.org/strain/007557; (Filliol et al., 2000)] and was backcrossed with wild-type C57BL/6N mice obtained from Envigo (formerly Harlan). Mice were maintained in-house as heterozygote pair or harem (one male, two female) breeding groups and were fed high-fat rodent chow and supplemented with γ-irradiated peanuts in the shell (S6711; Bio-Serv, Flemington, NJ) to enhance litter production (Dripps et al., 2018). Wild-type littermates (+/+) were used as controls in all experiments involving δ-receptor heterozygote (+/−) and homozygote (−/−) knockout mice. Since increasing receptor signaling enhances the efficacy of partial agonists, we used mice lacking regulators of G protein signaling 4 (RGS4) protein, which have been shown to enhance the activity of δ-receptor agonists (Wang et al., 2009; Stratinaki et al., 2013; Dripps et al., 2017). The Rgs4tm1Dgen/J mouse strain was obtained from The Jackson Laboratory (https://www.jax.org/strain/005833), backcrossed with wild-type C57BL/6N mice obtained from Envigo, and bred in-house from heterozygous breeding pairs to obtain knockout and wild-type littermates (Dripps et al., 2017). For all experiments utilizing transgenic mice, male and female mice were used.

Forced Swim Test. The forced swim test (FST) is a behavioral assay used to measure the antidepressant-like effects of drugs in rats and mice (Barkus, 2013). Our procedure was based on the original assay described by Porsolt et al. (1977) and has been described previously (Dripps et al., 2017). In brief, 60 min after SNC80 (0.32, 1, 3.2, 10 mg/kg s.c.), BU48 (1, 3.2, 10 mg/kg s.c.), or vehicle injection (s.c.), a mouse was placed in a 4-L beaker that was filled with approximately 15 cm of 25 ± 1°C water. These doses of SNC80 were selected based on previous studies (Dripps et al., 2017, 2018) demonstrating that SNC80 produces a U-shaped function; thus, only doses on the descending limb of the dose-effect curve were selected. The FST was conducted 60 min after administration of drug because of initial concerns about ability to swim after a convulsion. Swim sessions were recorded for 6 min using a Sony HDR-CX220 digital camcorder. Videos were analyzed by two different individuals who were blind to the experimental conditions across mice. Additionally, inter-rater reliability was confirmed every few weeks such that scores between the two observers differed by ≤10%. The amount of time the mice were immobile during the last 5 min and 30 sec of the 6-min swim was measured. Immobility was defined as the lack of active traveling around the swim tank with only small movements of the forelimbs or hind limbs.

Nitroglycerin-Induced Hyperalgesia. The NTG-induced hyperalgesia assay was conducted as previously described (Pradhan et al., 2014; Dripps et al., 2017). Hyperalgesia was assessed by immersing approximately 5 cm of the tail tip in a 46°C water bath and then measuring the latency to tail withdrawal from the water with a cutoff time of 60 sec. Tail withdrawal was identified by a strict definition—specifically, complete lift or removal of the tail out of the water or rapidly flicking the tail at least three times. After determining baseline withdrawal latencies (range 30–40 sec), each mouse received an injection of 10 mg/kg NTG (i.p.). Tail withdrawal latency was measured again 1 hr after NTG administration. At 90 min after NTG administration, mice received an injection of SNC80 (3.2, 10, 32, 100, 180 mg/kg s.c.), BU48 (3.2, 10, 32 mg/kg s.c.), or vehicle (subcutaneously) and were then continuously observed in separate cages for 20 min to watch for convulsions (see *SNC80-Induced Convulsions*). Finally, tail withdrawal latencies were assessed 30 min after SNC80 or BU48 administration, when peak drug effects were observed. Observers were not blind to the treatment conditions, because of the complexity of the experimental design.

SNC80-Induced Convulsions. Mice were observed continuously in separate cages for convulsions, catalepsy, myoclonic jerks, stop-and-stare behaviors, wet dog shakes, digging, and other normal or abnormal behaviors. Although experimenters were not blind to the treatment conditions as described above, all behaviors were logged in a detailed recording that occurred during the observation period. No more than two mice were observed simultaneously by any single observer, and these data were collected from at least two different observers across mice. Typically, convulsions comprised a single tonic phase (abrupt muscle tensing and forepaw extension) followed by repeated clonic contractions and frequent loss of balance. Convulsions

were followed by a period of post-ictal behavioral suppression or catalepsy-like behavior that started approximately 1 min after the end of a convulsion and lasted 2–5 min. Immediately after this period, mice were active but slightly uncoordinated in the observation cage environment. The severity of each convulsion was quantified using the following modified Racine scale: 1, teeth chattering or face twitching; 2, head bobbing or twitching; 3, tonic extension and/or clonic convulsion lasting less than 3 seconds; 4, tonic extension and/or clonic convulsion lasting longer than 3 seconds; 5, tonic extension and/or clonic convulsion lasting more than 3 seconds with loss of balance. In addition to measuring convulsion severity, the time to onset of convulsions and convulsion duration were also recorded (data shown in Fig. 2 or in Supplemental Figs.). Postconvulsion catalepsy-like behavior was assessed by placing a horizontal rod under the forepaws of the mouse to observe whether the mouse would remove its forepaws or climb off the rod. A positive catalepsy score was assigned if the mouse did not remove its forepaws or climb off after 30 sec.

δ-Receptor Saturation Binding. Mice were euthanized by cervical dislocation and decapitation without anesthesia at approximately 8–12 weeks old. The forebrain was removed, and membranes were freshly prepared as previously described (Broom et al., 2002a). The 5′-NTII was administered 24 hr prior to preparation of membranes. 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 using 10 μM of the opioid antagonist naloxone to define nonspecific binding as described previously (Broom et al., 2002a). Reactions were incubated for 60 min at 26°C and stopped by rapid filtration through GF/C-grade filter mats soaked in 0.1% polyethylenimine (PEI) using an MLR-24 harvester (Brandel, Gaithersburg, MD). Bound [^{3}H]DPDPE was determined by scintillation counting, and B_{max} and K_d values were 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.

[^{35}S]GTPγS Binding Assay: In Vitro. Mouse brain membranes (as prepared above, 10 μg per well) were incubated for 90 min at 26°C in buffer comprising 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 nM [^{35}S]GTPγS, 100 μM GDP, and 0.4 U/ml adenosine deaminase in a final volume of 200 μl. SNC80 or BU48 was also included at appropriate concentrations. SNC80 (10 μM) was used as the maximal standard, and assay buffer was used to assess basal [^{35}S]GTPγS binding. The reaction was terminated by filtration through glass microfiber GF/C-grade filters using a Brandel harvester. The filters were rinsed and dried, and radioactivity was determined by scintillation counting. To ensure the reliability of single values, membranes from each mouse (n = 5 per group) were assayed in duplicate.

[^{35}S]GTPγS Binding Assay: Ex Vivo Autoradiography. Brain tissue was collected after cervical dislocation and decapitation without anesthesia at ages 8–15 weeks and rapidly frozen on isopentane on dry ice. Coronal brain sections (20 μm) were cut on a cryostat maintained at −18°C, mounted onto gelatin-coated slides, and stored at −80°C until use.

Agonist-stimulated [^{35}S]GTPγS autoradiography was performed as previously described (Sim et al., 1995) with slight modifications. The brain slices were incubated in TME buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.1% bovine serum albumin, pH 7.4) for 10 min at room temperature and then incubated in secondary buffer (TME buffer with 2 mM GDP and 9.5 mU/ml adenosine deaminase) for 30 min at room temperature. Then, the slices were incubated in secondary buffer with 0.04 nM [^{35}S]GTPγS with or without varying doses of δ-receptor agonists (SNC80 or BU48) or with 10 μM unlabeled GTPγS for 1 h at room temperature. For antagonism tests, slices were incubated in secondary buffer with 10 μM BU48, the selective δ-receptor antagonist NTI, or vehicle for 30 min before they were incubated with 0.04 nM [^{35}S]GTPγS and 3 μM SNC80 or vehicle with or without the 10 μM pretreatment drugs for 1 h. After the 1-h incubation, the slices were rinsed three times in cold Tris buffer (50 mM Tris-HCl, pH 7.4) for 2, 2, and 1 min, respectively, and air dried at room temperature. The slices were then exposed to film (Carestream Health BioMax Maximum Resolution Film, 20.3 × 25.4 cm, 8 × 10 inches) for 24 h with ^{14}C standard. The images were digitized, and optical density (OD) values in the interested brain regions were measured with Image J software. Each condition was evaluated in brain slices from six different mice.

OD values of medial prefrontal cortex, caudate putamen, nucleus accumbens, hippocampus, substantia nigra, pontine nuclei, and median raphe nucleus were measured with Image J software and averaged within each treatment group. Nonspecific binding (determined by incubation with unlabeled GTPγS) in each brain region was subtracted from the basal and drug-stimulated binding. The agonist-stimulated increase in [^{35}S]GTPγS binding in each selected brain region was calculated as the percentage over region-specific basal stimulation values. The data were analyzed with GraphPad Prism software using nonlinear regression analysis using log(agonist) versus response equation.

Data Analysis. All data analyses were performed using GraphPad Prism version 6.02 (GraphPad, San Diego, CA). For all tests, level of significance (α) was set to 0.05. All analyses of dose-effect curves were two-way ANOVAs unless otherwise indicated. Post hoc analysis was conducted using the Tukey's post hoc test to correct for multiple comparisons. Post hoc analysis was only performed when F values achieved $P < 0.05$. Approximate ED_{50} values were calculated using GraphPad Prism version 6.02 by interpolating the 50% maximum effect from the straight-line analysis of the averaged treatment group data, including two to three points along the linear portion of the curve only. S.E.M. was not calculated for ED_{50} values, because different mice were present at each dose; thus, 50% maximum effects were calculated for the group-averaged data, which will not provide an interpolated value ± variation. Fold shifts in dose-effect curves were calculated by dividing the ED_{50} value of interest by the ED_{50} value calculated in wild-type mice or in absence of the antagonist. Measurement of pK_b values, the apparent affinity estimate for a single dose of antagonist, was calculated by comparing the groups receiving vehicle and 3.2 mg/kg NTI using the following equation: $pK_b = -\log[(\text{NTI mol/kg})/\text{dose ratio} - 1]$.

Materials. (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC80) was dissolved in 1 M HCl and diluted in sterile water to a final concentration of 3% HCl. *N*-Cyclopropylmethyl-[7α,8α,2′,3′]-cyclohexano-1′[S]-hydroxy-6,14-endo-ethenotetrahydronororipavine (BU48) (Broom et al., 2000) was dissolved in a solution comprising 10% ethanol, 10% Alkamuls EL-620 (Acros Organics, Morris Plains, NJ), and 80% sterile water. Stock solutions of SNC80 and BU48 were diluted with their appropriate vehicle solutions to obtain smaller concentrations. 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. 5′-NTII (Sigma-Aldritch, St. Louis, MO) was dissolved in 10% DMSO. NTI (Tocris Bioscience, Pittsburgh, PA) was dissolved in sterile water. 5′-NTII (32 mg/kg) or vehicle was injected 24 h prior to SNC80 administration. NTI (1, 3.2 mg/kg) or vehicle was injected 30 min prior to SNC80 administration. All drugs were injected at a volume of 10 ml/kg and given subcutaneously, except for NTG, which was administered via intraperitoneal injection.

Results

Characterization of BU48-Induced Behaviors. In vivo, BU48 failed to increase tail withdrawal latency in NTG-treated wild-type mice up to a dose of 32 mg/kg, whereas SNC80 significantly increased tail withdrawal latency at 10 and 32 mg/kg (Fig. 1A). Two-way ANOVA revealed significant effects of drug [$F(1,40) = 118.0$, $P < 0.0001$] and dose [$F(3,40) = 50.90$,

$P < 0.0001$] and a significant drug × dose interaction [F(3,40) = 45.41, $P < 0.0001$]. Additionally, pretreatment with BU48 30 min prior to SNC80 administration prevented SNC80-induced increases in tail withdrawal latency in wild-type mice (Fig. 1B). Two-way ANOVA revealed a significant interaction [BU48 dose × genotype, F(1,20) = 67.83, $P < 0.0001$] and significant main effects of BU48 dose [F(1,20) = 78.38, $P < 0.0001$] and SNC80 dose [F(1,20) = 57.97, $P < 0.0001$]. In mice lacking RGS4 proteins (R4/R4), 32 mg/kg of BU48 was able to increase tail withdrawal latencies relative to wild-type littermates (Fig. 1C). Two-way ANOVA revealed significant main effects of BU48 dose [F(1,21) = 17.67, $P = 0.0004$] and genotype [F(1,21) = 12.21, $P = 0.0022$], as well as a significant interaction [BU48 dose × genotype, F(1,21) = 23.46, $P < 0.0001$].

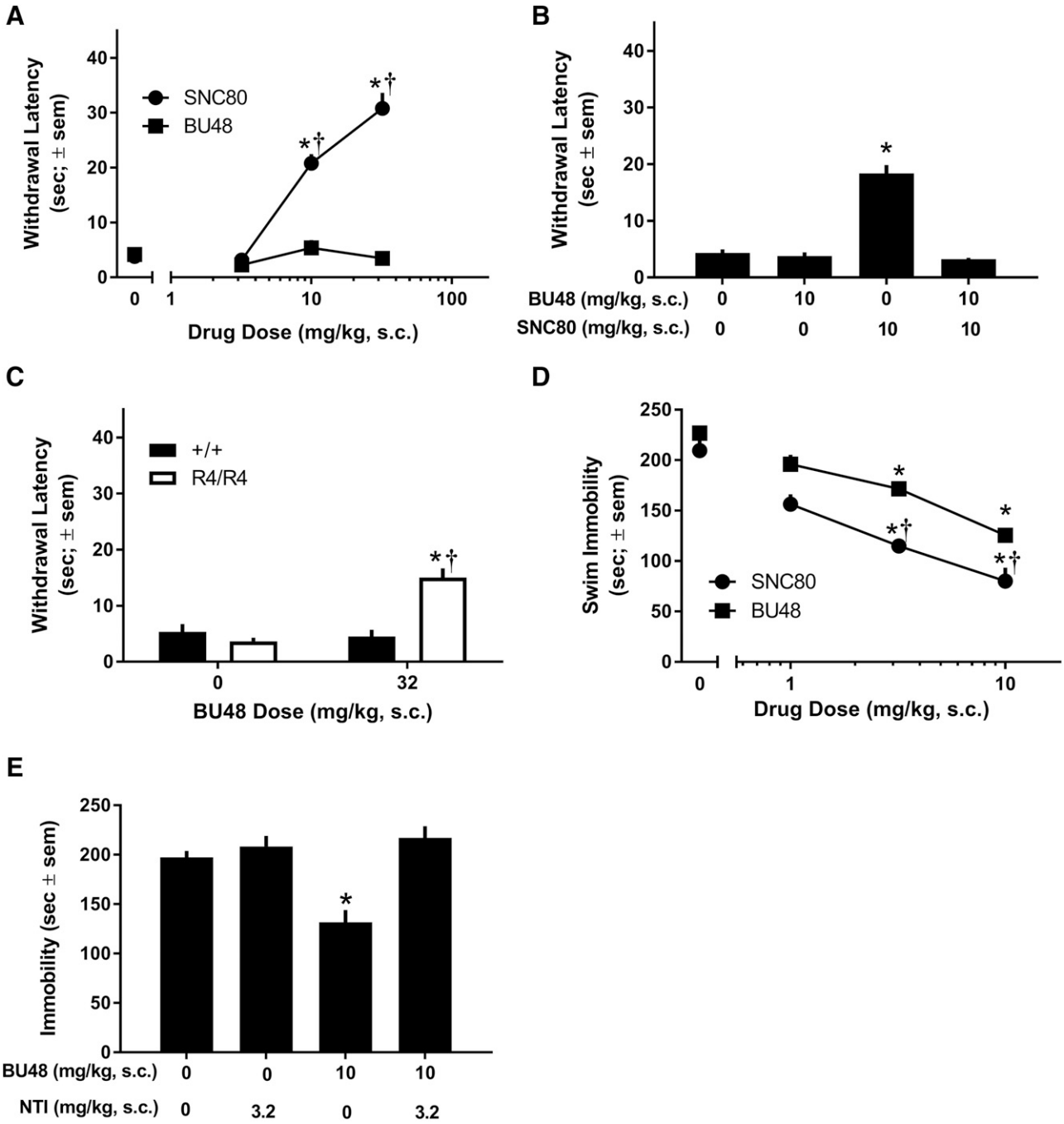

Fig. 1. Characterization of BU48-induced behaviors in C57BL/6N mice or RGS4 wild-type or knockout littermates. (A) Effects of different doses of BU48 or SNC80 on tail withdrawal latencies in NTG-treated mice. (B) Effect of 10 mg/kg SNC80 on tail withdrawal latencies in NTG-treated mice after pretreatment with 10 mg/kg BU48 or vehicle. (C) Effects of 32 mg/kg BU48 or vehicle on tail withdrawal latencies in NTG-treated RGS4 wild-type or knockout mice. (D) Effects of different doses of BU48 or SNC80 on immobility scores in the FST. (E) Effect of 10 mg/kg BU48 on immobility in the FST after pretreatment with 3.2 mg/kg NTI or vehicle. $n = 6$ mice per treatment group for all behavior experiments (in experiments with RGS4 transgenic mice, three male and three female mice were used per treatment group). Data are shown as averages per treatment condition with S.E.M. $^*P < 0.05$ compared with vehicle treatment; $^\dagger P < 0.05$ compared with wild-type mice with the same BU48 dose.

In the forced swim test, BU48 produced dose-dependent decreases in immobility (Fig. 1D). Two-way ANOVA revealed a significant main effect of drug [$F(1,42) = 38.48$, $P < 0.0001$] and significant main effects of dose [$F(3,42) = 60.3$, $P < 0.0001$], indicating that there was only a difference in potency and/or efficacy between BU48 and SNC80. To evaluate the role of δ-receptors in BU48-induced antidepressant-like effects, wild-type mice were pretreated with the δ-receptor selective antagonist NTI (Fig. 1E). BU48-induced decreases in immobility were blocked by pretreatment with 3.2 mg/kg of NTI [two-way ANOVA: BU48 dose × NTI dose, $F(1,21) = 13.04$, $P = 0.0016$], indicating a δ-receptor–mediated effect.

BU48 produced dose-dependent increases in convulsion severity in wild-type mice (Fig. 2A). As with those produced by SNC80, BU48-induced convulsions were comprised of tonic and clonic phases that were followed by a period of catalepsy-like behavior. For a given dose, there were no significant differences in the time of onset (Fig. 2B) or duration (Fig. 2C) of convulsions produced by BU48 and SNC80. BU48-induced convulsions were blocked by pretreatment with 3.2 mg/kg of NTI, indicating a δ-receptor–mediated effect (Fig. 2D).

Effects of BU48 in Mouse Brain Tissue. In forebrain homogenates, SNC80 produced robust, dose-dependent stimulation of [^{35}S]GTPγS binding (EC_{50}: 210 nM; Fig. 3A). BU48 did not produce significant stimulation of [^{35}S]GTPγS binding at concentrations up to 10 μM. Similarly, in mouse brain slices, SNC80 produced dose-dependent increases in [^{35}S]GTPγS binding in the medial prefrontal cortex [drug × dose interaction: $F(4,55) = 4.6$, $P = 0.003$; Fig. 3B], caudate putamen [drug × dose interaction: $F(5,87) = 8.7$, $P < 0.0001$; Fig. 3C], and nucleus accumbens [drug × dose interaction: $F(4,49) = 5.2$, $P = 0.002$; Fig. 3D], with EC_{50} values (±S.E.M.) of 92 (1.7), 538 (1.7), and 779 (1.7) nM, respectively. BU48 did not produce measurable G protein activation in any of these brain regions. Pretreatment with 10 μM BU48 [pretreatment × SNC80 interaction: $F(1,5) = 23.23$, $P = 0.005$; Fig. 3F] or 10 μM NTI [pretreatment × SNC80 interaction: $F(1,5) = 16.1$, $P = 0.01$; Fig. 3E] significantly decreased [^{35}S]GTPγS binding stimulated by 3 μM SNC80 in the caudate putamen.

SNC80- and BU48-stimulated [^{35}S]GTPγS binding was evaluated at a 10 μM agonist concentration across additional brain regions (Fig. 3G). Neither compound stimulated GTPγS binding above basal levels in the substantia nigra or raphe nuclei. Consistent with the concentration-response curve evaluations, SNC80 produced [^{35}S]GTPγS stimulation in the medial prefrontal cortex [$F(2,26) = 31.4$, $P < 0.0001$], caudate putamen [$F(2,31) = 26.6$, $P < 0.0001$], nucleus accumbens [$F(2,30) = 5.9$, $P = 0.007$], and pontine nuclei [$F(2,15) = 6.9$,

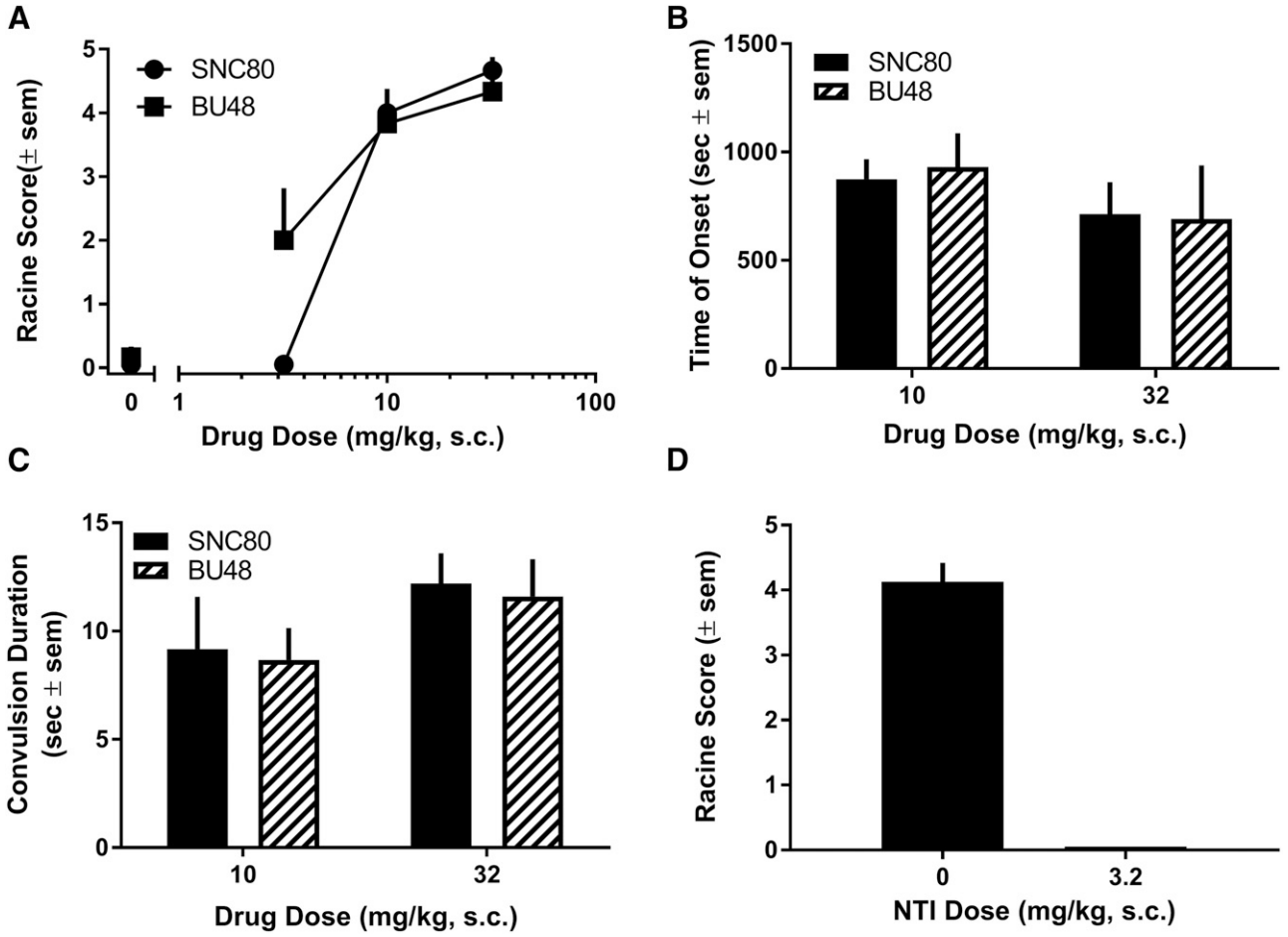

Fig. 2. Comparison of BU48- and SNC80-induced convulsions in C57BL/6N mice. (A) Severity as evaluated with a modified Racine score, (B) time of onset, and (C) duration of BU48- and SNC80-induced convulsions. (D) Effect of NTI on the severity of convulsions produced by 10 mg/kg BU48. $n = 6$ mice per treatment group for all experiments. Data are shown as averages per treatment condition with S.E.M.

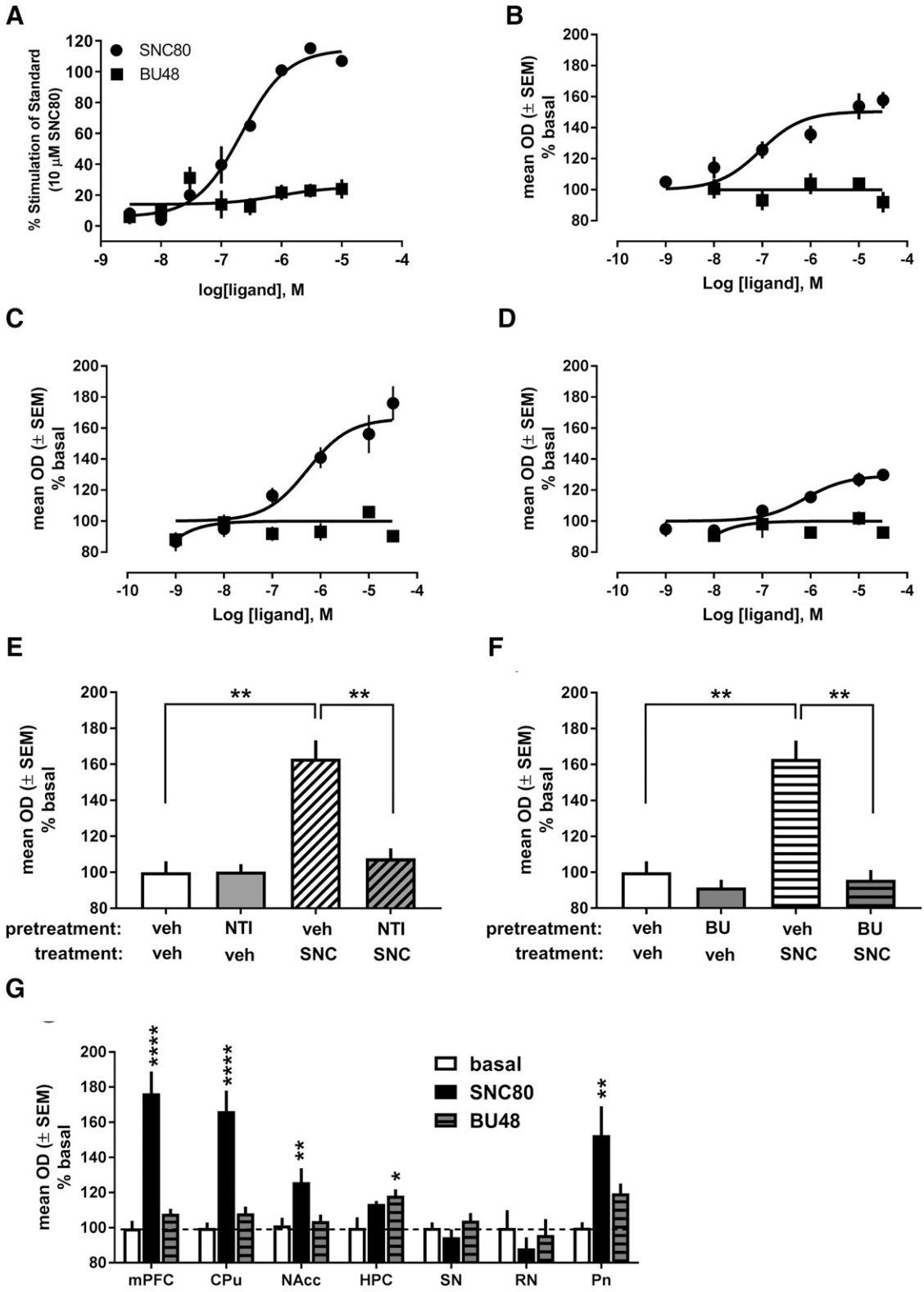

Fig. 3. Effect of increasing concentrations of BU48 or SNC80 on ^{35}[S]GTPγS binding in (A) forebrain tissue homogenates or coronal slices from the (B) medial prefrontal cortex, (C) caudate putamen, or (D) nucleus accumbens of C57BL/6N mice. The effects of 10 μM NTI (E) or 10 μM BU48 (F) on ^{35}[S]GTPγS binding stimulated by 3 μM SNC80 in brain slices from the caudate putamen. (G) [^{35}S]GTPγS stimulation by 10 μM of SNC80 or BU48 compared with basal [^{35}S]GTPγS binding in different brain regions: medial prefrontal cortex (mPFC), caudate putamen (CPu), nucleus accumbens (NAcc), hippocampus (HPC), substantia nigra (SN), median raphe nucleus (RN), and pontine nuclei (Pn). For experiments in tissue homogenates, each point represents the average values from five male C57BL/6N mice, each assayed in duplicate. For slice autoradiography experiments, each point or condition represents the average of values from six to nine male C57BL/6N mice, and data presented in the same graph were collected from consecutive slices for each mouse (analyzed by repeated measures). BU, BU48; SNC, SNC80; veh, vehicle. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$

$P = 0.008$] as compared with that produced by BU48 or basal conditions. In the hippocampus, SNC80 and BU48 produced small increases in [^{35}S]GTPγS binding over basal levels, but the effect was only statistically significant with BU48 treatment [$F(2,15) = 5.2$, $P = 0.02$].

δ-Receptor–Mediated Behaviors in δ-Receptor Knockout Mice. Changes in δ-receptor density and agonist affinity in δ-receptor mutant mice were assessed by saturation binding in brain tissue with the radiolabeled δ-receptor agonist [^{3}H]DPDPE (Fig. 4A). Total δ-receptor number in heterozygous knockout mice was approximately 40% of that measured in wild-type mice (Table 1). The affinity of [^{3}H]DPDPE for the δ-receptor did not differ significantly between wild-type and heterozygous knockout mice (Table 1). δ-Receptors could not be detected in δ-receptor homozygous knockout mice.

To evaluate agonist efficacy required for δ-receptor–mediated behaviors, we compared the potency of SNC80 to induce antihyperalgesia, antidepressant-like effects, and convulsions in δ-receptor wild-type and heterozygous (+/−) and homozygous (−/−) knockout mice. In the NTG-induced thermal hyperalgesia assay, there were no differences between genotypes in the baseline tail withdrawal latencies prior to NTG treatment

TABLE 1

δ-Receptor density and agonist affinity in δ-receptor knockout and 5′-NTII–treated mice

Group	B_{max} (fmol/mg ± S.E.M.)	[^{3}H]DPDPE K_d (nM ± S.E.M.)
δ-Receptor +/+	105 ± 7	2.3 ± 0.4
δ-Receptor +/−	42 ± 3	1.3 ± 0.3
δ-Receptor −/−	−3 ± 5	N/A
C57BL6 DMSO	127 ± 11	2.3 ± 0.4
C57BL6 NTII	86 ± 5	1.6 ± 0.3

(+/+, 35.7 ± 1.7 seconds; +/−, 35.6 ± 1.3 seconds; −/−, 38.9 ± 2.9 seconds). Administration of 10 mg/kg NTG (i.p.) significantly decreased tail withdrawal latency to a similar degree in all genotypes (+/+, 3.6 ± 0.3 seconds; +/−, 3.6 ± 0.2 seconds; −/−, 4.1 ± 0.4 seconds). The potency of SNC80 to increase tail withdrawal latency was significantly decreased in the δ-receptor heterozygous knockout mice as evidenced by a 7.1-fold rightward shift in the dose-effect curve relative to wild-type mice (Fig. 4B; ED_{50} values: +/+, 14 mg/kg; +/−, 100 mg/kg). Two-way ANOVA comparing the δ-receptor wild-type and heterozygous knockout groups revealed a significant interaction [SNC80 dose (0, 3.2–100 mg/kg only) × genotype, $F(4,51) = 7.99$, $P < 0.0001$], as well as significant main effects

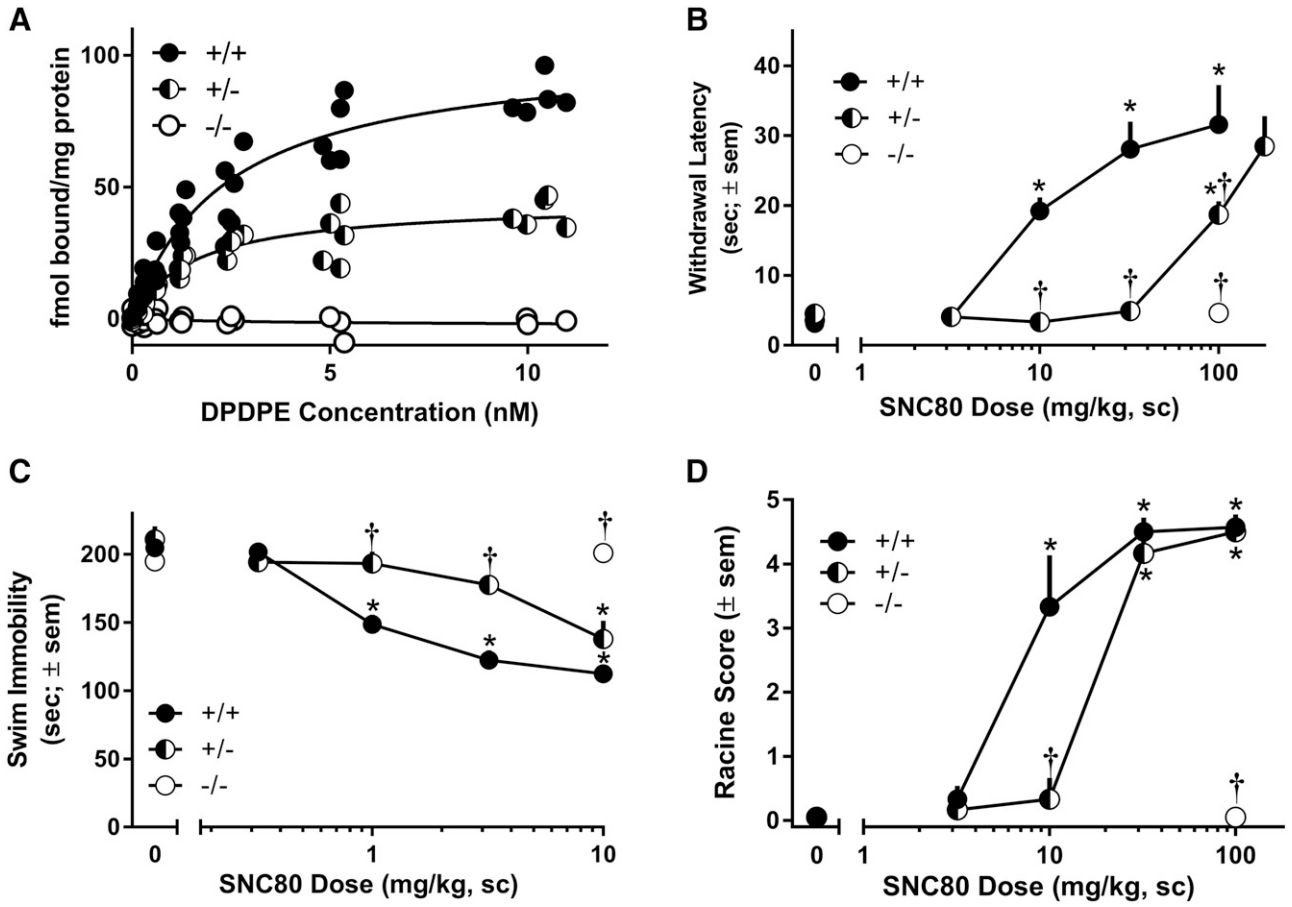

Fig. 4. Role of receptor density in δ-receptor–mediated behaviors. (A) Saturation binding of [^{3}H]DPDPE to membranes prepared from forebrains of δ-receptor wild-type or mutant mice. Each point represents tissue from a single mouse assayed in triplicate ($n = 5$ male C57BL/6N mice). (B) Effects of different doses of SNC80 on tail withdrawal latencies in NTG-treated δ-receptor wild-type (+/+), heterozygous (+/−), and null mutant (−/−) mice. (C) Effects of different doses of SNC80 on immobility scores in the FST in δ-receptor wild-type and mutant mice. (D) Severity of SNC80-induced convulsions in δ-receptor wild-type and mutant mice as measured by a modified Racine scale. Data are shown as average per treatment condition with S.E.M. $n = 6$ mice (three male, three female) per treatment group for all behavior experiments. *$P < 0.05$ compared with vehicle treatment in the same genotype; †$P < 0.05$ compared with wild-type mice with same drug dose.

of SNC80 dose [$F(4,51) = 23.97$, $P < 0.0001$] and genotype [$F(1,51) = 36.92$, $P < 0.0001$]. SNC80 failed to increase tail withdrawal latency in δ-receptor homozygous knockout mice at a dose of 100 mg/kg.

The potency of SNC80 to reduce immobility time in the forced swim test was evaluated in δ-receptor mutant mice (Fig. 4C). Two-way ANOVA comparing the wild-type and δ-receptor heterozygous knockout groups revealed significant main effects of SNC80 dose [$F(4,59) = 34.15$, $P < 0.0001$] and genotype [$F(1,59) = 22.20$, $P < 0.0001$] and a significant interaction effect [SNC80 dose × genotype, $F(4,59) = 4.74$, $P = 0.0022$]. The SNC80 dose-response curve in δ-receptor heterozygous knockout mice was shifted approximately 4.2-fold to the right relative to dose-effect curve in wild-type mice, indicating a decrease in the potency of SNC80 (ED_{50} values: +/+, 1.3 mg/kg; +/−, 5.5 mg/kg). SNC80 failed to reduce immobility in δ-receptor homozygous knockout mice at a dose of 10 mg/kg.

The potency of SNC80 to produce convulsions was also evaluated in δ-receptor mutant mice (Fig. 4D). The SNC80 dose-response curve for δ-receptor heterozygous knockout mice was shifted approximately 1.7-fold to the right relative to wild-type mice, indicating a decrease in the potency of SNC80 (ED_{50} values: +/+, 13 mg/kg; +/−, 22 mg/kg). SNC80 failed to produce convulsions in δ-receptor homozygous knockout mice at a dose of 100 mg/kg.

δ-Receptor–Mediated Behaviors in 5′-NTII–Treated Mice. To further explore efficacy requirements contributing to the behavioral effects of δ-receptor agonists, we aimed to decrease δ-receptor numbers by approximately 25%, which is less reduction than that observed in the δ-receptor heterozygous knockout mice, using the irreversible δ-receptor antagonist 5′-NTII. Pretreatment with 32 mg/kg of 5′-NTII for 24 hours reduced the B_{max} of [^{3}H]DPDPE by approximately 30% [$t(8) = 3.393$, $P = 0.0095$; Fig. 5A; Table 1]. There were no significant differences in the affinity of [^{3}H]DPDPE for δ-receptor between treatment groups (Table 1).

In the NTG-induced thermal hyperalgesia assay, there were no differences in the baseline tail withdrawal latencies 24 hours after 5′-NTII or vehicle pretreatment (vehicle: 37 ± 1.4 seconds; 5′-NTII: 41 ± 2.2 seconds). Administration of 10 mg/kg NTG (i.p.) significantly decreased tail withdrawal latency to a similar degree in both treatment groups (vehicle, 4.4 ± 0.5 seconds; 5′-NTII, 5.2 ± 0.4 seconds). Pretreatment with 32 mg/kg of 5′-NTII reduced the potency of SNC80 to increase tail withdrawal latency as evidenced by an approximately 3.3-fold rightward shift in the dose-response curve (Fig. 5B; ED_{50} values: vehicle, 13 mg/kg; 5′-NTII, 43 mg/kg).

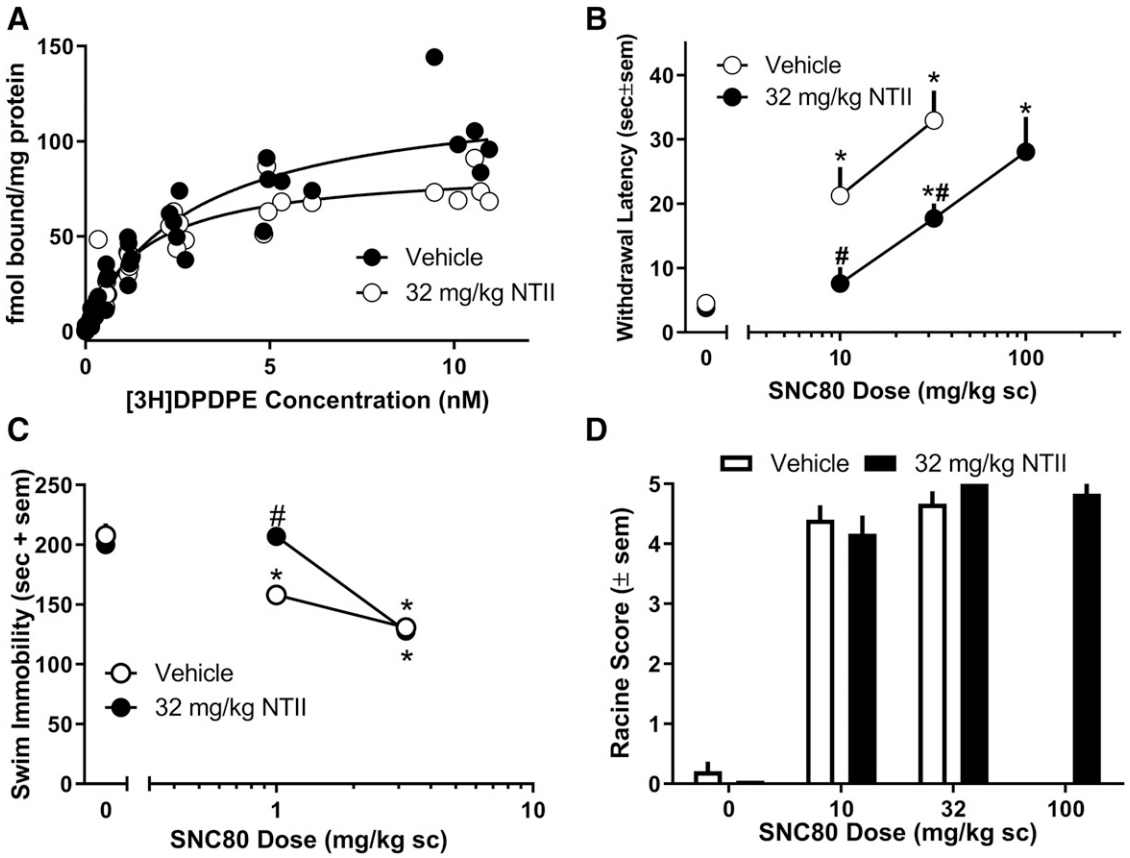

Fig. 5. Effects of 5′-NTII on δ-receptor density and δ-receptor–mediated behaviors. (A) Saturation binding of [^{3}H]DPDPE to membranes prepared from forebrains of C57BL/6N mice 24 hours after pretreatment with 32 mg/kg 5′-NTII or vehicle (10% DMSO). Each point represents tissue from a single mouse assayed in triplicate. (B) Effects of different doses of SNC80 on tail withdrawal latencies in NTG-treated mice 24 hours after pretreatment with 32 mg/kg 5′-NTII or vehicle. (C) Effects of different doses of SNC80 on immobility scores in the FST in mice 24 hours after pretreatment with 32 mg/kg 5′-NTII or vehicle. (D) Severity of SNC80-induced convulsions in mice as measured by a modified Racine scale 24 hours after pretreatment with 32 mg/kg 5′-NTII or vehicle. Data are shown as average per treatment condition with S.E.M. $n = 6$ male C57BL/6N mice per treatment group for all experiments.*$P < 0.05$ compared with vehicle SNC80 treatment in the same pretreatment group; $^{\dagger}P < 0.05$ compared with vehicle pretreatment with same SNC80 dose.

Two-way ANOVA revealed a significant interaction [SNC80 dose (0, 10, 32 mg/kg only) × 5′-NTII dose, $F(2,29) = 3.80$, $P = 0.034$], as well as significant main effects of SNC80 dose [$F(2,29) = 27.01$, $P < 0.0001$] and 5′-NTII dose [$F(1,29) = 17.04$, $P = 0.0003$].

In the forced swim test, 32 mg/kg 5′-NTII alone did not alter immobility scores relative to vehicle pretreatment. The effects of 1 mg/kg SNC80, but not 3.2 mg/kg SNC80, were blocked by pretreatment with 5′-NTII (Fig. 5C). Two-way ANOVA revealed a significant interaction [SNC80 dose × 5′-NTII dose, $F(2,30) = 9.22$, $P = 0.0008$], as well as significant main effects of SNC80 dose [$F(2,30) = 55.36$, $P < 0.0001$] and 5′-NTII dose [$F(1,30) = 4.62$, $P = 0.04$]. The severity of SNC80-induced convulsions was not altered by pretreatment with 5′-NTII (Fig. 5D), nor did 5′-NTII alter the frequency, duration, or time to onset of convulsions (see Supplemental Fig. 1).

δ-Receptor–Mediated Behaviors in NTI-Treated Mice. To further characterize the receptor populations mediating SNC80-induced behaviors, we evaluated SNC80 dose-effect curves following pretreatment with two doses (1 and 3.2 mg/kg) of the competitive δ-receptor antagonist NTI. In an NTG-induced thermal hyperalgesia assay, 10 and 32 mg/kg of SNC80 produced significant increases in tail withdrawal latency in C57BL6 wild-type mice (Fig. 6A). Pretreatment with either 1 or 3.2 mg/kg NTI decreased the potency of SNC80 to increase tail withdrawal latency as evidenced by 5- and 11-fold rightward shifts in the dose-response curve, respectively (SNC80 ED_{50} values: vehicle NTI, 13.2 mg/kg; 1 mg/kg NTI, 66.0 mg/kg; 3.2 mg/kg NTI, 145.4 mg/kg).

In the forced swim test, administration of 1 or 3.2 mg/kg SNC80 significantly decreased immobility in the absence of NTI (Fig. 6B). Pretreatment with 1 or 3.2 mg/kg NTI produced 1.6- and 11-fold rightward shifts in the dose-response curve, respectively, indicating a decrease in SNC80 potency (ED_{50} values: vehicle NTI, 1.3 mg/kg; 1 mg/kg NTI, 2.1 mg/kg; 3.2 mg/kg NTI, 14.3 mg/kg).

Administration of 10 or 32 mg/kg SNC80 alone produced convulsions in mice that did not receive NTI (Fig. 6C). Pretreatment with 1 mg/kg NTI failed to shift this dose-response relationship. However, pretreatment with 3.2 mg/kg NTI produced an approximately 10-fold rightward shift in the dose-response curve (ED_{50} values: vehicle NTI, 10.4 mg/kg; 1 mg/kg NTI, 10.5 mg/kg; 3.2 mg/kg NTI, 112.5 mg/kg). In the groups that received 3.2 mg/kg NTI, single-dose apparent affinity estimates (pK_b) for NTI were similar across all three behavioral endpoints (antihyperalgesia: 6.11; antidepressant-like effects: 6.11; convulsive effects: 6.10).

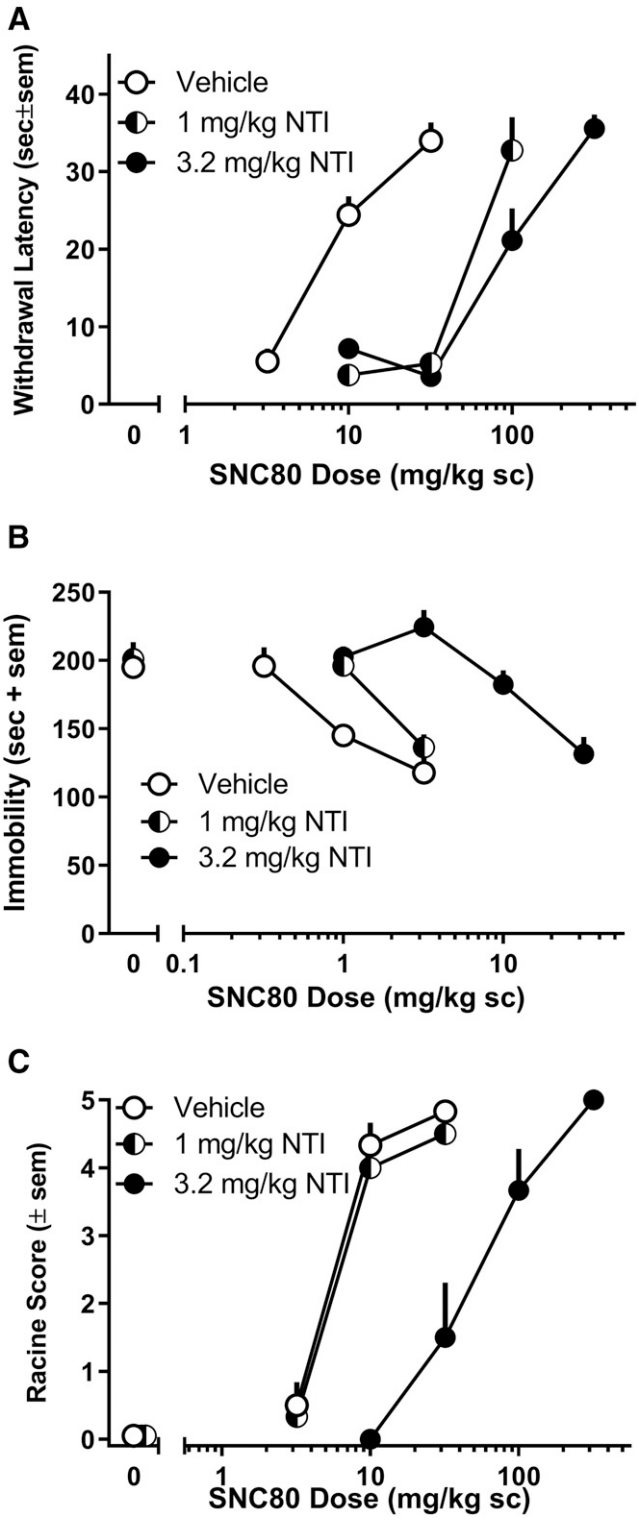

Fig. 6. Potency of NTI to antagonize δ-receptor–mediated behaviors. Effects of different doses of SNC80 after pretreatment with different doses of NTI on (A) tail withdrawal latencies in NTG-treated mice, (B) immobility scores in the FST, and (C) convulsion severity. Data are shown as average per treatment condition with S.E.M. $n = 6$ male C57BL/6N mice per treatment group for all experiments.

Discussion

In this report, we sought to explore the pharmacological characteristics differentiating the effects of δ-receptor agonists observed in vivo—specifically, antihyperalgesia, antidepressant-like, and convulsive effects. Overall, these data support the concept that δ-receptor–mediated behaviors demonstrate a rank order of efficacy requirement, with convulsions having the lowest requirement (largest receptor reserve), followed by antidepressant-like effects and then antihyperalgesia (lowest receptor reserve).

The δ-receptor partial agonist BU48 has previously been shown to elicit δ-receptor–mediated convulsions but not δ-receptor–mediated antinociception in NIH Swiss mice (Broom et al., 2000). Although BU48 produced partial stimulation of [^{35}S]GTPγS binding in C6 glioma cells expressing cloned δ-receptors (Broom et al., 2000), BU48 did not have

sufficient efficacy to stimulate G protein activation in mouse brain homogenates or slices in the current study. These disparate results may be due to lower δ-receptor density in mouse brain than in cultured cells (Akiyama et al., 1985). However, BU48 still bound to δ-receptors in mouse brain slices and attenuated SNC80-induced G protein activation. Interestingly, in the present study, BU48 produced G protein activation in some brain regions and, in the hippocampus, produced more G protein activation than SNC80. At this time, it is unclear why the patterns of G protein activation between SNC80 and BU48 should be different, but it could be due to coupling to different G protein subunits, different cellular environments, or activation of other opioid receptors, such as κ-opioid receptors (Broom et al., 2000). Although BU48 may be a very low-efficacy agonist (or no efficacy) at the level of G protein activation, BU48 may display higher apparent efficacy in assays in which greater signal amplification is involved or potentially in G protein–independent signaling pathways.

To further characterize the behavioral effects of this δ-receptor partial agonist, we evaluated the potency of BU48 in producing δ-receptor–mediated antihyperalgesia, antidepressant-like effects, and convulsions in the C57BL/6 mouse strain. BU48 produced dose-dependent increases in convulsion severity with similar potency and efficacy to SNC80. Although convulsive effects were not observed in mice treated with 3.2 mg/kg SNC80, the same dose of BU48 produced some preconvulsive behavior, such as head twitches and brief myoclonic jerks. BU48 also produced antidepressant-like effects in the forced swim test, albeit with reduced potency relative to SNC80. BU48 also appeared to be less efficacious than SNC80 in the forced swim test, consistent with a partial agonist profile; however, larger doses of BU48 would need to be tested to fully evaluate this claim. The reduced potency and/or efficacy of BU48 in the forced swim test may also be due to its κ-opioid agonist activity; κ agonists are known to produce prodepressant-like effects (Mague et al., 2003).

BU48 not only failed to reverse NTG-induced thermal hyperalgesia in wild-type mice, it also antagonized SNC80-induced antihyperalgesic effects. These data further support the claim that BU48 is a δ-receptor partial agonist in vivo. One alternative explanation is that BU48 is a biased δ-receptor agonist that cannot activate the intracellular signaling mechanisms needed to produce antihyperalgesia. However, a large (32 mg/kg) dose of BU48 did produce moderate antihyperalgesic effects in mice lacking RGS4 proteins. RGS4 acts as a negative regulator of $G\alpha_{i/o}$ signaling, and either genetic elimination and pharmacological inhibition of RGS4 has previously been shown to enhance the potency and efficacy of SNC80 (Wang et al., 2009; Dripps et al., 2017). These data further support the idea that δ-opioid receptor–mediated antihyperalgesia is mediated through a G protein–dependent pathway (Dripps et al., 2018) and that BU48 is a δ-receptor partial agonist. As such, BU48 should produce behaviors that require low agonist efficacy more readily than ones that require high-efficacy agonist stimulation. Overall, these findings suggest that BU48 is a partial agonist as compared with SNC80 and that δ-receptor–mediated behaviors display the following rank order of efficacy: convulsions < antidepressant-like effects < antihyperalgesia. Future studies should determine whether this rank order holds true for other structural classes of δ-receptor agonists.

To further probe the role of receptor reserve in δ-receptor–mediated behaviors, we considered the potency of SNC80 to produce these behaviors and evaluated SNC80 potency under conditions in which δ-receptors are expressed at lower levels, specifically in δ-receptor knockout mice and in mice treated with an irreversible δ-receptor antagonist. In wild-type littermates, SNC80 dose-dependently reversed NTG-induced hyperalgesia, consistent with previous reports (Pradhan et al., 2014; Dripps et al., 2017). SNC80 also produced decreases in immobility in the forced swim test and convulsions, which are well established behavioral effects generated by SNC80 (Broom et al., 2002b; (Saitoh et al., 2004); Dripps et al., 2017). When conside(Sim et al., 1995)ring the SNC80 dose that was administered, the antidepressant-like effects of SNC80 were the most potent, followed by the convulsive and antihyperalgesic effects of SNC80. However, SNC80-induced convulsions occur within minutes of drug administration prior to peak drug levels in circulation, whereas the antihyperalgesic and antidepressant-like effects were evaluated at much later time points (60 and 30 minutes post–drug administration, respectively; swimming behaviors were evaluated later because of initial concerns about swimming ability postconvulsions). Therefore, it is difficult to determine a true dose-effect curve for SNC80-induced convulsions and to relate potency alone to the receptor reserve or efficacy requirement of these SNC80-induced behaviors. Although in previous studies in which SNC80 was given by intravenous injection to bypass pharmacokinetic processes of absorption and distribution, the convulsive effects of SNC80 were slightly more potent than its antidepressant-like effects (Jutkiewicz et al., 2005). Thus, to probe the role of receptor reserve in δ-receptor–mediated behaviors, we had to alter receptor numbers by genetic deletion and by using an irreversible antagonist.

SNC80 failed to produce any of these behaviors in δ-receptor homozygous knockout mice, supporting previous evidence that these behaviors are specifically mediated by δ-receptors. The potency of SNC80 to produce all three of these behaviors was significantly reduced in δ-receptor heterozygous knockout mice, with the following rank order of efficacy requirement: convulsions (1.7-fold loss of potency) < antidepressant-like effects (4.2-fold) < antihyperalgesia (7.1-fold). Consistent with these findings, a 30% reduction in δ-receptor number induced by the irreversible δ-receptor antagonist was sufficient to decrease the potency of SNC80 to elicit antihyperalgesia and antidepressant-like effects but failed to shift the dose-response curve for convulsions. The minimal inhibition of SNC80-induced convulsions after a significant reduction in receptor number suggests that δ-receptor–mediated convulsions have a large receptor reserve and that low agonist efficacy is required to produce convulsions (i.e., low efficacy requirement). Conversely, SNC80-induced antihyperalgesia was particularly sensitive to changes in δ-receptor number, suggesting a low receptor reserve and high efficacy requirement. The decrease in potency of SNC80-induced antidepressant-like effects was moderate, suggesting an intermediate efficacy requirement.

These apparent differences in efficacy requirement could be potentially explained in part by distinct populations of δ-receptor mediating these behaviors. Against this, we found that a large dose of 3.2 mg/kg of the δ-receptor competitive antagonist NTI produced similar shifts in the dose-response curves of SNC80-induced behaviors. Indeed, the estimated in vivo pK_b values of NTI were similar across all

three behaviors that were tested (antihyperalgesia: 6.11; antidepressant-like effects: 6.11; convulsive effects: 6.10), suggesting that these behaviors are mediated by similar types of δ-receptors.

Interestingly, a small dose of 1 mg/kg NTI differentially shifted the dose-response curves of the observed δ-receptor–mediated behaviors. Differences in the apparent effectiveness of NTI may be due to the inability to conduct the in vivo experiments at times when the agonist and antagonist are at equilibrium. This discrepancy could also be due, in part, to differences in the times at which these behaviors are observed (as natural progression or occurrence of the behavior) or measured (as determined by assay design)—specifically, antihyperalgesic effects at 30 min, antidepressant-like effects at 60 min, and convulsive effects between 0 and 30 min. Alternatively, it is possible that the receptor reserve for δ-receptor–mediated convulsions and antidepressant-like effects is sufficiently large that the small dose of NTI is unable to occupy a large enough portion of receptors (at any single point in time) to observe significant antagonism. It is thought that a competitive antagonist needs to occupy 50% of receptor sites before its effects on an agonist can be noticed (Kenakin, 2009). This hypothesis is consistent with the previously discussed findings that a 30% loss in δ-receptor (by 5′-NTII) does not alter SNC80-induced convulsions, but an approximately 60% loss (in δ-receptor heterozygous knockouts) produces mild inhibition. Furthermore, Broom et al. (2002a) showed that a 75% reduction in δ-receptor number abolished BW373U86-induced antinociception but still produced convulsions in a majority of NIH Swiss mice.

However, differences in the effectiveness of an antagonist across separate behavioral endpoints could suggest that different receptor populations or different ligand-receptor interactions mediate these behaviors. There are several possibilities regarding what these different receptor populations may represent. The putative δ-receptor 1 and 2 subtypes have previously been implicated in mediating the behavioral effects of SNC80 (Pacheco et al., 2005; Rawls et al., 2005). The existence of δ-μ and δ-κ receptor heterodimers that engage unique signaling mechanisms and possibly produce distinct behaviors has been proposed (Jordan and Devi, 1999; Rozenfeld and Devi, 2007). Differences in the subcellular localization of receptors or agonist-induced internalizing properties of δ-receptors could also lead to differences in downstream signaling (Pradhan et al., 2009). Future studies should evaluate differences in δ-receptor antagonist potency and effectiveness or potential non–δ-receptor mechanisms that may contribute to these effects.

In conclusion, the current report demonstrates that the convulsive, antidepressant-like, and antihyperalgesic effects of the δ-receptor agonist SNC80 are mediated through the same receptor, but the receptor reserve for these SNC80-induced behavioral effects varies. Since receptor reserve is considered both a property of the amplification of the receptor stimulus within a tissue (or brain region) and of agonist efficacy (Kenakin, 2009), other δ-receptor agonists may have different magnitudes of receptor reserve. It should be noted that multiple δ-receptor agonists have been developed that do not produce convulsions when given systemically in large doses (Naidu et al., 2007; Le Bourdonnec et al., 2008; Vergura et al., 2008; Saitoh et al., 2011). Thus, it is critical to determine why these agonists do not produce convulsions. It is possible that nonconvulsive δ-receptor agonists induce receptor signaling amplification in such a way as to not produce convulsions, potentially through different signaling mechanisms [e.g., signaling localization or biased signaling (Pradhan et al., 2009)]. Alternatively, the pharmacokinetic properties of these nonconvulsive δ-receptor agonists could inhibit their ability to produce convulsions. It has been shown that rapid intravenous infusion of SNC80 increases the potency of SNC80 to produce convulsions, whereas slow (20 or 60 min) infusions of SNC80 greatly diminish potency to produce convulsions (Jutkiewicz et al., 2005). Future studies should evaluate the pharmacokinetic properties of these different δ-receptor agonists, signaling molecules, and pathways contributing to the convulsive effects and the receptor reserves associated with each agonist.

Authorship Contributions

Participated in research design: Dripps, Traynor, Jutkiewicz.

Conducted experiments: Dripps, Chen, Shafer, Livingston.

Contributed new reagents or analytic tools: Disney, Husbands, Rice, Traynor, Jutkiewicz.

Performed data analysis: Dripps, Chen, Livingston, Traynor, Jutkiewicz.

Wrote or contributed to the writing of the manuscript: Dripps, Chen, Jutkiewicz.

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