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Delta Opioid Receptor-Mediated Antidepressant-Like Effects of Diprenorphine in Mice[S]

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ABSTRACT

Major depressive disorder is a highly common disorder, with a lifetime prevalence in the United States of approximately 21%. Traditional antidepressant treatments are limited by a delayed onset of action and minimal efficacy in some patients. Ketamine is effective and fast-acting, but there are concerns over its abuse liability. Thus, there is a need for safe, fast-acting antidepressant drugs. The opioid buprenorphine shows promise but also has abuse liability due to its mu-agonist component. Preclinical evidence indicates that the delta-opioid system contributes to mood disorders, and delta-opioid agonists are effective in preclinical models of depression- and anxiety-like states. In this study, we test the hypothesis that the mu-opioid antagonist diprenorphine by virtue of its partial delta opioid agonist activity may offer a beneficial profile for an antidepressant medication without abuse liability. Diprenorphine was confirmed to bind with high affinity to all three opioid receptors, and functional experiments for G protein activation verified diprenorphine to be a partial agonist at delta- and kappa-opioid receptors and a mu-antagonist. Studies in C57BL/6 mice demonstrated that an acute dose of diprenorphine produced antidepressant-like effects in the tail suspension test and the novelty-induced hypophagia test that were inhibited in the presence of the delta-selective antagonist, naltrindole. Diprenorphine did not produce convulsions, a side effect of many delta agonists but rather inhibited convulsions caused by the full delta agonist SNC80; however, diprenorphine did potentiate pentylenetetrazole-induced convulsions. Diprenorphine, and compounds with a similar pharmacological profile, may provide efficient and safe rapidly acting antidepressants.

SIGNIFICANCE STATEMENT

The management of major depressive disorder, particularly treatment-resistant depression, is a significant unmet medical need. Here we show that the opioid diprenorphine, a compound with mu-opioid receptor antagonist activity and delta- and kappa-opioid receptor partial agonist activities, has rapid onset antidepressant-like activity in animal models. Diprenorphine and compounds with a similar pharmacological profile to diprenorphine should be explored as novel antidepressant drugs.

Introduction

Major depressive disorder is a common mood disorder worldwide. Approximately 21 million adults in the United States (8.4% of the population) had one or more major depressive episodes in 2020 (National Institute of Mental Health, 2020: https://www.nimh.nih.gov/health/statistics/major-depression) with a lifetime prevalence of approximately 20.6% (Hasin et al., 2018). Major depressive disorder is typically treated with serotonin or serotonin/norepinephrine reuptake inhibitors. Unfortunately, these drugs are ineffective in approximately 50% of major depressive disorder patients and require 4 to 12 weeks of treatment before symptom relief in patients that show a response (Warden et al., 2007); such drugs can also produce significant monoamine-mediated side effects (Wang et al., 2018). The discovery of the rapid onset of antidepressant action of ketamine was a major breakthrough in the management of depression (Browne and Lucki, 2013) and led to the Food and Drug Administration (FDA) approval in 2019

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ABBREVIATIONS: CHO, Chinese hamster ovary; DAMGO, [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin; DOPr, delta-opioid receptor; DPN, diprenorphine; FDA, Food and Drug Administration; DOPr, delta-opioid receptor; HEK, human embryonic kidney; KOPr, kappa-opioid receptor; MOPr, mu-opioid receptor; NTI, naltrindole; SNC80, (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-di-methyl-1-piperazinyl)-3-methoxybenzyl]-N, N-diethylbenzamide; TST, tail suspension test; NIH, novelty-induced hypophagia; PTZ, pentylenetetrazole; U69593, N-methyl-2-phenyl-N-(7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]decan-8-yl)acetamide.

of esketamine, the S(+) enantiomer of ketamine, as a nasal spray for treatment-resistant depression. The drug acts rapidly, but there are concerns over its abuse liability and potential for misuse (Hillhouse and Porter, 2015; Witkin et al., 2019) such that it can only be administered in a certified medical office. Consequently, there remains a significant unmet medical need to identify rapid-onset, effective, and safe antidepressants.

The delta-opioid receptor (DOPr) is a member of the opioid peptide receptor family of 7-transmembrane G-protein coupled receptors and a target for the development of novel antidepressant drugs. Mice lacking DOPr show increased depressive- and anxiogenic–like behaviors in preclinical models, including the forced-swim test, dark–light box test, and elevated plus maze test (Filliol et al., 2000). In support of this, DOPr agonists work in preclinical models used to evaluate novel antidepressant drugs (Broom et al., 2002b, 2002c; Hudzik et al., 2011) without producing significant gastrointestinal (Porreca et al., 1984), respiratory (Negus et al., 1994; Su et al., 1998), or abuse liability (Negus et al., 1994; Do Carmo et al., 2009; Hudzik et al., 2014) associated with mu-opioid receptor (MOPr) agonists or dysphoria associated with kappa-opioid receptor (KOPr) agonists (Chavkin and Koob, 2016). Many DOPr agonists produce convulsions, which limits their clinical potential (Hong et al., 1998; Broom et al., 2002d), although DOPr agonist-mediated antidepressant-like effects and convulsive activity can be separated (Jutkiewicz et al., 2005). Furthermore, the convulsive activity of DOPr agonists is not required to observe antidepressant-like actions (Broom et al., 2002a). In support of this, several nonconvulsive selective DOPr agonists have been reported including JNJ-20788560, KNT127, ADL5859, ARM390, and several AZD compounds (Pradhan and Clarke, 2005; Le Bourdonnec et al., 2008, 2009; Saitoh et al., 2011; Nozaki et al., 2012), and at least one of these (AZD2327) was evaluated in phase II clinical trials for antidepressant and anxiolytic activity (Richards et al., 2016).

Diprenorphine (DPN) has traditionally been recognized as a nonselective opioid antagonist, although it is reported to exhibit partial agonist activity at DOPr as well as KOPr (Traynor et al., 1987; Szekeres and Traynor, 1997), in addition to potent MOPr antagonism (Lee et al., 1999). Here, we test the hypothesis that DPN will produce antidepressant-like effects through a DOPr-mediated mechanism. We confirm the opioid receptor profile of DPN as a DOPr and KOPr partial agonist and MOPr antagonist. Further, we show that in mice, DPN produces potent antidepressant-like activity when measured using the tail suspension test (TST), and rapid onset antidepressant-like effects in the novelty-induced hypophagia (NIH) test, which requires chronic treatment with traditional antidepressant drugs. The antidepressant-like activity of DPN is fully reversed by the DOPr selective antagonist naltrindole. Additionally, DPN alone did not produce typical DOPr-mediated convulsions but rather inhibited convulsions caused by the full DOPr agonist SNC80.

Materials and Methods

In Vitro Assays

Cell Culture and Membrane Preparation. Chinese hamster ovary (CHO) expressing human (h) DOPr, MOPr, or KOPr were cultured in Dulbecco's modified Eagle's medium containing 10% FBS and 1% penicillin–streptomycin to 80% confluency. Cells were harvested, and membrane homogenates were prepared as previously described (Nastase et al., 2018) and stored at −80°C at a protein concentration of 0.5 to 1.5 mg/ml.

Saturation Binding. Cell membranes (10 μg protein) were incubated for 60 minutes at 30°C in 50 mM Tris-HCl (pH 7.4) with various concentrations of [^{3}H]-DPN with or without 10 μM naloxone to determine the degree of nonspecific binding or total binding, respectively (Hillhouse et al., 2021). Assays were stopped by filtration through glass microfiber GF/B filters (Whatman), filters washed three times with ice-cold assay buffer, dried, treated with EcoLume liquid scintillation fluid (MP Biomedicals), and radioactivity retained on the filters determined using a Wallac 1450 MicroBeta2 counter (PerkinElmer).

[^{35}S]GTPγS Binding. Agonist stimulation of [^{35}S]GTPγS binding was measured as previously described (Traynor and Nahorski, 1995; Hillhouse et al., 2021). Briefly, cell membranes (15–20 μg protein/well) were incubated in GTPγS buffer (50 mM Tris-HCl, 100 mM NaCl, 5mM MgCl2, pH 7.4) containing 0.1 nM [^{35}S]GTPγS, 30 μM guanosine diphosphate and varying concentrations of DPN for 60 minutes in a shaking water bath at 30°C. SNC80, DAMGO, and U69,593 were used as standards for DOPr, MOPr, and KOPr, respectively. Reactions were terminated, and radioactivity was measured as described in the previous text.

β-Arrestin2 Recruitment. The PathHunter β-galactosidase enzyme-complementation assay (DiscoverRx, Fremont, CA, USA) was employed to determine β-arrestin2 recruitment to MOPr and DOPr in CHO cells (Burford et al., 2013). Cells were incubated with various concentrations of drugs (DPN or DAMGO and SNC80 as positive controls) for 60 minutes. β-galactosidase activity was detected by luminescence measured with a Synergy 2 plate reader (Biotech, Winooski, VT, USA).

DOPr Internalization. Human embryonic kidney (HEK) cells stably expressing N-terminal FLAG-tagged hDOPr (Bradbury et al., 2009) were plated in 24-well plates coated with poly-D-Lysine. When cells reached 80% confluency, they were treated with vehicle (1% DMSO), 10 μM SNC80, or 10 μM DPN for 1 hour at room temperature. Cells were fixed with formaldehyde and then washed with Tris-buffered saline and blocked for 1 hour with 1% bovine serum albumin. After washing, cells were incubated with FLAG M2 alkaline phosphatase antibody at a 1:625 dilution for 60 minutes and absorption read at 405 nm on VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Precent internalization was measured by loss of surface receptors using (1 – [(O.D. drug − O.D. background)/(O.D. vehicle − O.D. background)] × 100), where O.D. is optical density. The absorbance of wild-type HEK293 cells without a transfected receptor was used as the background value.

In Vivo Assays

Animals. Adult male and female C57BL/6 mice (8–16 weeks of age) were employed for all experiments unless otherwise stated. Mice were bred at the University of Michigan; breeder mice were from Envigo (Indianapolis, IN, USA). All mice were maintained on a 12-hour light/dark cycle; experiments were performed during the light phase. Mice were group-housed (five animals per cage according to sex, unless stated otherwise) in clear polypropylene cages with corncob bedding and had free access to food and water as well as enrichment in their home cage. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the University of Michigan Committee on the Institutional Animal Care and Use Committee.

Tail Suspension Test. Experiments were conducted in male mice as previously described (Steru et al., 1985; Talbot et al., 2010; Casal-Dominguez et al., 2013) using a 6-minute test session. A trained observer blind to the treatment conditions scored immobility time (seconds). Immobility was defined as hanging motionless with no escape-related behaviors, defined as running-like movements with paws and forelimbs, strong shakes of the body, and attempts to

reach the suspension bar. Drugs were administered intraperitoneally 30 minutes before test sessions. For the antagonist experiments, naltrindole was administered subcutaneously 30 minutes prior to DPN.

Novelty-Induced Hypophagia. Procedures as previously described (Talbot et al., 2010) were followed. Male and female mice were group-housed (four per cage, according to sex) and acclimated to sipper tubes (Med Associates Inc; supply number PHM-127-15) by providing overnight access to water on days 1 and 2. On days 3 and 4, mice were allowed access to a sweetened solution (Vanilla Ensure at a water:Ensure ratio of 1:2) for 2 to 4 hours. Mice were singly housed at the end of day 4 for the remainder of the study. During days 5 to 7 inclusive, mice had 30 minutes access to the sweetened solution in their home cage. On day 8, a home cage test session was conducted, and on day 9, a novel cage test session was conducted in which mice were placed into a test cage (42 × 22 × 16 cm) of the same material and color as the home cage (28 × 17 × 13 cm), but without bedding. The home cage and novel cage test sessions were 30 minutes in duration, and the latency to drink and volume of sweetened solution consumed were recorded. A trained, blinded observer scored test sessions. DPN or SNC80 was administered intraperitoneally 30 minutes prior to novel cage test sessions. For the antagonist experiments, naltrindole was administered subcutaneously 30 minutes before DPN or SNC80.

Locomotor Activity. Male and female C57BL/6 mice bred in-house were used at 8 to 10 weeks of age with four mice per sex per treatment condition (eight mice total per condition). Mice were removed from their home cage and administered an intraperitoneal injection of saline, 10 mg/kg DPN, 10 mg/kg morphine, or 32 mg/kg morphine and then placed immediately into Plexiglass chambers (44.5 cm width × 44.5 cm depth × 20.5 cm height) with an XY grid of infrared light beams spaced 1 inch apart and 2 to 2.5 cm from the floor of the chamber (Columbus Instruments, Columbus, OH, USA). Data were collected for 60 minutes in 5-minute bins. Mice were not habituated to locomotor chambers prior to these measurements, consistent with the behavioral measures collected in the TST or novel environment in the NIH test.

Convulsive Activity. Experiments were conducted as previously described (Hong et al., 1998; Dripps et al., 2020). Male and female mice were used for most experiments, although certain experiments used only male mice as noted in the results and figure legends. Mice were placed in clear, clean cages with bedding immediately following subcutaneous DPN, SNC80, or vehicle administration. Mice were observed for 60 minutes. A trained, blinded observer measured the latency to convulse, percentage of mice convulsing and severity of convulsions by use of a modified Racine score as follows: (i) teeth chattering/face twitching; (ii) head bobbing/twitching; (iii) tonic extension and/or repeated clonic contractions lasting <3 seconds; (iv) tonic extension and/or repeated clonic contractions lasting >3 seconds; and (v) tonic extension and/or repeated clonic contractions (convulsion) lasting >3 seconds with loss of balance. Racine scores of 4 or 5 were considered a full convulsion while Racine scores of 1 to 3 were considered preconvulsive behavior. To examine the effect of DPN or naltrindole on SNC80-induced convulsions, mice were pretreated with the antagonists (subcutaneously) 30 minutes prior to SNC80 administration. To examine the ability of DPN or SNC80 to potentiate PTZ-induced convulsions, mice were pretreated with either drug or vehicle (subcutaneously) 30 minutes before 32 mg/kg PTZ (subcutaneously).

Drugs and Materials. [^{3}H]-DPN and [^{35}S]GTPγS were purchased from Perkin Elmer Life Sciences (Cambridge, MA, USA). DPN HCI was from the National Institute on Drug Abuse Research Resource Drug Supply Program (Bethesda, MD, USA). SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-di-methyl-1-piperazinyl)-3-methoxybenzyl]-N, N-diethylbenzamide) was synthesized as previously described (Calderon et al., 1994). Morphine sulfate solution was from Hospira, Inc (Lake Forest, IL, USA). Naltrindole HCI was from Tocris (Minneapolis, MN, USA), and desipramine, FLAG M2 alkaline phosphatase antibody, and all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). For behavioral experiments, desipramine, DPN, and morphine were dissolved in physiologic saline, naltrindole HCl was dissolved in sterile water, and SNC80 was dissolved in 1 M HCl and then diluted in sterile water to a concentration of 3% to 5% HCI. All drugs were administered at a volume of 10.0 mL/kg.

Data Analysis. Results were analyzed in GraphPad Prism 7.0 (La Jolla, CA, USA) and expressed as mean ± S.E.M. For saturation binding experiments, a one-site saturation binding analysis was used to determine affinity (K_D) values and maximal binding. For the [^{35}S]GTPγS assays, potency (EC_{50}) and degree of stimulation were determined using nonlinear regression analysis. Comparison of internalization was made by t test. For the TST experiments, immobility time (seconds) was analyzed using either a t test (desipramine) or a between-subjects one-way ANOVA (DPN). For the NIH experiments, volume consumed (ml) and latency to drink (seconds) were analyzed by mixed factor two-way ANOVA with environment (home or novel cage) as the within-subject factor and drug treatment as the between-subjects factor. For the locomotor measurements, total X and Y beam breaks for each 5-minute interval over 60 minutes of measurement were averaged per treatment group. Locomotor activity data are also shown as the total X and Y beam breaks for 60 minutes per treatment group. Locomotor activity time course data were analyzed by repeated measures two-way ANOVA with Tukey's post hoc test (with treatment as the between-subjects factor and time as the within-subjects factor), and total beam breaks were analyzed by one-way ANOVA with Tukey's post hoc test. For the convulsion experiments, data are the percentage of mice that convulsed, latency to convulse, and severity of convulsion for each treatment group. Significant ANOVAs were followed by Tukey post hoc test. The criterion for significance was at $P < 0.05$.

Results

In Vitro Assays

Affinity and Functional Activity of Diprenorphine at Opioid Receptors. DPN displayed high (nM) affinity at all three receptors expressed in CHO cell membranes in the rank order MOPr = KOPr > DOPr (Table 1). DPN was seen to be a partial agonist of high potency at DOPr in the [^{35}S]GTPγS assay in membranes from CHO cells compared with the standard full agonist SNC80 and a more potent partial agonist at KOPr when compared with the standard agonist U69,593 (Table 1). In contrast, at MOPr DPN did not stimulate [^{35}S]GTPγS binding but acted as a potent antagonist of the MOPr full agonist DAMGO (Table 1), with an antagonist affinity constant (0.18 nM) that matches its ligand binding affinity (0.3nM). To further characterize activity at MOPr and DOPr, we tested the ability of DPN to recruit β-arrestin2 and to drive DOPr internalization. DPN did not recruit β-arrestin2 at any detectable levels to MOPr or DOPr expressed in CHO cells (Table 1), despite its partial agonist activity at DOPr in the [^{35}S]GTPγS assay. In accordance with its inability to recruit β-arrestin2, DPN did not cause internalization of DOPr tagged with a FLAG epitope expressed in HEK 293 cells (not shown).

Behavioral Assays

Antidepressant-Like Activity in the Tail Suspension Test. DPN significantly reduced immobility time in the TST [$F(4,25) = 6.30$, $P = 0.0012$] (Fig. 1A). Specifically, 10.0 mg/kg reduced the immobility time compared with saline ($P < 0.05$). The positive control desipramine (32 mg/kg) significantly decreased the time spent immobile as compared with the saline control [$t(10) = 4.63$, $P = 0.0009$] (Fig. 1B) as did the selective DOPr agonist SNC80 [$F(3, 21) = 4.56$, $P = 0.013$]

TABLE 1

In vitro profile of DPN at opioid receptors

Experiments were performed in CHO cells (β-arrestin) or CHO cell membranes (saturation binding and [^{35}S]-GTPγS binding) expressing either hMOPr, hDOPr, or hKOPr. Saturation-binding experiments were performed with ^{3}H-DPN. Naloxone was used to define non-specific binding. In the [^{35}S]-GTPγS assay and β-arrestin assays, standard agonists were used to define the maximum response (100%). The K_B value for DPN at MOPr was determined against the agonist DAMGO. Potency values for the standard agonists are as follows. in the [^{35}S]-GTPγS assay: (MOPr: DAMGO EC_{50} = 26 ± 2.4 nM; DOPr: SNC80 EC_{50} = 2.2 ± 0.6 nM; KOPr: U69593 EC_{50} = 9.6 ± 3.8 nM; in the β-arrestin assay: MOPr: DAMGO EC_{50} = 120 ± 2.4 nM; DOPr: SNC80 EC_{50} = 84 ± 17 nM. Values are means of three experiments ± S.E.M. each performed in duplicate.

	Saturation binding		[^{35}S]-GTPγS binding		
Receptor	K_D (nM)	B_{Max} (fmols/mg protein)	EC50 (nM)	Max response (%)	β-arrestin2 recruitment
MOPr	0.31 ± 0.04	4.0 ± 0.2	K_B: 0.18 ± 0.05	NR	NR
DOPr	1.1 ± 0.06	2.2 ± 0.5	5.0 ± 1.1	55 ± 5	NR
KOPr	0.35 ± 0.09	1.3 ± 0.1	0.71 ± 0.21	31 ± 5	NT

NR, no response; NT, not tested.

(Fig. 1C); SNC80 at 3.2 mg/kg lowered immobility time compared with vehicle ($P < 0.01$). The effects of DPN were observed to occur after 30 minutes, similar to desipramine and SNC80. To examine whether the observed effect of DPN was DOPr-mediated, mice were treated with the DOPr selective antagonist naltrindole 30 minutes before DPN administration. When pretreated with vehicle, 10.0 mg/kg DPN significantly decreased time spent immobile [F (4,39) = 10.87 P = 0.0006] (Fig. 1D). Pretreatment with either 3.2 or 10.0 mg/kg naltrindole significantly lessened the antidepressant-like effects of 10 mg/kg DPN ($P < 0.05$). Naltrindole (3.2 mg/kg) alone had no effect on immobility time (Fig. 1D).

Antidepressant-Like Activity in the Novelty-Induced Hypophagia Test. DPN reduced the latency to drink with a significant main effect of treatment [F(3,34) = 10.22, $P <$ 0.0001] and environment [F(1,34) = 264.9, $P < 0.0001$] and a significant interaction [F(3, 34) = 11.79, $P < 0.0001$]. Post hoc analysis revealed that DPN dose-dependently decreased latency to drink in the novel environment with significant decreases at 3.2 mg/kg ($P < 0.01$) and 10 mg/kg ($P < 0.001$) (Fig. 2A). DPN increased the volume consumed with a significant main effect of treatment [F(3,34) = 6.49, P = 0.0021] and environment [F (1, 34) = 18.29, P = 0.0002] but not a significant interaction [F(3,34) = 0.64, P = 0.67] (Fig. 2B). Overall, treatment with 10 mg/kg DPN, regardless of test environment, significantly increased the volume consumed ($P < 0.01$). The positive control, SNC80, also reduced latency to drink with a significant main effect of treatment [F(1,16) = 3.74, P = 0.032] and environment [F(1,16) = 50.85, $P < 0.0001$] and a significant interaction [F(2,16) = 3.45, P = 0.043]; SNC80 at 10 mg/kg significantly reduced the latency to drink in the novel environment ($P < 0.01$) (Fig. 2C), but, unlike DPN, SNC80 did not significantly alter the volume consumed in the home cage or novel environment (main effect of treatment [F(1,16) = 0.72, P = 0.77] (Fig. 2D). The action of DPN to increase the amount of sweetened solution ingested in both the novel and home cages was unexpected. However, effects of opioids on food intake are complex (Bodnar, 2019), such that any aspect of the multifaceted pharmacology of DPN could be responsible.

To evaluate whether the action of DPN in the NIH test required activation of DOPr, mice were pretreated with naltrindole or vehicle (Fig. 3). There was a significant main effect of treatment [F(3, 33) = 6.02, P = 0.0022] and environment [F(1, 33) = 185.4, $P< 0.0001$] and a significant interaction [F(3, 33) = 6.40, P = 0.0015], such that the ability of 10 mg/kg DPN to decrease latency to drink in the novel cage was completely blocked by 3.2 mg/kg naltrindole ($P < 0.001$) (Fig. 3A). For volume consumed, there was a significant main effect of treatment [F(3, 33) = 3.19, P = 0.03] and environment [F(1,33) = 31.03, $P < 0.0001$] but no significant interaction [F(3, 33)=1.42, P = 0.26], such that more liquid was taken in the home cage as compared with the novel environment (Fig. 3B). Naltrindole (3.2 mg/kg) did not affect either latency to drink or volume of sweetened solution consumed in the vehicle control mice. Supplementary Tables 1 and 2 provide a breakdown of the NIH results by sex.

Locomotor Activity

To evaluate DPN-induced locomotor activity, mice were placed into chambers with infrared beams immediately following intraperitoneal injections of saline, 10 mg/kg DPN, 10 mg/kg morphine, or 32 mg/kg morphine. There was a significant effect of locomotor activity over time [time × treatment interaction; F(33, 308) = 5.15, $P < 0.0001$] (Fig. 4A); however, DPN and 10 mg/kg morphine failed to significantly increase locomotor activity over saline control at any time interval. In contrast, 32 mg/kg morphine significant increased

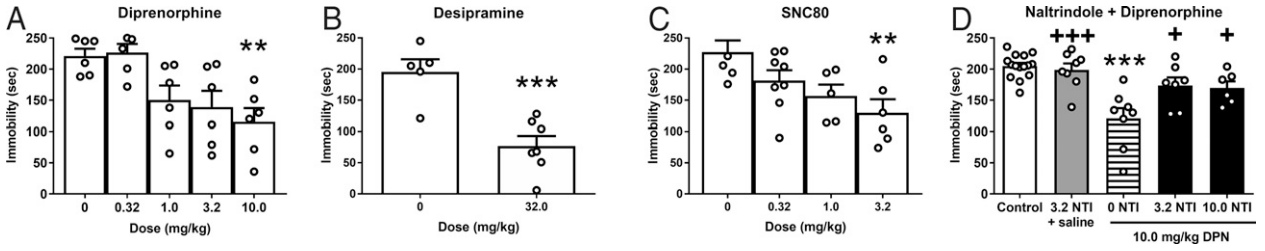

Fig. 1. Effects of DPN on immobility time in the TST. Reduction in immobility time by (A) DPN, (B) desipramine, and (C) SNC80. (D) The inhibition of the effect of DPN following pretreatment with 3.2 or 10 mg/kg naltrindole (NTI). All points shown represent means ± S.E.M. for 6 to 8 male mice for each treatment condition and 15 male mice for the control group (D). Data were analyzed by ANOVA followed by a Tukey post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle/saline/control group; +$P < 0.05$, +++ $P < 0.001$ vs. 0 NTI + 10 mg/kg DPN.

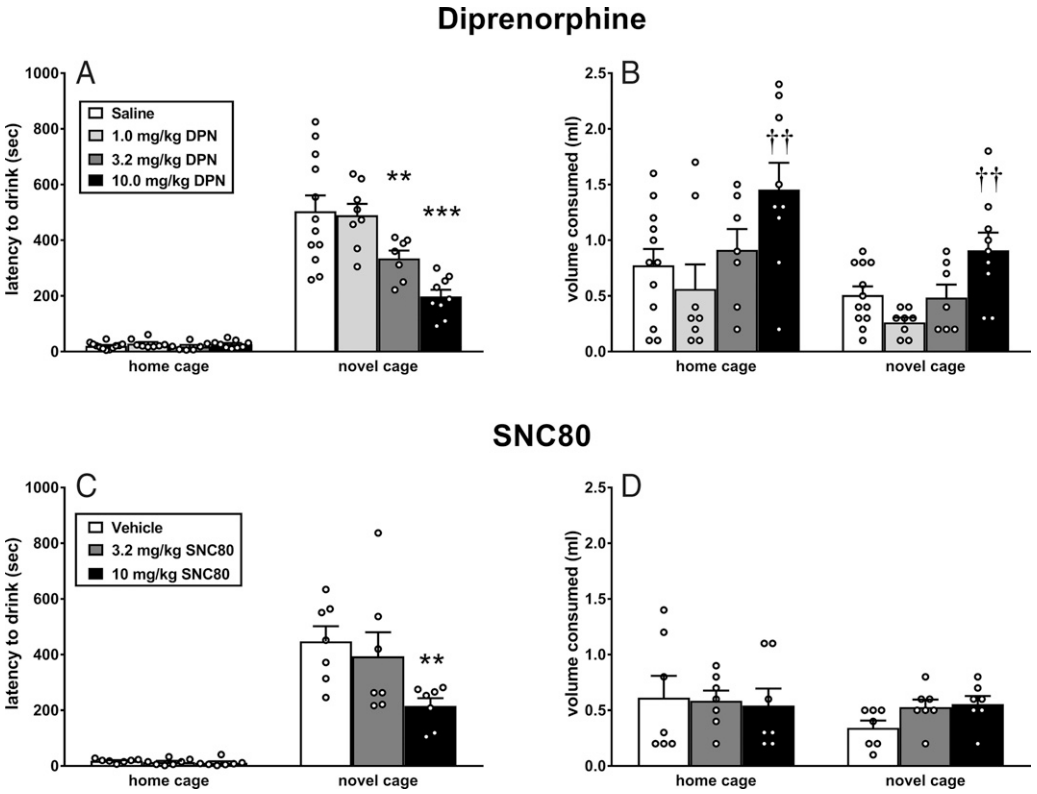

Fig. 2. Effects of DPN and SNC80 on latency to drink and volume consumed in the novelty-induced hypophagia test. DPN dose-dependently decreased the latency to drink in the novel cage (A) and increased volume consumed in both home the cage and the novel environment (B). SCN80 decreased latency to drink (C) but not volume consumed (D). Measurements were analyzed by ANOVA followed by a Tukey post hoc test, and the data shown represent means ± S.E.M. for 7 to 9 male or female mice for each treatment condition, with 12 control animals. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle/saline; $\dagger\dagger P < 0.01$ main effect of treatment vs. saline.

locomotor activity as compared with saline, 10 mg/kg DPN, and 10 mg/kg morphine at 20 to 60 minutes postinjection ($P < 0.05$ for all time points vs. the three other treatments). Consistently, there was a significant effect of total beam breaks over 60 minutes [$F(3,28)=8.44$, $P = 0.004$] (Fig. 4B); only 32 mg/kg morphine ($P < 0.001$), but not 10 mg/kg DPN or 10 mg/kg morphine, significantly increased beam breaks as compared with saline control. Interestingly, there were no observable sex differences (○ females, ● males) at saline, 10 mg/kg DPN, or 10 mg/kg morphine treatments, but 32 mg/kg morphine appears to induce higher levels of beam breaks in female than male mice (Supplementary Table 3).

Convulsive Activity

The propensity of DPN to cause convulsive behavior in mice is shown in Fig. 5. As expected, SNC80 (10 and 32 mg/kg) produced convulsions in every mouse (Fig. 5A). In contrast, DPN did not produce overt convulsions at any dose tested up to 32 mg/kg (Fig. 5A). Supplementary Tables 4 and 5 provide a breakdown of these results by sex. In fact, using male mice, we saw that pretreatment with 10 mg/kg DPN blocked the convulsions produced by 32 mg/kg SNC80 (Fig. 5B). To further explore the potential convulsive activity of DPN, we examined whether the convulsive agent pentylenetetrazole (PTZ) lowers the threshold for DPN-mediated convulsive activity, since PTZ does enhance DOPr agonist-mediated convulsive activity

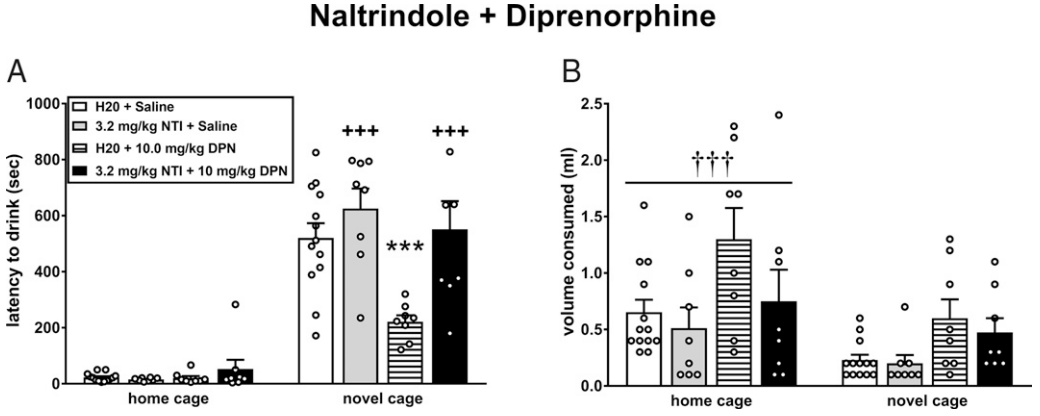

Fig. 3. Naltrindole inhibits the action of DPN in the NIH test. (A) Pretreatment with 3.2 mg/kg naltrindole (NTI) blocked the decrease in latency to drink produced by 10 mg/kg DPN in a novel environment and (B) the volume consumed in the home cage. Significant ANOVAs were followed by a Tukey post hoc test. All data are means ± S.E.M. for 8 male or female mice for each treatment condition (data for 3.2 mg/kg naltrindole was obtained using female mice only) and 13 control animals. $^{***}P < 0.001$ vs. H_2O + saline; $+++P < 0.001$ vs. H_2O + 10 mg/kg DPN; $\dagger P < 0.05$ main effect of treatment vs. novel cage.

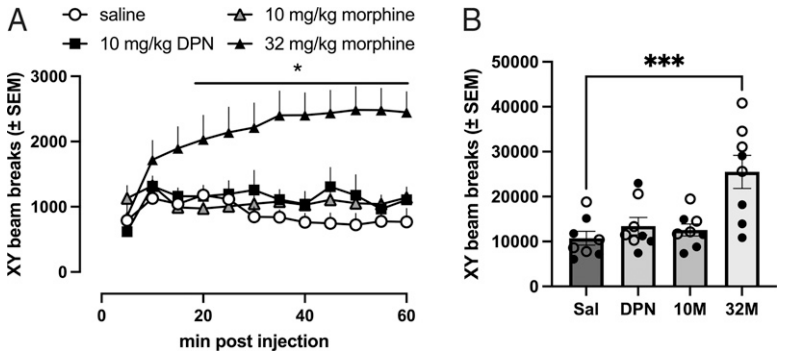

Fig. 4. DPN does not stimulate locomotor activity. (A) Treatment with 10 mg/kg i.p. DPN and 10 mg/kg i.p. morphine did not increase locomotor activity over the course of the 60-minute recording period as compared with saline; however, 32 mg/kg morphine increased activity as compared with either saline, 10 mg/kg DPN, or 10 mg/kg morphine at 20 to 60 minutes postinjection ($P < 0.01$). (B) Treatment with 10 mg/kg DPN and 10 mg/kg morphine (10M) did not increase the total locomotor activity (as measured in beam breaks), and there did not appear to be any sex differences (○ shows data points from female mice, ● shows data points from male mice). Consistent with the time course data, 32 mg/kg morphine (32M) increased total activity measured over 60 minutes as compared with either saline ($P < 0.0001$), or DPN or 10mg/kg morphine ($P < 0.01$ for both); this effect appears to be larger in magnitude in female than in male mice.

(Hudzik et al., 2011), including partial agonists (Dripps et al., 2020). As a control, we confirmed in male mice that SNC80-mediated convulsions were potentiated in the presence of a subconvulsive dose (32 mg/kg) of PTZ, as seen by the leftward shift in the SNC80 dose–effect curve (EC_{50} = 12 mg/kg in the absence of PTZ but 0.5 mg/kg in the presence of PTZ) (Fig. 6A). This effect was completely prevented following pretreatment with 3.2 mg/kg naltrindole [$t(10)=17$, $P < 0.0001$)] (Fig. 6B). In the presence of the 32 mg/kg PTZ, DPN produced convulsions that occurred 10 to 15 minutes after PTZ administration (Fig. 6C and 6D), with a dose-dependent increase in the severity of convulsions as determined by Racine scores [significant interaction: $F(5,66)= 3.8$, $P = 0.0047$] (Fig. 6C). It is noticeable that PTZ afforded a greater increase in the potency of DPN versus SNC80. Pretreatment with naltrindole (3.2 mg/kg) fully inhibited the convulsive behavior induced by 0.01 mg/kg DPN in the presence of 32 mg/kg PTZ ($P < 0.0001$) and partially inhibited the effect of 10 mg/kg DPN in the presence of 32mg/kg PTZ ($P < 0.05$) [main effect of naltrindole treatment $F(1.21) = 38.8$, $P < 0.0001$] (Fig. 6D).

Discussion

This study has identified DPN as a potential rapid-acting antidepressant medication via a DOPr-mediated mechanism. DPN is effective following a single dose in the NIH assay, which requires chronic dosing with traditional antidepressants and can therefore detect rapidly acting antidepressants (Dulawa et al., 2004; Saavedra et al., 2020), including ketamine (Louderback et al., 2013). Moreover, unlike many DOPr agonists, it did not produce overt convulsions in mice, even at three times the dose that produced antidepressant-like effects. DPN is not approved for human use although it has been employed for positron emission tomography imaging in humans (Jones et al., 1988; Frost et al., 1990;Dougherty et al., 2008) and is approved for the reversal of opioid immobilization in large animals (Ducker and Boyd, 1972; Alford et al., 1974; Meyer et al., 2018).

In vitro DPN acts as a MOPr antagonist but a partial agonist at DOPr and KOPr and is likely to exert similar properties in vivo although this will depend on the levels of receptor reserve, and the partial agonist activity will manifest as antagonism of higher efficacy agonists. The antidepressant-like activity of DPN was fully blocked in the TST and NIH assays by the DOPr selective antagonist naltrindole, demonstrating the DOPr partial agonist component of DPN's complex pharmacology is an absolute requirement for this effect. Then again, we cannot discount a supporting role for KOPr partial agonism, based on the effectiveness of the KOPr partial agonist nalbuphine in the forced-swim test (Browne et al., 2020) or MOPr antagonism based on data showing involvement of MOPr antagonism by buprenorphine in the anxiolytic component of the NIH test (Robinson et al., 2017). Indeed, it is feasible that MOPr antagonist activity of DPN may contribute to its higher potency in the NIH test.

The observed actions of DPN in both tests resemble the antidepressant-like effects of selective DOPr agonists (Jutkiewicz et al., 2005; Broom et al., 2002b). DPN is a low-efficacy DOPr agonist but produced effects equivalent to the full DOPr agonist SNC80, showing only a low level of efficacy is sufficient to

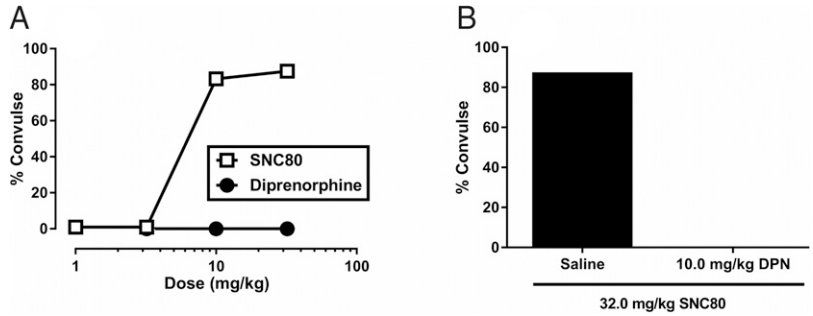

Fig. 5. DPN does not cause overt convulsions in mice. (A) Treatment with 10 or 32 mg/kg SNC80 produced convulsions in >80% of mice. DPN (1–32 mg/kg) did not produce convulsion in mice. (B) Pretreatment with 10 mg/kg DPN prevented SNC80-induced convulsions. Data are means ± S.E.M. for 6 to 8 male or female mice per condition (A) or 6 male mice (B).

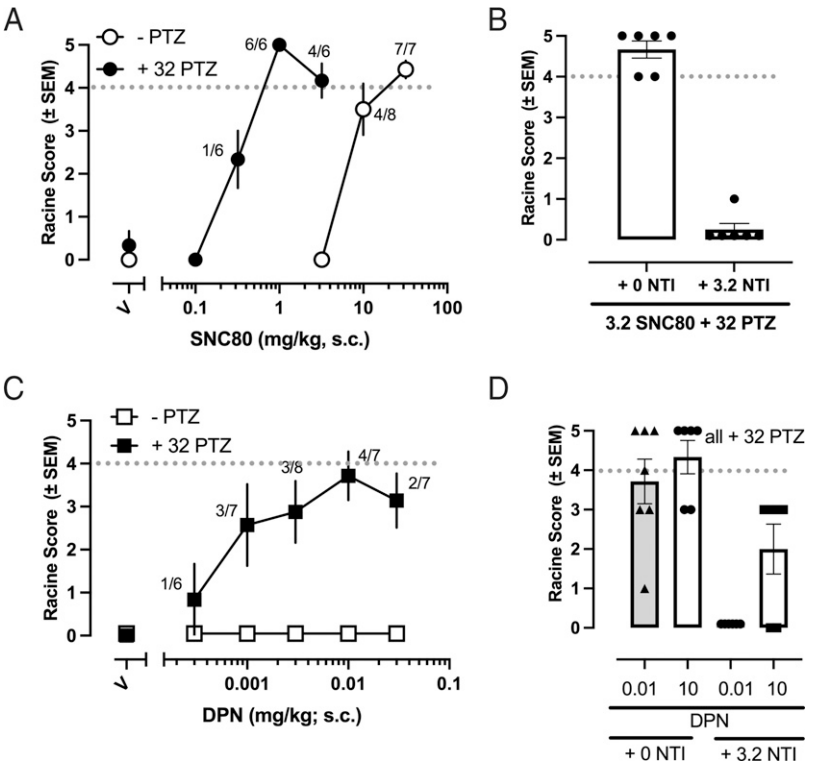

Fig. 6. DPN shows convulsive behavior in the presence of PTZ. The convulsive effects of SNC80 or DPN alone or in combination with a subconvulsive dose (32 mg/kg) of PTZ in male mice. Severity of convulsive behaviors as Racine score is displayed on the *y* axes in (A) and (C) and presented as the average score across all mice per each treatment condition or as individual scores in (B) and (D). The gray, dotted line highlights the Racine score assigned for overt clonic and/or tonic contractions lasting >3 second in duration; the number of mice with a Racine score > 4 as a fraction of the total mice tested per condition is shown in the ratio above each data point or bar. (A) Dose–response for SNC80 alone or in combination with PTZ. (B) Effect of pretreatment with 3.2 mg/kg naltrindole (NTI) or vehicle (0 NTI) on the convulsive effect induced by 3.2 mg/kg SNC80 in combination with 32 mg/kg PTZ. (C) Dose–response for DPN alone or in combination with 32 mg/kg PTZ. (D) Inhibition by 3.2 mg/kg NTI of the convulsive effects produced by 10 mg mg/kg DPN in combination with 32 mg/kg PTZ following pretreatment with 3.2 mg/kg NTI.

afford antidepressant-like activity. This is supported by data with the DOPr partial agonist BU48 (Dripps et al., 2020). Drug-induced increases in locomotor activity can make interpretation of effects in the TST problematic, and previous studies found that DPN can increase locomotor activity in mice (Parker, 1974; DeRossett and Holtzman, 1982; Parker, 1974). However, in the current study using C57BL/6 mice, DPN did not stimulate locomotor activity (Fig. 4). Additionally, it is important to note that (i) not all drugs that stimulate locomotor activity in mice (e.g., morphine) (Berrocoso et al., 2013; Ostadhadi et al., 2016; Rosa et al., 2017; Anand et al., 2018; but see Steru et al., 1985) have antidepressant-like effects in the TST, and (ii) drugs with locomotor-stimulating properties typically increase latencies to consume a sweetened solution in the NIH assay. Overall, these data suggest that DPN produces rapid antidepressant-like effects independent of locomotor-stimulating properties.

The clinical utility of DOPr agonists as antidepressants has been limited by their propensity to cause convulsions in rodents (Hong et al., 1998; Broom et al., 2002a; Jutkiewicz et al., 2005) and nonhuman primates (Danielsson et al., 2006). DPN did not produce convulsions in mice and furthermore inhibited the convulsive effects of the full DOPr agonist SNC80 yet was more potent at enhancing the convulsive activity PTZ than SNC80. The reasons for this are not clear, but the effect is DOPr-mediated since it was fully inhibited by 3.2 mg/kg naltrindole. Nonetheless, this activity of DPN might not limit its clinical utility as the FDA-approved antidepressant, bupropion produces seizures in 0.4% to 2.8% of patients (Hill et al., 2007).

The reasons why DPN and several other DOPr agonists (Pradhan and Clarke, 2005; Le Bourdonnec et al., 2008; Le Bourdonnec et al., 2009; Saitoh et al., 2011; Nozaki et al., 2012) do not produce convulsions in preclinical models are unclear. Like DOPr-mediated antidepressant-like activity, DOPr-mediated convulsive behavior is a low efficacy requiring response (Broom et al., 2002d; Dripps et al., 2020); thus, it is unlikely that the partial agonist activity of DPN at DOPr explains its lack of convulsive activity. The potency of SNC80 to generate convulsions is greater in β-arrestin-1 (arrestin 2) knockout mice, indicating a protective role for this arrestin (Dripps et al., 2018). We did not measure the ability of DPN to recruit β-arrestin-1, but this is unlikely given the low efficacy of DPN, plus our finding that it did not cause recruitment of β-arrestin-2. This is in line with studies indicating the higher efficacy requirement for arrestin recruitment (Gillis et al., 2020). Speed of delivery to the brain may be the determining factor in whether DOPr agonists cause convulsions (Jutkiewicz et al., 2005). However, DPN has rapid brain penetration and, as mentioned earlier, is used to reverse opioid overdose/immobilization of large animals in veterinary medicine (Ducker and Boyd, 1972; Alford et al., 1974; Meyer et al., 2018) and reverses fentanyl-mediated respiratory depression in mice (Hill et al., 2020). It is possible that the reasons why DPN and several selective DOPr agonists do not produce convulsions are different, and the polypharmacology of DPN explains both its effectiveness and its preclinical safety, since MOPr antagonists are not seizurogenic (Tortella et al., 1987) and the partial agonist activity of DPN at KOPr may reduce or limit the risk of convulsions (Tortella et al., 1986; Loacker et al., 2007). KOPr agonists are known to cause dysphoria in rodents and humans (Mysels and Sullivan, 2009; Lalanne et al., 2014) so partial KOPr agonist activity may limit the clinical utility of DPN. Although the low KOPr efficacy of DPN in vitro may indicate the compound would function as a KOPr antagonist in vivo, not all KOPr agonists cause dysphoria (Brust et al., 2016); for example, the KOPr agonist nalfurafine produced only a low incidence of dysphoria during clinical trials (Wikstrom et al., 2005; Kumagai et al., 2010). Future

investigations will need to examine DPN for KOPr-mediated dysphoric activity, but even if observed, this could be ameliorated with a KOPr antagonist, which may provide additional antidepressant action (Reed et al., 2022).

Buprenorphine, a close analog of DPN, exhibits antidepressant-like activity in animal models and humans by virtue of its KOPr antagonist activity (Falcon et al., 2016). No convulsive activity has been reported with buprenorphine, presumably because it is a DOPr antagonist in vitro and in vivo (Lee et al., 1999; Negus et al., 2002). However, since it is a MOPr partial agonist, buprenorphine is open to abuse and diversion (Lavonas et al., 2014; Lofwall and Walsh, 2014; Chilcoat et al., 2019; Han et al., 2021). To combat this, buprenorphine has been packaged with samidorphan, a selective potent MOPr antagonist (Chaudhary et al., 2019) as the combination medication ALK-5461 (Zajecka et al., 2019). ALK-5461 has shown antidepressant activity across several trials (Fava et al., 2016, 2020; Thase et al., 2019), but to date, FDA approval has not been obtained due to concerns regarding the drug's benefit–risk profile, including potential for misuse and abuse (Yavi et al., 2021). DPN offers the potential therapeutic benefit of the buprenorphine–samidorphan combination (Bidlack et al., 2018) by a different mechanism and without the abuse liability associated with buprenorphine.

Because of its MOPr antagonist action DPN would not be suitable for people currently taking opioids or those requiring opioid medication for pain. In addition, major depressive disorder is associated with increased coupling efficiency of MOPr in the anterior insular cortex together with evidence for increased opioid peptide release (Nummenmaa et al., 2020; Lutz et al., 2021), and so chronic inhibition of MOPr might mitigate successful treatment if these effects are compensatory, rather than causative, responses. On the other hand, there is evidence that the MOPr antagonist component of DPN may not be problematic. For example, 52-week administration of the buprenorphine–samidorphan combination, together with antidepressant therapy, to patients with major depressive disorder did not report any problems or a favorable profile of suicidal thoughts and behavior (Thase et al., 2019). Similarly, a 24-week pilot study of a bupropion—naltrexone combination, which is FDA-approved for weight loss showed improvement in depressive symptoms in overweight and/or obese women with major depression (McElroy et al., 2013). Chronic naltrexone is generally well tolerated in patients with alcohol use disorder (Anton, 2008), and in a randomized controlled trial, in subjects with opioid dependence, naltrexone-treated patients tended to exhibit an improvement in their depressive symptoms over time compared with the control group (Dean et al., 2006).

The present study identifies DPN as a prospective antidepressant treatment with several key advantages, rapid onset, and minimal concerns regarding convulsive side effects, and as a MOPr antagonist, DPN would not be expected to have abuse liability, thus providing a potentially improved therapeutic window over other available and preclinical rapidly acting antidepressant drugs. Although shown to be a MOPr antagonist in many studies, DPN is a Drug Enforcement Administration schedule II compound subject to special procedures. However, given there are no reports of MOPr agonist activity with DPN, this scheduling could be changed.

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Authorship Contributions

Participated in research design: Olson, Hillhouse, Jutkiewicz, Traynor.

Conducted experiments: Olson, Hillhouse, Burgess, West, Hallahan, Dripps, Ladetto.

Contributed new reagents or analytic tools: Rice.

Performed data analysis: Olson, Hillhouse, Burgess, Jutkiewicz, Traynor.

Wrote or contributed to the writing of the manuscript: Olson, Hillhouse, Jutkiewicz, Traynor.

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