

Sensory and Motor Systems

Paroxetine Increases δ Opioid Responsiveness in Sensory Neurons

Allison Doyle Brackley¹ and  Nathaniel A. Jeske^{1,2,3}<https://doi.org/10.1523/ENEURO.0063-22.2022>

¹Departments of Physiology, University of Texas Health San Antonio, TX 78229, ²Oral and Maxillofacial Surgery, University of Texas Health San Antonio, TX 78229, and ³Pharmacology, University of Texas Health San Antonio, TX 78229

Abstract

There are currently no Food and Drug Administration (FDA)-approved δ -opioid receptor (DOR)-selective agonists, despite having fewer side effects in rodents and nonhuman primates compared with traditional μ -opioid receptor (MOR) therapeutics (Vanderah, 2010). Targeting peripheral receptors is an attractive strategy to reduce abuse potential. However, peripheral opioid receptors do not readily respond to agonists unless primed by inflammation, which would limit their efficacy in noninflammatory pain patients (Stein et al., 1989). It was recently identified that G-protein-coupled receptor kinase 2 (GRK2) maintains DOR incompetence in noninflamed nociceptors (Brackley et al., 2016, 2017). Here, we report that paroxetine, a selective serotonin reuptake inhibitor (SSRI) and potent GRK2 inhibitor (Thal et al., 2012), reduces chronic GRK2 association with membrane DOR, thereby enhancing peripheral DOR-mediated analgesic competence in the absence of inflammation. Interestingly, paroxetine's effects on GRK2 *in vivo* are limited to peripheral tissues in the male rat. The effects of paroxetine on DOR competence are notably antagonized by GRK2 overexpression. This is the first study to suggest that paroxetine induces peripheral DOR analgesic competence through a GRK2-dependent mechanism, improving analgesic efficacy in noninflamed tissue. Because paroxetine targets the protein that governs peripheral opioid receptor responsiveness, and does so in the absence of inflammation, we propose that paroxetine may be suitable as a co-therapy with peripherally-restrictive doses of opioids to improve analgesic efficacy in noninflammatory pain conditions.

Key words: GRK2; opioid; pain; paroxetine

Significance Statement

Opioids that target MOR represent the gold-standard for analgesic health care, despite widespread abuse potential and the ongoing opioid-epidemic. Work herein uncovers the therapeutic potential of targeting peripheral δ -opioid receptor (DOR) for analgesic utility with a Food and Drug Administration (FDA)-approved G-protein-coupled receptor kinase 2 (GRK2) inhibitor paroxetine to boost efficacy and reduce side effect profiles. Analgesic pain management targeting DOR with increased efficacy through adjuvant paroxetine treatment could reduce over-reliance on MOR agonist opioids for pain relief and usher in new options for analgesia.

Introduction

Opioids remain a pillar for severe pain management, despite debilitating central side effects that contribute to an ever-growing opioid epidemic in America. One analgesic strategy that has been gaining traction in recent years

involves targeting peripheral opioid receptors to circumvent debilitating central side effects associated with systemically administered opioids. Traditional therapeutics systemically target μ -opioid receptor (MOR) and/or κ -opioid receptor (KOR) throughout the body. Interestingly, agonists that

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target the δ -opioid receptor Delta Opioid Receptor (DOR) produce analgesia as effectively as MOR agonists, but have a reduced side effect profile that includes less gastrointestinal dysfunction, tolerance, dependence and abuse potential (Vanderah, 2010). DOR is the most highly expressed opioid receptor subtype in peripheral pain-sensing neurons (Wang and Wessendorf, 2001; Bao et al., 2003). Perhaps for this reason, DOR has greater potential to treat inflammatory, thermal, mechanical and neuropathic pain modalities across genders compared with MOR and KOR, as evidenced by genetic deletion studies of individual opioid receptors (Nadal et al., 2006; Gavériaux-Ruff et al., 2008). However, peripheral DOR must be primed by inflammation before rendered analgesically competent (Stein et al., 1989; Patwardhan et al., 2005; Rowan et al., 2009; Brackley et al., 2016, 2017), which would limit the therapeutic efficacy of peripherally-restricted DOR agonist treatments alone to severe inflammatory pain patients only.

Mechanisms that regulate peripheral DOR incompetence have only recently been identified. *In vitro* and *in vivo* studies demonstrate that DOR incompetence in peripheral sensory neurons is governed by a chronic interaction between membrane-associated DOR and G-protein-coupled receptor kinase 2 (GRK2) maintained by constitutive protein kinase A (PKA)-dependent phosphorylation of GRK2 at Ser685 (Brackley et al., 2016, 2017). Novel therapies that disrupt these protein-protein interactions would be expected to enhance DOR functional competence in the periphery. The National Institutes of Health (NIH)'s National Center for Advancing Translational Sciences is currently pushing forward a new initiative that focuses on repurposing Food and Drug Administration (FDA)-approved drugs, supporting preclinical recycling of preexisting drugs for new indications that establish rationale for clinical trial implementation. In the past several years, the FDA-approved selective serotonin reuptake inhibitor (SSRI) paroxetine (Paxil) has been found to directly bind to GRK2, acting as a potent *in vitro* and *in vivo* inhibitor (Thal et al., 2012; Schumacher et al., 2015). In mice, paroxetine dose dependently enhances endogenous opioid antinociception (Gray et al., 1998; Kesim et al., 2005), mediated by DOR (Gray et al., 1998). In rats, paroxetine enhances antinociception produced by systemically-administered low-dose morphine, presumably targeting peripheral opioid receptors, in multiple pain modalities including neuropathic, mechanical, thermal, and cold allodynia (Lee et al., 2012). However, the mechanism that underlies paroxetine priming of opioid receptor analgesic competence has yet to be explored.

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Correspondence should be addressed to Nathaniel A. Jeske at jeske@uthscsa.edu.

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Given that paroxetine enhances opioid analgesia (Lee et al., 2012) and directly inhibits GRK2 (Thal et al., 2012), which constitutively maintains peripheral DOR incompetence (Brackley et al., 2016, 2017), we hypothesized that paroxetine-induced DOR competence in the periphery is mediated by sequestration of GRK2. This proof-of-concept study in male rats uses a drug recycling approach to target protein-protein interactions that govern peripheral opioid receptor responsiveness in the absence of inflammation. Using biochemical, molecular, functional, and behavioral techniques, we establish a physiological mechanism that provides rationale for a combination therapy between the SSRI paroxetine and peripherally-acting opioids. Repurposing this FDA-approved drug as an analgesic adjuvant would be expected to enhance opioid-mediated analgesia in noninflammatory pain patients with reduced incidence of debilitating systemic side effects.

Materials and Methods

Animals

Procedures using animals were approved by University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee. Studies were conducted in accordance with the policies for the ethical treatment of animals established by the NIH with every effort made to limit animal discomfort and number of animals used.

Neuronal cultures

For biochemistry, trigeminal ganglia (TG) were dissected bilaterally from adult male Sprague Dawley rats (200–250 g; Charles River Laboratories). TG were dissociated by collagenase treatment (30 min; Worthington), followed by trypsin treatment (30 min; Sigma-Aldrich). Dissociated TG were re-suspended in complete media (DMEM, Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 ng/ml nerve growth factor (NGF; Harlan Laboratories), mitotic inhibitors (Sigma), 1% penicillin/streptomycin (Invitrogen), and 1% glutamine (Sigma) and plated on poly-D-lysine-coated plates (Corning). Similarly, for functional studies (Ca^{2+} imaging), dorsal root ganglia (DRG) dissected bilaterally at L4–L6 were dissociated by 40-min co-treatment with collagenase and dispase II (Sigma). Next, cells were re-suspended in complete media and plated on poly-D-lysine/laminin-coated coverslips (BD Biosciences). Cultures were maintained at 37°C and 5% CO_2 and grown for 1–2 d for functional studies and 5–6 d for biochemistry with media changed the following day and every 2 d thereafter. TG were used for biochemical experiments to satisfy NIH requirements to reduce animal use in research.

Cell/tissue processing and co-immunoprecipitation (Co-IP/IP)

Primary TG cultures were pretreated as indicated. Cells were harvested and homogenized in homogenization buffer [25 mM HEPES, 25 mM sucrose, 1.5 mM MgCl_2 , 50 mM NaCl (pH 7.4), 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate (Sigma), 1 $\mu\text{g/ml}$ pepstatin (Sigma), 1 $\mu\text{g/ml}$ leupeptin (Sigma), 1 $\mu\text{g/ml}$ aprotinin

(Sigma), and 100 mM phenylmethylsulfonyl fluoride (PMSF; Sigma)] with 20 strokes using a Potter-Elvehjem pestle and glass homogenizer tube. Homogenates were placed on ice for 15 min and centrifuged at $1000 \times g$ for 1 min to remove nuclei and un-lysed cells from the homogenate. Resulting supernatant was centrifuged at $16,000 \times g$ for 30 min at 4°C to separate membrane proteins from cytosolic proteins. Cytosolic supernatant was separated from the pellet (crude membrane fraction), which was re-suspended in 250- μl homogenization buffer containing 1% Triton X-100 (Fisher Scientific).

For tissue analysis following *in vivo* injection, four tissues were collected following behavioral time course described below. TG and DRG (L4–L6) were bilaterally dissected, along with the corresponding lumbar spinal cord (SC). We also collected midbrain tissue that included midbrain and striato-pallidal regions. Dissected tissue samples were cut a minimum of 20 times, then homogenized in homogenization buffer with 40 strokes. As described above, homogenates were fractionated to separate membrane proteins from cytosolic proteins.

Total protein from membrane (PM) and/or cytosolic (CYTO) lysates were quantified (Bradford, Sigma), followed by Co-IP/IP⁹⁻¹⁰. Briefly, equal amounts of protein (125 μg) were immunoprecipitated with 1 μg anti-GRK2 (C-15, Santa Cruz Biotechnology) or anti-DOR (ab66317/ab66318, Abcam) antiserum for IP and Co-IP, respectively. Protein samples were eluted at 95°C for 5 min and placed at -20°C for Western blot (WB) analysis.

WB analysis

Protein samples were resolved by 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF; Millipore). Membranes were then blocked with 5% nonfat dried milk in Tris-buffered saline/Tween 20 (TBS-T: 15.35 mM Tris/HCl, 136.9 mM NaCl, pH 7.6, with 0.1% Tween 20) or 5% bovine serum albumin (Sigma) in TBS-T containing phosphatase inhibitor sodium orthovanadate (1 μM) for phosphorylation-specific antibodies. WBs were visualized using anti-phospho-GRK2-Ser685 (#12397-1, SAB Signalway Antibody; Brackley et al., 2017), anti-GRK2 (C-15, Santa Cruz Biotechnology; Wang et al., 2011), anti-DOR (ab66317/ab66318, Abcam; Brackley et al., 2016, 2017), anti-Caveolin-1 (N-20, Santa Cruz Biotechnology; Gomez et al., 2011), anti- β -1-integrin (Santa Cruz Biotechnology; Por et al., 2012), or anti- β -actin (I-19-R, Santa Cruz Biotechnology; Gomez et al., 2011), followed by appropriate horseradish-peroxidase-conjugated secondary antisera (GE Healthcare) and ECL (enhanced chemiluminescence or prime) detection following the manufacturer's protocol (GE Healthcare). Antibody specificities were verified by the manufacturers, BLAST sequence analysis, and used in previous publications as indicated. Integrated density measurement values, equivalent to the product of area and mean gray value by histogram analysis, were performed using NIH ImageJ software.

siRNA transfection

Specific FITC-labeled siRNA duplexes custom-designed to target GRK2 were previously designed and characterized

(QIAGEN; Brackley et al., 2016). The sequence for the sense strand of GRK2 siRNA was 5'-GCAGAAGUAUCUAGAGG AUUU-3' and antisense strand of GRK2 siRNA was 5'-AUCCUCUAGAUACUUCUGCUU-3'. For Ca^{2+} imaging experiments, FITC-labeled siRNA duplexes (45 ng/coverslip) were transfected into cultured sensory neurons using HiPerFect (QIAGEN), following manufacturer's directions as described previously (Brackley et al., 2016). Additional cells were treated with no siRNA (mock), used as a negative control.

cDNA nucleofection

DRG were cultured as described above and nucleofected with Effectene nucleofection reagent (QIAGEN) following manufacturer's instruction, maintaining a cDNA to enhancer reagent ratio of 1:8, for 10 h, as described previously (Brackley et al., 2016). For these experiments, empty vector (E.V.) pcDNA3.1 or GRK2 (Jeffrey L. Benovic, Thomas Jefferson University) cDNA (500 ng/coverslip) were nucleofected into sensory neurons. GFP cDNA (250 ng/coverslip) was co-nucleofected to identify positive transfection.

Single-cell Ca^{2+} imaging

Following 2-h serum-starvation, cultured DRG were loaded with fura-2 A.M. (1 μM ; Invitrogen) and pluronic F-127 (0.04%; Invitrogen) for 1 h at 37°C in the dark, in standard extracellular solution (SES) containing: 140 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, and 10 mM D-(+)-glucose, pH 7.40. Neurons were observed on an inverted Nikon Eclipse T_i-U microscope fitted with a 20 \times /0.75 numerical aperture Fluor objective and imaged using MetaFluor System for Ratio Fluorescence (MetaMorph). Fluorescent images were taken as previously described (Brackley et al., 2016). The following criteria were used to indicate positive sensory neuronal phenotype within a heterogenous culture: (1) bright round cell bodies with clear nuclei (Goldenberg and De Boni, 1983; Liu et al., 2013); (2) depolarization in response to 50 mM KCl (Khasabova et al., 2004; Pettinger et al., 2013); and (3) sensitivity to capsaicin [CAP; 1 μM ; 25% above baseline (BL), Sigma; Brackley et al., 2016]. Corresponding filters were used to restrict analysis to FITC-siRNA-positive or GFP-positive DRG.

DOR activity was quantified as a measure of [D-Pen^{2,5}]-enkephalin (DPDPE, 1 μM ; Sigma) inhibition of 50 mM KCl-evoked Ca^{2+} transients in CAP (1 μM)-sensitive DRG as previously described (Brackley et al., 2016). A perfusion valve controller (<0.1 psi) and multi-barrel glass pipette were used to apply 3 s exposures of KCl at 60 s, 270 s, 740 s, 950 s, and 1440 s, followed by CAP at 1770 s. To assess DOR competence, the first two exposures to KCl were in SES and the second two in the presence of bath-perfused DPDPE, followed by a fifth exposure to KCl following DPDPE washout to confirm neuron remained viable. In CAP-sensitive DRG, single-cell recording traces were used to obtain the area under the curve (AUC), calculated as an integral across 3 min for the average response to KCl in

the absence or presence of a DOR agonist. The following equations were used to calculate DOR activity:

$$KCI = \frac{(KCI_1, KCI_2)}{3}$$

$$KCI + DPDPE = \frac{(KCI_3, KCI_4)}{2}$$

$$DPDP \text{ Agonist Inhibition}(\%)$$

$$= 100 - \left(\frac{AUC_{KCI + DPDPE}}{AUC_{KCI}} \times 100\% \right).$$

For Ca^{2+} imaging, stock solution of DOR agonist DPDPE (1 mM) was prepared in sterile water. CAP (1 mM) stock solution was prepared in EtOH. Paroxetine (25 mM; Sigma/Toronto Research Chemicals), CMPD101 (2.5 mM; Tocris), and fluoxetine hydrochloride (25 mM; Sigma) stock solution was prepared in DMSO. KCI was prepared fresh in SES the day of experimentation. All drugs and appropriate vehicles were diluted in SES to doses determined by IC_{50} values in this study and others. The IC_{50} value of paroxetine concentration response curve (3.8 μM) yielded similar values to that observed for GRK2 inhibition (4.7 μM ; Thal et al., 2012), 5 μM was the *in vitro* dose used for paroxetine the remainder of the study. GRK2/3 inhibitor CMPD101, which binds the same site as paroxetine, served as a positive control (Thal et al., 2011). Because doses $>2 \mu M$ CMPD101 reduces selectivity for GRK2 over PKA, 0.5 μM was for *in vitro* studies. Fluoxetine, a typical SSRI that does not inhibit GRK2 at doses exceeding 100 μM , was used as a negative control at equimolar doses to paroxetine (Thal et al., 2012).

Behavioral test for paroxetine priming of DOR functional competence

Electronic von Frey (IITC Life Science Inc.) was used to assess mechanical allodynia in male Sprague Dawley rats (250–300 g), measured by paw withdrawal threshold (PWT; g) to a rigid tip. Rats were given 2 h to acclimate to environment and 30 min to the raised von Frey mesh stand. For testing, the rigid tip was applied to the hind paw. Upon reaction, the system displayed an electronic reading for PWT. A minimum of six BL readings were taken from ipsilateral and contralateral hind paws with duplicate measurements for each time point, then averaged for statistical analysis.

DOR analgesic competence was measured as DPDPE (20 μg) inhibition of prostaglandin E_2 (PGE_2 ; 0.3 μg ; Cayman Chemicals)-induced allodynia (Rowan et al., 2009; Brackley et al., 2016). Following BL readings, animals were injected intraperitoneally with vehicle [10% dimethylsulfoxide (DMSO)/90% Dulbecco's PBS (DPBS)], paroxetine (5 mg/kg), CMPD101 (0.5 mg/kg), or fluoxetine (5 mg/kg); 25 min after intraperitoneal injection, an intraplantar (i.pl.) co-injection of DPDPE and PGE_2 was given into the hind paw. PWT readings were recorded by a blinded observer every 5 min for 20 min following each injection. Intraperitoneal drugs were administered at a final

volume of 1 ml and i.pl. co-injection at a final volume of 50 μl .

Statistics

GraphPad Prism 5.0 was used for statistical analyses (GraphPad Software.). Quantitative data expressed as mean \pm SEM. Statistical significance was determined by one-way ANOVA or two-way ANOVA with Bonferroni *post hoc* analyses as needed; $p < 0.05$ was considered statistically significant.

Results

Paroxetine reduces GRK2 association with DOR to enhance receptor competence

Previous studies establish that the SSRI paroxetine directly interacts with and inhibits GRK2, which promotes G-protein-coupled receptor (GPCR) signaling *in vitro* and *in vivo* (Thal et al., 2012; Schumacher et al., 2015). Under naive conditions, GRK2 constitutively associates with membrane-bound DOR, rendering the receptor unresponsive to agonist stimulation in peripheral sensory neurons (Brackley et al., 2016, 2017). Given that paroxetine-induced analgesia can be dose dependently antagonized by a DOR-selective antagonist (Gray et al., 1998; Kesim et al., 2005), we sought to determine whether paroxetine disrupts membrane DOR-GRK2 association. Paroxetine dose dependently reduces GRK2 Co-IP with DOR in peripheral sensory neuron membrane fractions (Fig. 1A). Additional controls monitored whether paroxetine's effects on GRK2 are shared by a chemically related compound, CMPD101, a selective GRK2 and GRK3 inhibitor, or unrelated compound, fluoxetine, in the same drug class (Fig. 1B). Compared with vehicle-treated cells, GRK2 association with membrane-bound DOR is reduced to $55.48 \pm 3.46\%$ and $53.62 \pm 17.89\%$ by paroxetine and CMPD101, respectively. In contrast, fluoxetine yields levels comparable to vehicle. These data verify that paroxetine reduces GRK2 association with DOR in peripheral sensory membrane fractions.

In peripheral sensory neurons, PKA-dependent phosphorylation of GRK2 drives the constitutive association between membrane-bound DOR and GRK2 (Brackley et al., 2017). Therefore, we tested whether paroxetine affects PKA-dependent phosphorylation of GRK2 at Ser685 in immunoprecipitated lysates using a site-specific antibody. Paroxetine treatment reduces GRK2 phosphorylation at Ser685 to $45.50 \pm 11.09\%$ of vehicle-treated cells. Whereas, CMPD101 reduces PKA-dependent GRK2 phosphorylation by about half that to $74.40 \pm 12.58\%$. Again, fluoxetine yields results comparable to vehicle (Fig. 1C). Thus, paroxetine reduces PKA phosphorylation of GRK2 at Ser685 in peripheral nociceptors.

To build on this, functional experiments were conducted to determine whether paroxetine enhances functional DOR competence. For this purpose, we measured opioid inhibition of voltage-gated Ca^{2+} channels (VGCCs) evoked by KCI-induced Ca^{2+} influx in cultured sensory neurons (Brackley et al., 2016, 2017). Given that paroxetine reduces phosphorylation of GRK2 at Ser685, an

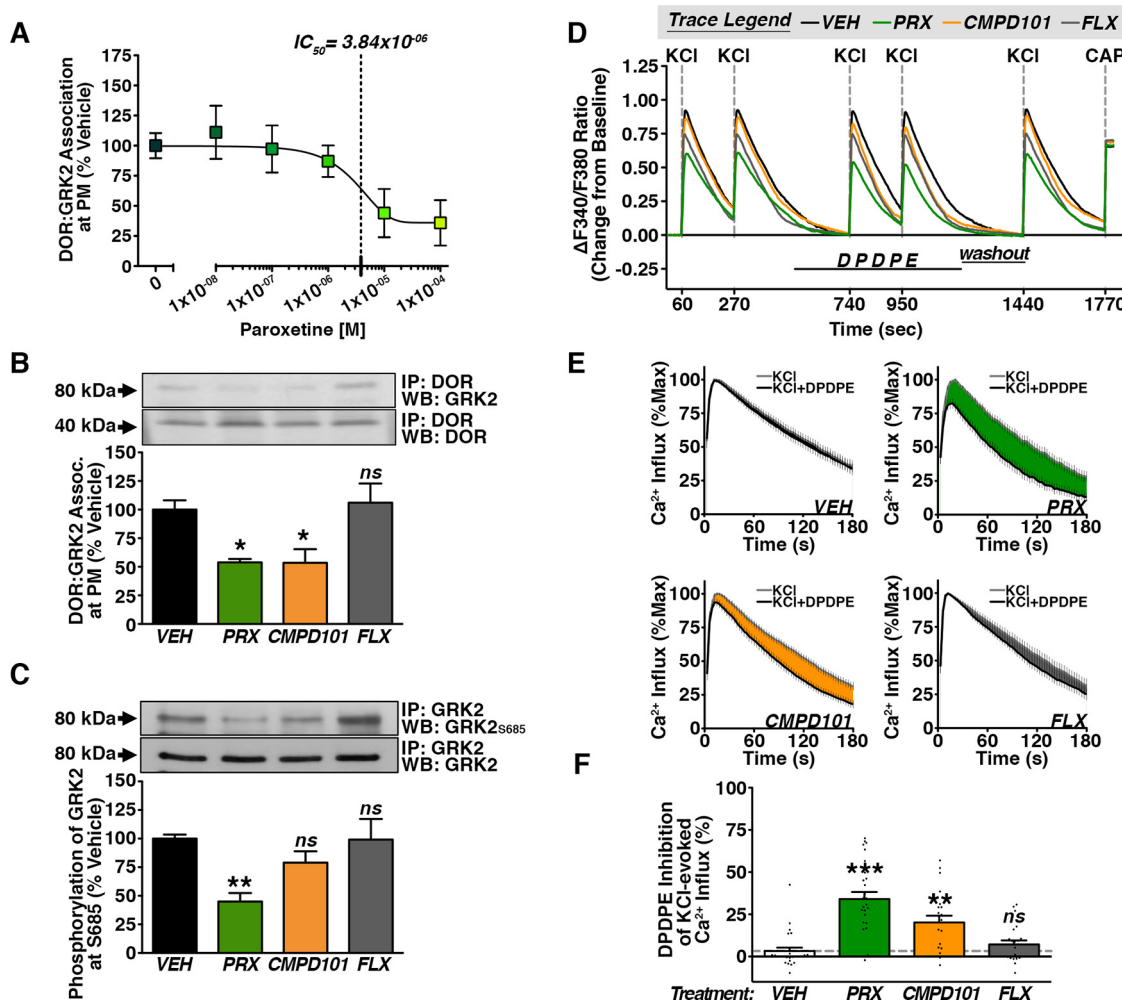


Figure 1. Paroxetine enhances Delta Opioid Receptor (DOR) functional competence in sensory neurons. **A**, Concentration response curve for paroxetine ([M], 10 min) effect on GRK2 Co-IP with DOR in crude membrane fractions from serum-starved TG cultures [$IC_{50} = 3.840 \mu M$ (vertical black dotted line), least squares fit (best-fit) variable slope curve (black line), ANOVA summary: $F_{(5,18)} = 2.932, p = 0.415$, one-way ANOVA with Bonferroni *post hoc*, mean \pm SEM, $n = 4$ independent trials from 24 TG/12 total rats]. **B**, GRK2 Co-IP with membrane-associated DOR in serum-starved TG cultures treated for 10 min with vehicle (VEH; DMSO), paroxetine (PRX; $5 \mu M$), CMPD101 ($0.5 \mu M$), or fluoxetine (FLX; $5 \mu M$); $*p < 0.05$, ns = not significant, ANOVA summary: $F_{(3,12)} = 8.805, p = 0.0023$, one-way ANOVA with Bonferroni *post hoc*, mean \pm SEM, $n = 4$ independent trials from 24TG/12 total rats). **C**, GRK2 phosphorylation at Ser685 isolated by GRK2 IP in serum-starved TG cultures treated for 10 min with vehicle (VEH; DMSO), paroxetine (PRX; $5 \mu M$), CMPD101 ($0.5 \mu M$), or fluoxetine (FLX; $5 \mu M$); $**p < 0.01$, ns = not significant, ANOVA summary: $F_{(3,12)} = 7.328, p = 0.0047$, one-way ANOVA with Bonferroni *post hoc* test, mean \pm SEM, $n = 4$ independent trials from 24 TG/12 total rats). **D–F**, Cumulative (**C**) mean experimental traces, (**D**) average KCl response traces, and (**E**) quantification of DPDPE ($1 \mu M$) inhibition of KCl (50 mM)-evoked Ca^{2+} influx in CAP ($1 \mu M$)-sensitive serum-starved DRG pretreated for 10 min with vehicle (VEH; DMSO), paroxetine (PRX; $5 \mu M$), CMPD101 ($0.5 \mu M$), or fluoxetine (FLX; $5 \mu M$); $***p < 0.005$, $**p < 0.01$, ns = not significant, ANOVA summary: $F_{(3,92)} = 18.83, p < 0.0001$, one-way ANOVA with Bonferroni *post hoc*, mean \pm SEM, $n = 21–27$ DRG/group collected from a minimum of 5 rats).

important site for GRK2 maintenance of functional DOR incompetence (Brackley et al., 2017), we next tested whether paroxetine enhances DOR responsiveness to agonist stimulation. Recapitulating published population findings in primary sensory neurons, DOR agonist DPDPE, which has ~100-fold selectivity for DOR over other opioid receptor subtypes, fails to inhibit KCl-evoked Ca^{2+} transients in CAP-sensitive DRG (Fig. 1D–F). Paroxetine pretreatment, on the other hand, produces a robust response to DPDPE and inhibits KCl-evoked Ca^{2+} transients collectively by $34.59 \pm 4.30\%$. Pretreatment with CMPD101 produces DPDPE inhibition of KCl-evoked Ca^{2+} transients by $20.19 \pm 4.11\%$. Fluoxetine, which does not reduce PKA-dependent

phosphorylation of GRK2, simultaneously fails to enhance DOR competence in peripheral sensory neurons (Fig. 1D–F). Taken together, paroxetine enhances DOR functional competence in CAP-sensitive sensory neurons.

Paroxetine enhances functional receptor competence by antagonizing GRK2-DOR interaction

In sensory neurons, paroxetine reduces DOR association with GRK2 by way of reduced PKA-dependent phosphorylation of GRK2 and, thus, increases functional DOR competence (Fig. 1). Because paroxetine binds GRK2 (Thal et al., 2012), we hypothesized that paroxetine

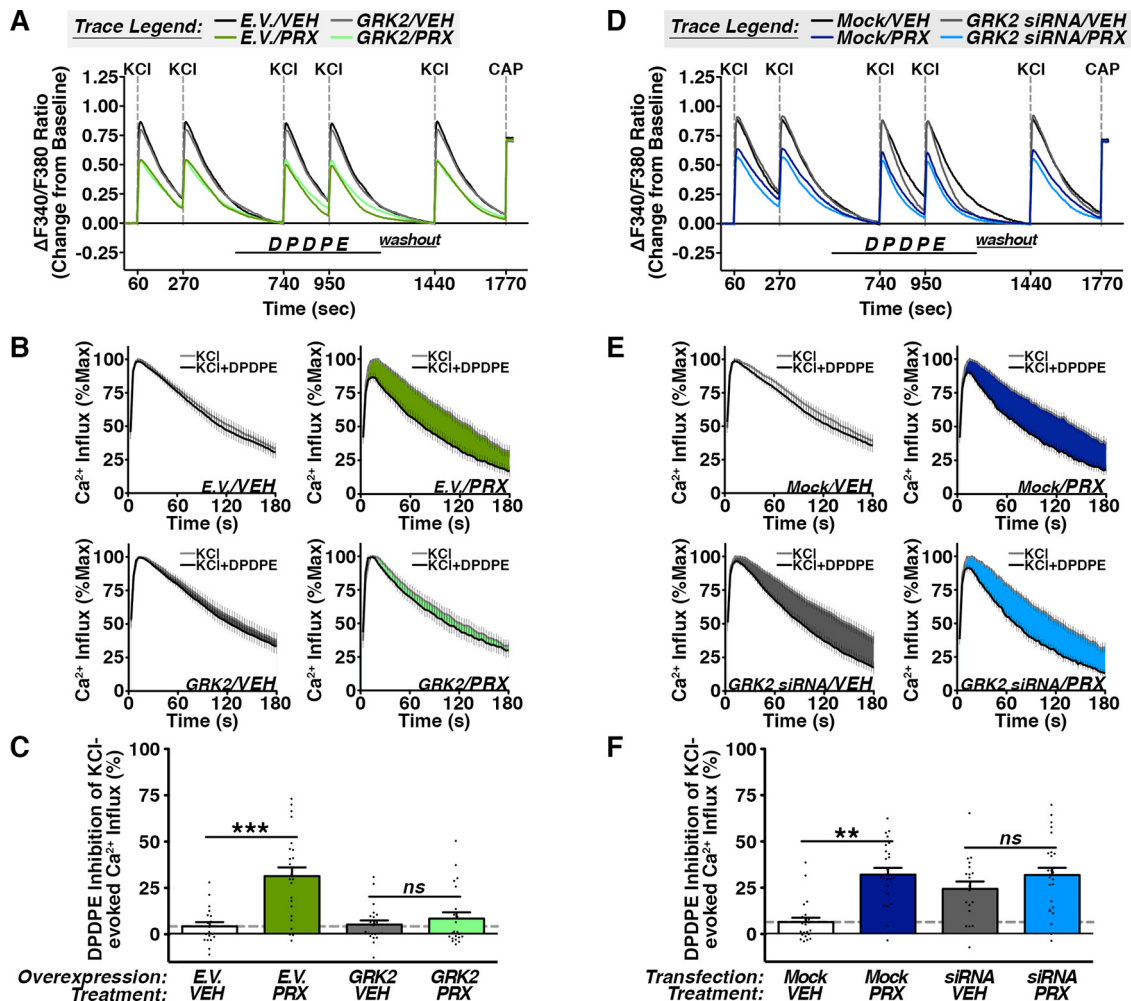


Figure 2. Paroxetine enhances Delta Opioid Receptor (DOR) functional competence via GRK2-dependent mechanism. **A–C**, Cumulative (**A**) mean experimental traces, (**B**) average KCl response traces, and (**C**) quantification of DPDPE (1 μ M) inhibition of KCl (50 mM)-evoked Ca²⁺ influx in CAP (1 μ M)-sensitive serum-starved nucleofected DRG pretreated with vehicle (VEH; DMSO) or paroxetine (PRX; 5 μ M) for 10 min [$***p \leq 0.005$, ns = not significant, ANOVA summary: Interaction: $F_{(1,82)} = 11.72$, $p = 0.0010$, Overexpression (E.V. vs GRK2): $F_{(1,82)} = 9.900$, $p = 0.0023$, Pretreatment (VEH vs PRX): $F_{(1,82)} = 19.12$, $p < 0.0001$, two-way ANOVA with Bonferroni *post hoc*, mean \pm SEM, $n = 19$ –24 DRG/group collected from a minimum of 5 rats]. **D–F**, Cumulative (**D**) mean experimental traces, (**E**) average KCl response traces, and (**F**) quantification of DPDPE (1 μ M) inhibition of KCl (50 mM)-evoked Ca²⁺ influx in CAP (1 μ M)-sensitive serum-starved transfected DRG pretreated with vehicle (VEH; DMSO) or paroxetine (PRX; 5 μ M) for 10 min [$**p \leq 0.01$, ns = not significant, ANOVA summary: Interaction: $F_{(1,87)} = 6.377$, $p = 0.0134$, Transfection (Mock vs FITC-GRK2 siRNA): $F_{(1,87)} = 6.141$, $p = 0.0151$, Pretreatment (VEH vs PRX): $F_{(1,87)} = 21.27$, $p < 0.0001$, two-way ANOVA with Bonferroni *post hoc*, mean \pm SEM, $n = 19$ –26 DRG/group collected from a minimum of 5 rats].

mechanistically targets GRK2 to functionally enhance DOR competence. To assess this, we overexpressed GFP with E.V. or wild-type GRK2 and measured DOR responsiveness to DPDPE in pretreated with vehicle or paroxetine. GFP-positive CAP-sensitive DRG nucleofected with E.V. or GRK2 are similarly insensitive to DPDPE, producing only $4.26 \pm 2.27\%$ and $5.22 \pm 2.22\%$ inhibition of KCl-evoked Ca²⁺ transients, respectively (Fig. 2A–C). Paroxetine pretreatment in control DRG produces significant DPDPE sensitivity and collectively inhibits KCl-induced Ca²⁺ influx by $31.37 \pm 4.69\%$. However, overexpression of GRK2 in CAP-sensitive DRG paroxetine fails to induce functional DOR competence with only $8.52 \pm 3.35\%$ DPDPE inhibition of KCl-evoked Ca²⁺ transients (Fig. 2A–C). Thus, GRK2

overexpression antagonizes paroxetine-induced DOR competence in primary sensory neurons.

Paroxetine, like most clinically efficacious SSRI's, has off-target affinity for receptors and proteins other than the serotonin transporter (Owens et al., 1997, 2002; Thal et al., 2012). To ascertain whether a GRK2-independent mechanism may account for any paroxetine-induced DOR competence in sensory neurons, we conducted the same Ca²⁺ imaging paradigm following GRK2 knock-down in DRG using previously validated FITC-GRK2 siRNA (Brackley et al., 2016). In line with published population findings in primary cultures, vehicle pretreatment in mock-treated DRG yields only $6.44 \pm 2.35\%$ DPDPE inhibition of KCl-evoked Ca²⁺ influx (Fig. 2D–F). On the other

hand, mock-treated DRG exposed to paroxetine collectively produce a significant response to DPDPE and inhibit KCl-evoked Ca^{2+} influx by $32.05 \pm 3.67\%$. DRG transfected with FITC-GRK2 siRNA robustly respond to the DOR agonist DPDPE and readily inhibits KCl-evoked Ca^{2+} transients by $24.40 \pm 3.96\%$ following vehicle pretreatment. Similarly, DPDPE inhibits $31.88 \pm 3.88\%$ of KCl-evoked Ca^{2+} transients in FITC-GRK2 siRNA DRG following paroxetine pretreatment (Fig. 2D–F). Notably, in GRK2 knock-down DRG, paroxetine pretreatment does not significantly improve DOR competence in vehicle-treated neurons. Taken together with our biochemical observations, data presented herein identify that paroxetine-induced DOR competence is driven by its sequestration of GRK2 from membrane-bound DOR in sensory neurons.

Paroxetine enhances DOR-mediated analgesia *in vivo* by targeting peripheral GRK2

Paroxetine's selective interaction with GRK2 *in vitro* and *in vivo* enhances GPCR signaling (Thal et al., 2012; Schumacher et al., 2015). After we identified that paroxetine facilitates opioid signaling in sensory neurons *in vitro*, we measured its physiological effect on peripheral DOR-mediated analgesia *in vivo*. To assess whether systemic (Fig. 3A,B) or peripheral (Fig. 3C,D) paroxetine pretreatment enhances DOR analgesic competence, we assessed DPDPE inhibition of PGE_2 -induced mechanical allodynia in rats. In vehicle (intraperitoneally, 10% DMSO/90% DPBS)-treated animals, a peripherally restrictive dose of DPDPE (20 μg , i.pl.; Rowan et al., 2009) co-injected into the hind paw does not elicit DOR-mediated analgesia, which is measured as neutralization of PGE_2 (0.3 μg , i.pl.)-induced allodynia. However, paroxetine (5 mg/kg, i.p.) and CMPD101 (0.5 mg/kg, i.p.) systemically produce equivalent DOR-mediated analgesia *in vivo*, as measured by a return to BL in the ipsilateral hind paw. Congruent with biochemical and functional data, fluoxetine (0.5 mg/kg, i.p.) fails to reverse peripheral DOR incompetence (Fig. 3A). Interestingly, a similar dose of paroxetine (150 μg , i.pl.) injected into the hind paw produces allodynia and edema, whereas a dose two log units lower (1.50 μg , i.pl.) not only produces less allodynia, but primes DOR analgesic competence (Fig. 3C). No changes were observed across treatment groups in contralateral hind paws (Fig. 3B,D). These data demonstrate that systemic paroxetine treatment reverses peripheral DOR analgesic incompetence *in vivo*. The results of these experiments also suggest that concentrated local inhibition of GRK2 produces allodynia that supersedes DOR-mediated analgesia.

Next, we sought to determine whether drug-induced molecular changes occur at the doses that result in DOR-mediated analgesia, peripheral (TG, DRG) and central tissues (brain, SC). Based on posttranslational changes governing functional DOR competence (Brackley et al., 2017), we assessed PKA-mediated phosphorylation of GRK2 (Fig. 3E), membrane-targeting of GRK2 (Fig. 3F), and GRK2 association with plasma membrane DOR (Fig. 3G). Surprisingly, paroxetine only induced changes in

peripheral tissues (DRG and TG), not central (SC or midbrain; Fig. 3E,F). In TG and DRG, only paroxetine and CMPD101 significantly reduce GRK2 phosphorylation at Ser685 and GRK2 translocation to the plasma membrane. Accordingly, similar effects occur in respect to GRK2 association with membrane-bound DOR with significant reductions on GRK2-DOR association in DRG (Fig. 3G). Collectively, these findings suggest that paroxetine reverses peripheral opioid receptor incompetence selectively through changes to GRK2 in the periphery.

Discussion

Efforts to manage severe pain without disabling side effects including respiratory depression, tolerance and substance abuse, increasingly point toward peripheral opioid receptors as potential therapeutic targets. One challenge to this approach is that these receptors are analgesically incompetent unless primed by inflammation, which is conserved in animals (Stein et al., 1989; Obara et al., 2009; Rowan et al., 2009; Brackley et al., 2016) and humans (Stein et al., 1991; Likar et al., 2001). Despite the analgesic potential of this receptor subpopulation, the therapeutic effectiveness of peripherally-restrictive opioid agonists would be limited to severe inflammatory pain. The catalyst that restores functional opioid receptor competence depends on the induction of a signaling cascade that results in the summation of GRK2 sequestration from the receptor (Brackley et al., 2016). FDA-approved drug paroxetine, which binds and inhibits GRK2 (Thal et al., 2012), may have analgesic potential by enhancing peripheral opioid receptor competence in multiple pain modalities (Lee et al., 2012). In this study, we identify a GRK2-dependent mechanism that establishes a rationale for clinical trial implementation of repurposing of the FDA-approved drug paroxetine as a potential co-treatment to restore peripheral opioid competence.

Paroxetine has long been FDA approved and is currently indicated for the treatment of major depressive disorder, obsessive compulsive disorder, panic disorder, social anxiety disorder, generalized anxiety disorder, posttraumatic stress disorder, and was recently approved to treat vasomotor symptoms associated with menopause. Although not currently indicated for the treatment of pain, multiple studies indicate that paroxetine, on its own or as a co-therapy, is analgesically efficacious for a variety of human pain conditions. These pain conditions include headache and migraine (Foster and Bafaloukos, 1994; Langemark and Olesen, 1994; Holroyd et al., 2003; Park et al., 2006; Davanzo et al., 2014), fibromyalgia (Patkar et al., 2007; Pae et al., 2009a,b; Ramzy, 2017), diabetic neuropathy (Sindrup et al., 1990), irritable bowel syndrome (Marks et al., 2008), burning mouth syndrome (Maina et al., 2002), rheumatoid arthritis (Bird and Broggin, 2000), temporomandibular disorder (Inagaki et al., 2007), noncardiac chest pain (Doraiswamy et al., 2006), phantom limb pain (Nagoshi et al., 2012), and somatic pain comorbid with the disorders for which paroxetine is indicated

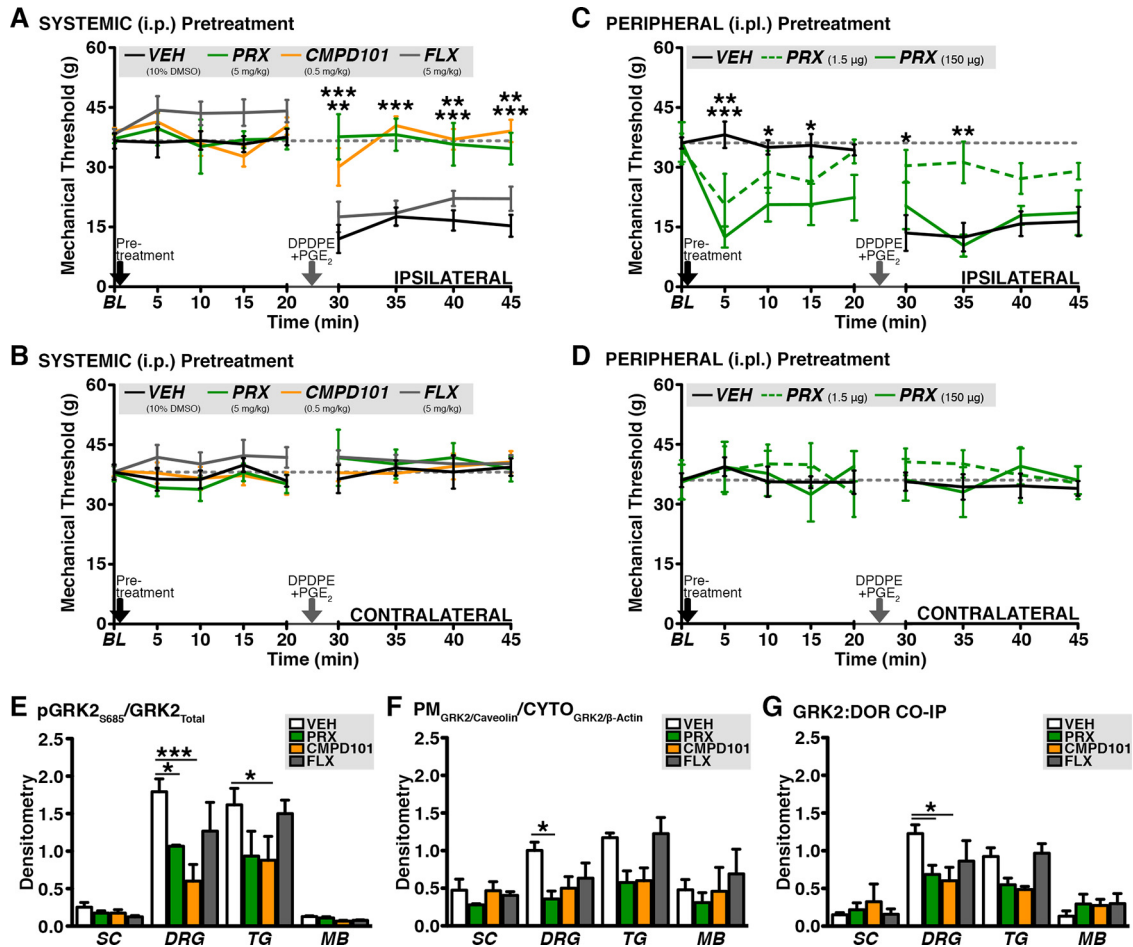


Figure 3. Paroxetine targets GRK2 to modulate of DOR-mediated antinociception. **A, B**, DPDPPE (20 µg) inhibition of PGE₂ (0.3 µg)-induced mechanical allodynia in **(A)** ipsilateral and **(B)** contralateral hindpaws following systemic treatment. Readings were collected at 5-min intervals for 20 min following initial (intraperitoneal) injection [black arrow, vehicle (VEH; 10% DMSO/90% DPBS), paroxetine (PRX; 5.0 mg/kg), CMPD101 (0.5 mg/kg), or fluoxetine (FLX; 5.0 mg/kg)] and a second (i.pl.) injection (gray arrow, co-injection DPDPPE (20 µg)/PGE₂ (0.3 µg)); DPDPPE inhibition of PGE₂-induced allodynia: **A**, VEH versus PRX (***p* < 0.005 at 30, 35 min, ***p* < 0.01 at 40, 45 min) or CMPD101 (***p* < 0.01 at 30 min, ****p* < 0.005 at 35, 40, 45 min), FLX versus PRX (***p* < 0.01 at 30 and ****p* < 0.005 at 35) and CMPD101 (***p* < 0.005 at 35); ipsilateral ANOVA summary: Interaction: $F_{(24,180)} = 4.358$, $p = 0.0001$, Treatment: $F_{(3,180)} = 19.16$, $p = 0.0001$, Time: $F_{(8,180)} = 14.72$, $p = 0.0001$; repeated measures two-way ANOVA Bonferroni *post hoc*; mean ± SEM, $n = 6$ rats/group. **C, D**, DPDPPE (20 µg) inhibition of PGE₂ (0.3 µg)-induced mechanical allodynia in **(C)** ipsilateral and **(D)** contralateral hindpaws following the timeline described above, but initial injection was peripherally-administered [i.pl., VEH or PRX (1.50 µg or 150 µg)]; **C**, PRX-induced allodynia: VEH vs 1.50 µg (***p* < 0.01 at 5 min) or 150 µg (***p* < 0.005 at 5 min; **p* < 0.05 at 10–15 min); DPDPPE inhibition of PGE₂-induced allodynia: VEH vs 1.50 µg (**p* < 0.05 at 30 min, ***p* < 0.01 at 35 min); ipsilateral ANOVA summary: Interaction: $F_{(16,135)} = 2.915$, $p = 0.0004$, Treatment: $F_{(2,135)} = 11.21$, $p = 0.0001$, Time: $F_{(8,135)} = 5.410$, $p = 0.0001$; repeated measures two-way ANOVA Bonferroni *post hoc*; mean ± SEM, $n = 6$ rats/group. **E–G**, Molecular changes 50 min following systemic (intraperitoneal) injection [vehicle (VEH; 10% DMSO/90% DPBS), paroxetine (PRX; 5.0 mg/kg), CMPD101 (0.5 mg/kg), or fluoxetine (FLX; 5.0 mg/kg)] in spinal cord (SC), dorsal root ganglia (DRG), trigeminal ganglia (TG), and midbrain (MB). PKA-dependent phosphorylation of GRK2 at Ser685 (**E**), GRK2 translocation (**F**), and GRK2 association with membrane-bound DOR [**G**; **p* < 0.05, ***p* < 0.01, ANOVA summary (phosphoGRK2-Ser685): Interaction: $F_{(9,27)} = 1.830$, $p = 0.1085$, Tissue: $F_{(3,9)} = 25.17$, $p = 0.0001$, Treatment: $F_{(3,27)} = 8.393$, $p = 0.0004$, Matching: $F_{(9,27)} = 2.272$, $p = 0.0480$, ANOVA summary (GRK2 translocation): Interaction: $F_{(9,27)} = 1.441$, $p = 0.2202$, Tissue: $F_{(3,9)} = 4.558$, $p = 0.0332$, Treatment: $F_{(3,27)} = 7.930$, $p = 0.0006$, Matching: $F_{(9,27)} = 3.103$, $p = 0.0108$, ANOVA summary (DOR:GRK2 association): Interaction: $F_{(9,27)} = 2.025$, $p = 0.0758$, Tissue: $F_{(3,9)} = 5.469$, $p = 0.0204$, Treatment: $F_{(3,27)} = 3.468$, $p = 0.0299$, Matching: $F_{(9,27)} = 4.691$, $p = 0.0008$, $n = 3–4$ independent trials of tissue collected from 16 total rats, matched two-way ANOVA with Bonferroni correction]. See Extended Data Figure 3-1 for representative WB images.

(Aikens et al., 2008; Wise et al., 2008; Hollander et al., 2010). However, one lumbar chronic back pain study found a nonsignificant 25% reduction in pain intensity (Atkinson et al., 1999), and case studies reveal that

paroxetine may not be appropriate for rare pain conditions (Zhu et al., 2008). Nevertheless, overwhelming evidence supports paroxetine’s potential to be repurposed for the treatment of pain.

Paroxetine human studies vary greatly in dose and treatment time. Pharmacokinetic properties of paroxetine were investigated in humans before its approval by the FDA⁵⁷⁻⁶⁰ (Lund et al., 1979, 1982; Greb et al., 1989; Kaye et al., 1989). Paroxetine is highly bioavailable and undergoes quick, extensive first-pass metabolism. Its lipophilic properties facilitate wide distribution throughout the body with only 1% remaining in the plasma. Of the preclinical studies for its use in human pain conditions, plasma levels of paroxetine have only been reported for diabetic neuropathy (Sindrup et al., 1990). Interestingly, paroxetine treatment at doses 10–50 mg requires plasma levels of 200–660 and 300–800 nM, respectively, for maximal relief in most patients. This dose range is within one log unit of our concentration response curve (Fig. 1A). Given that paroxetine can ipsilaterally affect proteins, including GRK2, in the periphery under painful conditions (Gai et al., 2014), it is likely that a substantial portion of the remaining 99% of paroxetine is present at higher concentrations locally in tissues that require it. Indeed, human brain SSRI concentrations up to 20:1 compared with plasma levels have been reported (Karson et al., 1993). Thus, the paroxetine concentration that targets GRK2 and prime the peripheral opioid system in animals may be within the physiological range present in tissues of human pain patients.

This study was performed in male rats and, given that GRK2 phosphorylation is reportedly higher in female rodents (Abraham et al., 2018), paroxetine could be more therapeutically effective at increasing peripheral DOR competence in females compared with males. Indeed, selective GRK2 inhibition in female mice increased analgesic effects of systemic MOR and KOR agonists (Abraham et al., 2018). These findings warrant additional studies in female rodents to determine whether paroxetine could maximally prime the peripheral opioid system, circumventing estrogen regulation of GRK2.

Although highly selective for GRK2/3, CMPD101 is less selective of GRK2 over GRK3 (Thal et al., 2011) compared with paroxetine (Thal et al., 2012). Both GRK inhibitors were able to attenuate GRK2 association with membrane-bound DOR (Fig. 1B) and enhance DPDPE inhibition of KCl-evoked Ca^{2+} influx (Fig. 1D–F). Although there were no significant differences observed between paroxetine and CMPD101, it is apparent in the summary data that paroxetine showed both a greater inhibition of GRK2 phosphorylation at Ser365 (Fig. 1C) and DPDPE inhibition of KCl-evoked Ca^{2+} influx (Fig. 1F). Therefore, CMPD101 cross-inhibition of GRK3, which is also expressed in sensory neurons (Diverse-Pierluissi et al., 1996), could explain the subtle differences observed between GRK inhibitors in these cultured neuron experiments.

CMPD101 largely produces analogous effects to paroxetine *in vivo* (Fig. 3) and in sensory neuron cultures (Fig. 1) and tissue (Fig. 3), but we identify only one functional similarity between the SSRIs fluoxetine and paroxetine. Each partially inhibits VGCCs in the absence of opioid ligand (Fig. 1D). While such a phenomenon has been reported in neurons of the central nervous system (Stauderman et al., 1992; Deák et al., 2000), to our knowledge, this is the first

report of such an effect in peripheral sensory neurons. According to our data, this does not predict the priming capacity of functional (Fig. 1D–F) or physiological (Fig. 3A) DOR competence. Given that paroxetine's effect on KCl-evoked Ca^{2+} transients in the absence of opioid was unchanged following GRK2 knock-down or overexpression (Fig. 2A,D), this SSRI effect is GRK2-independent. Interestingly, paroxetine's effect is twice that of fluoxetine at equimolar doses. This may be due, in part, to its unique molecular structure relative to other SSRIs. Paroxetine is a phenylpiperidine and nearly all phenylpiperidines used clinically in research capacities are opioids. Interestingly, this structure is part of the morphine and fentanyl molecules. Furthermore, paroxetine-induced analgesia is dose dependently reversed by opioid receptor antagonists (Gray et al., 1998; Kesim et al., 2005). This raises the possibility that paroxetine may have some affinity for opioid receptors in addition to its known target proteins (Owens et al., 1997, 2002; Thal et al., 2012).

In conclusion, experimental results demonstrate that paroxetine induces peripheral DOR analgesic competence through a GRK2-dependent mechanism. Within this framework, paroxetine mimics the inflammatory priming cascade by scaffolding GRK2. Consequently, constitutive phosphorylation of GRK2 at Ser685 is impaired and chronic GRK2 association with plasma membrane DOR reduced, thus freeing DOR from its incompetent state in sensory neurons. This study provides proof-of-concept that the pathways regulating peripheral opioid receptor incompetence can be targeted to enhance opioid-mediated analgesia in the absence of inflammation. Because paroxetine targets the protein that governs peripheral opioid receptor responsiveness, and does so in the absence of inflammation, we propose that paroxetine may be suitable as a repurposed FDA drug that can be used as a co-therapy with peripherally-restrictive doses of opioids to improve analgesic efficacy in noninflammatory pain conditions. The findings within this study support the preclinical recycling of paroxetine for an unrecognized indication and establishes rationale for clinical trial implementation.

References

- Abraham AD, Schattauer SS, Reichard KL, Cohen JH, Fontaine HM, Song AJ, Johnson SD, Land BB, Chavkin C (2018) Estrogen regulation of GRK2 inactivates kappa opioid receptor signaling mediating analgesia, but not aversion. *J Neurosci* 38:8031–8043.
- Aikens JE, Kroenke K, Nease DE Jr, Klinkman MS, Sen A (2008) Trajectories of improvement for six depression-related outcomes. *Gen Hosp Psychiatry* 30:26–31.
- Atkinson JH, Slater MA, Wahlgren DR, Williams RA, Zisook S, Pruitt SD, Epping-Jordan JE, Patterson TL, Grant I, Abramson I, Garfin SR (1999) Effects of noradrenergic and serotonergic antidepressants on chronic low back pain intensity. *Pain* 83:137–145.
- Bao L, Jin SX, Zhang C, Wang LH, Xu ZZ, Zhang FX, Wang LC, Ning FS, Cai HJ, Guan JS, Xiao HS, Xu ZQ, He C, Hökfelt T, Zhou Z, Zhang X (2003) Activation of delta opioid receptors induces receptor insertion and neuropeptide secretion. *Neuron* 37:121–133.
- Bird H, Brogini M (2000) Paroxetine versus amitriptyline for treatment of depression associated with rheumatoid arthritis: a randomized, double blind, parallel group study. *J Rheumatol* 27:2791–2797.

- Brackley AD, Gomez R, Akopian AN, Henry MA, Jeske NA (2016) GRK2 constitutively governs peripheral delta opioid receptor activity. *Cell Rep* 16:2686–2698.
- Brackley AD, Sarrami S, Gomez R, Guerrero KA, Jeske NA (2017) Identification of a signaling cascade that maintains constitutive delta-opioid receptor incompetence in peripheral sensory neurons. *J Biol Chem* 292:8762–8772.
- Davanzo R, Bua J, Paloni G, Facchina G (2014) Breastfeeding and migraine drugs. *Eur J Clin Pharmacol* 70:1313–1324.
- Deák F, Lasztóczy B, Pacher P, Petheö GL, Kecskeméti V, Spät A (2000) Inhibition of voltage-gated calcium channels by fluoxetine in rat hippocampal pyramidal cells. *Neuropharmacology* 39:1029–1036.
- Diverse-Pierluissi M, Inglese J, Stoffel RH, Lefkowitz RJ, Dunlap K (1996) G protein-coupled receptor kinase mediates desensitization of norepinephrine-induced Ca²⁺ channel inhibition. *Neuron* 16:579–585.
- Doraiswamy PM, Varia I, Hellegers C, Wagner HR, Clary GL, Beyer JL, Newby LK, O'Connor JF, Beebe KL, O'Connor C, Krishnan KR (2006) A randomized controlled trial of paroxetine for noncardiac chest pain. *Psychopharmacol Bull* 39:15–24.
- Foster CA, Bafaloukos J (1994) Paroxetine in the treatment of chronic daily headache. *Headache* 34:587–589.
- Gai BM, Bortolotto CF, Brüning CA, Zborowski VA, Stein AL, Zeni G, Nogueira CW (2014) Depression-related behavior and mechanical allodynia are blocked by 3-(4-fluorophenylselenyl)-2,5-diphenylselenophene in a mouse model of neuropathic pain induced by partial sciatic nerve ligation. *Neuropharmacology* 79:580–589.
- Gavériaux-Ruff C, Karchewski LA, Hever X, Matifas A, Kieffer BL (2008) Inflammatory pain is enhanced in delta opioid receptor-knockout mice. *Eur J Neurosci* 27:2558–2567.
- Goldenberg SS, De Boni U (1983) Pure population of viable neurons from rabbit dorsal root ganglia, using gradients of Percoll. *J Neurobiol* 14:195–206.
- Gomez R, Por ED, Berg KA, Clarke WP, Glucksman MJ, Jeske NA (2011) Metalloproteinase inhibition potentiates bradykinin-induced hyperalgesia. *Pain* 152:1548–1554.
- Gray AM, Spencer PS, Sewell RD (1998) The involvement of the opioidergic system in the antinociceptive mechanism of action of antidepressant compounds. *Br J Pharmacol* 124:669–674.
- Greb WH, Brett MA, Buscher G, Dierdorf HD, von Schrader HW, Wolf D, Mellows G, Zussman BD (1989) Absorption of paroxetine under various dietary conditions and following antacid intake. *Acta Psychiatr Scand Suppl* 350:99–101.
- Hollander E, Stein DJ, Fineberg NA, Marteau F, Legault M (2010) Quality of life outcomes in patients with obsessive-compulsive disorder: relationship to treatment response and symptom relapse. *J Clin Psychiatry* 71:784–792.
- Holroyd KA, Labus JS, O'Donnell FJ, Cordingley GE (2003) Treating chronic tension-type headache not responding to amitriptyline hydrochloride with paroxetine hydrochloride: a pilot evaluation. *Headache* 43:999–1004.
- Inagaki T, Miyaoka T, Shinno H, Horiguchi J, Matsuda S, Yoshikawa H (2007) Treatment of temporomandibular pain with the selective serotonin reuptake inhibitor paroxetine. *Prim Care Companion J Clin Psychiatry* 9:69–70.
- Karson CN, Newton JE, Livingston R, Jolly JB, Cooper TB, Sprigg J, Komoroski RA (1993) Human brain fluoxetine concentrations. *J Neuropsychiatry Clin Neurosci* 5:322–329.
- Kaye CM, Haddock RE, Langley PF, Mellows G, Tasker TC, Zussman BD, Greb WH (1989) A review of the metabolism and pharmacokinetics of paroxetine in man. *Acta Psychiatr Scand Suppl* 350:60–75.
- Kesim M, Duman EN, Kadioglu M, Yaris E, Kalyoncu NI, Erciyes N (2005) The different roles of 5-HT(2) and 5-HT(3) receptors on antinociceptive effect of paroxetine in chemical stimuli in mice. *J Pharmacol Sci* 97:61–66.
- Khasabova IA, Harding-Rose C, Simone DA, Seybold VS (2004) Differential effects of CB1 and opioid agonists on two populations of adult rat dorsal root ganglion neurons. *J Neurosci* 24:1744–1753.
- Langemark M, Olesen J (1994) Sulpiride and paroxetine in the treatment of chronic tension-type headache. An explanatory double-blind trial. *Headache* 34:20–24.
- Lee BS, Jun IG, Kim SH, Park JY (2012) Interaction of morphine and selective serotonin receptor inhibitors in rats experiencing inflammatory pain. *J Korean Med Sci* 27:430–436.
- Likar R, Koppert W, Blatnig H, Chiari F, Sittl R, Stein C, Schäfer M (2001) Efficacy of peripheral morphine analgesia in inflamed, non-inflamed and perineural tissue of dental surgery patients. *J Pain Symptom Manage* 21:330–337.
- Liu R, Lin G, Xu H (2013) An efficient method for dorsal root ganglia neurons purification with a one-time anti-mitotic reagent treatment. *PLoS One* 8:e60558.
- Lund J, Lomholt B, Fabricius J, Christensen JA, Bechgaard E (1979) Paroxetine: pharmacokinetics, tolerance and depletion of blood 5-HT in man. *Acta Pharmacol Toxicol (Copenh)* 44:289–295.
- Lund J, Thayssen P, Mengel H, Pedersen OL, Kristensen CB, Gram LF (1982) Paroxetine: pharmacokinetics and cardiovascular effects after oral and intravenous single doses in man. *Acta Pharmacol Toxicol (Copenh)* 51:351–357.
- Maina G, Vitalucci A, Gandolfo S, Bogetto F (2002) Comparative efficacy of SSRIs and amisulpride in burning mouth syndrome: a single-blind study. *J Clin Psychiatry* 63:38–43.
- Marks DM, Han C, Krulewicz S, Pae CU, Peindl K, Patkar AA, Masand PS (2008) History of depressive and anxiety disorders and paroxetine response in patients with irritable bowel syndrome: post hoc analysis from a placebo-controlled study. *Prim Care Companion J Clin Psychiatry* 10:368–375.
- Nadal X, Baños JE, Kieffer BL, Maldonado R (2006) Neuropathic pain is enhanced in delta-opioid receptor knockout mice. *Eur J Neurosci* 23:830–834.
- Nagoshi Y, Watanabe A, Inoue S, Kuroda T, Nakamura M, Matsumoto Y, Fukui K (2012) Usefulness of milnacipran in treating phantom limb pain. *Neuropsychiatr Dis Treat* 8:549–553.
- Obara I, Parkitna JR, Korostynski M, Makuch W, Kaminska D, Przewlocka B, Przewlocki R (2009) Local peripheral opioid effects and expression of opioid genes in the spinal cord and dorsal root ganglia in neuropathic and inflammatory pain. *Pain* 141:283–291.
- Owens MJ, Morgan WN, Plott SJ, Nemeroff CB (1997) Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J Pharmacol Exp Ther* 283:1305–1322.
- Owens JM, Knight DL, Nemeroff CB (2002) [Second generation SSRIS: human monoamine transporter binding profile of escitalopram and R-fluoxetine]. *Encephale* 28:350–355.
- Pae CU, Masand PS, Marks DM, Krulewicz S, Peindl K, Mannelli P, Patkar AA (2009a) History of depressive and/or anxiety disorders as a predictor of treatment response: a post hoc analysis of a 12-week, randomized, double-blind, placebo-controlled trial of paroxetine controlled release in patients with fibromyalgia. *Prog Neuropsychopharmacol Biol Psychiatry* 33:996–1002.
- Pae CU, Masand PS, Marks DM, Krulewicz S, Han C, Peindl K, Mannelli P, Patkar AA (2009b) History of early abuse as a predictor of treatment response in patients with fibromyalgia: a post-hoc analysis of a 12-week, randomized, double-blind, placebo-controlled trial of paroxetine controlled release. *World J Biol Psychiatry* 10:435–441.
- Park HJ, Lee ST, Shim JY, Kim B, Hwang SH, Kim SH, Park JE, Park JH, Jung SH, Ahn JY, Chu K, Kim M (2006) The effect of paroxetine on the reduction of migraine frequency is independent of its anxiolytic effect. *J Clin Neurol* 2:246–251.
- Patkar AA, Masand PS, Krulewicz S, Mannelli P, Peindl K, Beebe KL, Jiang W (2007) A randomized, controlled, trial of controlled release paroxetine in fibromyalgia. *Am J Med* 120:448–454.
- Patwardhan AM, Berg KA, Akopain AN, Jeske NA, Gamper N, Clarke WP, Hargreaves KM (2005) Bradykinin-induced functional competence and trafficking of the delta-opioid receptor in trigeminal nociceptors. *J Neurosci* 25:8825–8832.

- Pettinger L, Gigout S, Linley JE, Gamper N (2013) Bradykinin controls pool size of sensory neurons expressing functional δ -opioid receptors. *J Neurosci* 33:10762–10771.
- Por ED, Bierbower SM, Berg KA, Gomez R, Akopian AN, Wetsel WC, Jeske NA (2012) β -Arrestin-2 desensitizes the transient receptor potential vanilloid 1 (TRPV1) channel. *J Biol Chem* 287:37552–37563.
- Ramzy EA (2017) Comparative efficacy of newer antidepressants in combination with pregabalin for fibromyalgia syndrome: a controlled, randomized study. *Pain Pract* 17:32–40.
- Rowan MP, Ruparel NB, Patwardhan AM, Berg KA, Clarke WP, Hargreaves KM (2009) Peripheral delta opioid receptors require priming for functional competence in vivo. *Eur J Pharmacol* 602:283–287.
- Schumacher SM, Gao E, Zhu W, Chen X, Chuprun JK, Feldman AM, Tesmer JJ, Koch WJ (2015) Paroxetine-mediated GRK2 inhibition reverses cardiac dysfunction and remodeling after myocardial infarction. *Sci Transl Med* 7:277ra231.
- Sindrup SH, Gram LF, Brøsen K, Eshøj O, Mogensen EF (1990) The selective serotonin reuptake inhibitor paroxetine is effective in the treatment of diabetic neuropathy symptoms. *Pain* 42:135–144.
- Stauderman KA, Gandhi VC, Jones DJ (1992) Fluoxetine-induced inhibition of synaptosomal [3H]5-HT release: possible Ca(2+)-channel inhibition. *Life Sci* 50:2125–2138.
- Stein C, Millan MJ, Shippenberg TS, Peter K, Herz A (1989) Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of mu, delta and kappa receptors. *J Pharmacol Exp Ther* 248:1269–1275.
- Stein C, Comisel K, Haimerl E, Yassouridis A, Lehrberger K, Herz A, Peter K (1991) Analgesic effect of intraarticular morphine after arthroscopic knee surgery. *N Engl J Med* 325:1123–1126.
- Thal DM, Yeow RY, Schoenau C, Huber J, Tesmer JJ (2011) Molecular mechanism of selectivity among G protein-coupled receptor kinase 2 inhibitors. *Mol Pharmacol* 80:294–303.
- Thal DM, Homan KT, Chen J, Wu EK, Hinkle PM, Huang ZM, Chuprun JK, Song J, Gao E, Cheung JY, Sklar LA, Koch WJ, Tesmer JJ (2012) Paroxetine is a direct inhibitor of G protein-coupled receptor kinase 2 and increases myocardial contractility. *ACS Chem Biol* 7:1830–1839.
- Vanderah TW (2010) Delta and kappa opioid receptors as suitable drug targets for pain. *Clin J Pain* 26 Suppl 10:S10–S15.
- Wang H, Wessendorf MW (2001) Equal proportions of small and large DRG neurons express opioid receptor mRNAs. *J Comp Neurol* 429:590–600.
- Wang H, Heijnen CJ, Eijkelkamp N, Carbajal AG, Schedlowski M, Kelley KW, Dantzer R, Kavelaars A (2011) GRK2 in sensory neurons regulates epinephrine-induced signalling and duration of mechanical hyperalgesia. *Pain* 152:1649–1658.
- Wise TN, Meyers AL, Desai D, Mallinckrodt CH, Robinson MJ, Kajdasz DK (2008) The significance of treating somatic symptoms on functional outcome improvement in patients with major depressive disorder: a post hoc analysis of 2 trials. *Prim Care Companion J Clin Psychiatry* 10:270–275.
- Zhu KY, Huang Y, Zhong SS, Bao ZY, Tian XL (2008) [Nummular headache: 21 new cases and therapeutic results]. *Zhonghua Yi Xue Za Zhi* 88:2935–2937.