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Allosteric interactions between delta and kappa opioid receptors in peripheral sensory neurons.

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Abbreviations:

AA, arachidonic acid; **AT1R**, type 1 angiotensin II receptor; **BK**, bradykinin; **CB1R**, type 1 cannabinoid receptor; **CB2R**, type 2 cannabinoid receptor; **CGRP**, calcitonin gene-related peptide; **DAMGO**, [D-Ala²,N-MePhe⁴,Gly-ol⁵]enkephalin; **ERK**, Extracellular signal-Regulated Kinase; **G protein**, guanine nucleotide binding protein; **MOR**, μ opioid receptor; **KOR**, κ opioid receptor; **DOR**, δ opioid receptor; **DPDPE**, [D-Pen^{2,5}]-Enkephalin; **DADLE**, [D-Ala², D-Leu⁵]-Enkephalin ; **5'-GNTI**; 5'-guanidinonaltrindole, **6'-GNTI**; 6'-guanidinonaltrindole **NTI**, naltrindole; **nor-BNI**; nor-binaltorphimine; **NGF**, nerve growth factor; **PGE₂**, prostaglandin E₂; **PLA₂**, phospholipase A₂; **PLC**, phospholipase C; **PKC**, protein kinase C; **PWL**, paw withdrawal latency; **TG**, trigeminal ganglion

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Abstract

The peripheral delta opioid receptor (DOR) is an attractive target for analgesic drug development. There is evidence that DOR can form heteromers with the kappa opioid receptor (KOR). As drug targets, heteromeric receptors offer an additional level of selectivity and, as a consequence of allosteric interactions between protomers, functionality. Here we report that selective KOR antagonists differentially altered the potency and/or efficacy of DOR agonists in primary cultures of adult rat peripheral sensory neurons and in a rat behavioral model of thermal allodynia. *In vitro*, the KOR antagonist, nor-BNI, enhanced the potency of DPDPE, decreased the potency of DADLE, and decreased the potency and efficacy of SNC80 to inhibit prostaglandin E₂ (PGE₂)-stimulated adenylyl cyclase activity. *In vivo*, nor-BNI enhanced the effect of DPDPE and decreased the effect of SNC80 to inhibit PGE₂-stimulated thermal allodynia. In contrast to nor-BNI, the KOR antagonist, 5'-GNTI, reduced the response of DPDPE both in cultured neurons and *in vivo*. Evidence for DOR-KOR heteromers in peripheral sensory neurons included co-immunoprecipitation of DOR with KOR, a DOR-KOR heteromer selective antibody augmented the antinociceptive effect of DPDPE *in vivo*, and the DOR-KOR heteromer agonist, 6'-GNTI, inhibited adenylyl cyclase activity *in vitro* as well as PGE₂-stimulated thermal allodynia *in vivo*. Taken together, these data suggest that DOR-KOR heteromers exist in rat primary sensory neurons and that KOR antagonists can act as modulators of DOR agonist responses most likely through allosteric interactions between the protomers of the DOR-KOR heteromer.

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Introduction

Management of pain by opioid analgesics is confounded by central adverse effects that limit clinical dosages and treatment paradigms. Consequently, increased attention has been given to analgesia mediated by peripheral opioid receptors. Opioid receptors are expressed in peripheral primary sensory neurons that transduce pain information (nociceptors). Interestingly, peripherally-restricted opioids generally do not elicit an analgesic response when administered to normal tissue, but can produce antinociception when administered into injured or inflamed tissue (Ferreira and Nakamura, 1979; Stein et al., 1989). This finding suggests that some stimulus from the inflamed tissue interacts with opioid receptor systems in nociceptors to make them functionally competent to inhibit nociceptor signaling.

We have shown previously that functional competence of rat peripheral opioid receptor systems can be induced by brief pre-treatment with inflammatory mediators, such as bradykinin (BK). When applied locally to the rat hindpaw (i.pl.), the DOR agonist, [D-Pen^{2,5}]-Enkephalin (DPDPE), does not alter PGE₂-induced thermal allodynia. However, when administered 15 min after local injection of BK, DPDPE produces a profound anti-allodynic response (Rowan et al., 2009). Similarly, in primary sensory neuronal cultures of adult rat trigeminal ganglion (TG), the DOR agonists, DPDPE or [D-Ala², D-Leu⁵]-Enkephalin (DADLE), are ineffective at reducing PGE₂-stimulated cAMP accumulation or BK/PGE₂-stimulated neuropeptide release. However, following brief (15 min) pre-treatment with BK (or other activators of Gq-mediated signaling), DOR agonists become capable of inhibiting adenylyl cyclase activity and neuropeptide release (Patwardhan et al., 2005; Patwardhan et al., 2006). We found similar effects of BK on induction of functional competence to inhibit adenylyl cyclase activity and neuropeptide release by

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activation of the mu opioid receptor (MOR) system (Berg et al., 2007a; Berg et al., 2007b) as well as for the kappa opioid receptor (KOR) system (Berg et al., 2011).

Several studies have demonstrated that DOR and KOR can form heterodimers in heterologous expression systems (Jordan and Devi, 1999; Waldhoer et al., 2005; Xie et al., 2005). Heteromers offer intriguing possibilities for drug development in that heteromer-selective ligands would be expected to have greater tissue specificity as such drugs would be effective only in tissues that co-express the heteromer receptor pairs. Interestingly, 6'-guanidinonaltrindole (6'-GNTI), originally developed as a KOR agonist (Sharma et al., 2001), appears to have selective DOR/KOR heteromer agonist properties (Waldhoer et al., 2005). 6'-GNTI does not activate DOR and has only weak efficacy at KOR when expressed individually in HEK cells. However, it is a potent and efficacious agonist when both receptors are co-expressed and its effects can be fully blocked by occupancy of DOR with the antagonist, naltrindole, or KOR with the antagonist, nor-binaltorphimine (nor-BNI). 6'-GNTI has been shown to produce analgesia when administered into the spinal cord, but not the brain, supporting the notion that heteromer-selective ligands will provide greater tissue specificity of action (Waldhoer et al., 2005).

An interesting property of heteromers with respect to drug development is the potential for allosteric interactions between the protomers (Fuxe et al., 2010; Keov et al., 2011; Smith and Milligan, 2010). The interaction between two protomers of a heteromeric pair could influence the affinity, efficacy or both of a ligand for one of the protomers. For example, the potency of orexin A to promote activation of Extracellular signal-Regulated Kinase (ERK) in CHO cells was increased 100-fold by the presence of the CB1 receptor (Hilairret et al., 2003). Ligand binding to the orthosteric site of one protomer of a heteromeric pair can alter the function of a second ligand that binds to the orthosteric site of the second protomer of the pair. With respect to

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DOR-KOR heteromers expressed in HEK-293 cells, the DOR-selective antagonist, naltrindole, increased the binding of the KOR-selective antagonist, nor-BNI, and vice-versa (Xie et al., 2005). Further, Javitch's group recently showed that the conformational state of one protomer of a dopamine D2 receptor heteromer could alter agonist efficacy at the second protomer (Han et al., 2009).

An understanding of the allosteric interactions between protomers of heteromeric pairs of receptors may lead to the development of new drugs with improved efficacy and specificity for treatment of disease. However, although heteromers have been well-studied using heterologous expression systems, there is little known of the role of heteromers *in vivo*. Here we studied the effect of KOR antagonist ligands on the function of DOR agonist ligands in a behavioral model of inflammatory peripheral pain (PGE₂-induced thermal allodynia) and in a primary cell culture model of nociceptor function.

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Materials and Methods

Materials: The following compounds were purchased from Sigma-Aldrich (St Louis, MO); DPDPE, DAMGO, DADLE, 17-Cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7,2',3'-indolomorphinan (naltrindole), 5'-Guanidiny-17-(cyclopropylmethyl)-6,7-dehydro-4,5 α -epoxy-3,14-dihydroxy-6,7-2',3'-indolomorphinan(5'-GNTI), 6-Guanidiny-17-(cyclopropylmethyl)-6,7-dehydro-4,5 α -epoxy-3,14-dihydroxy-6,7-2',3'-indolomorphinan (6'-GNTI) and 17,17'-(dicyclopropylmethyl)-6,6',7,7'-6,6'-imino-7,7'-binorphinan-3,4',14,14'-tetrol (norBNI). PGE₂ was purchased from Cayman Chemicals (Ann Arbor, MI). [¹²⁵I]-cAMP was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Collagenase was from Worthington (Lakewood, NJ). Fetal bovine serum and all other tissue culture reagents were purchased from Invitrogen Corp (Carlsbad, CA). All other drugs and chemicals (reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals: Adult male Sprague-Dawley rats (Charles River, Wilmington, MA, USA), weighing 250-300 gm, were used in this study. The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to International Association for the Study of Pain (IASP) and federal guidelines. Animals were housed for one week, with food and water available *ad libitum*, prior to behavioral testing or harvesting of TG cells.

Rat trigeminal ganglia culture: Primary cultures of rat TG cells were prepared as described previously (Berg et al., 2007a; Berg et al., 2007b; Patwardhan et al., 2005; Patwardhan et al., 2006). In brief, fresh TG were washed with Hank's balanced salt solution (HBSS; Ca⁺⁺, Mg⁺⁺ free), digested with 3 mg/ml collagenase for 30 min at 37° C, and centrifuged (1000 rpm for 1

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min). The pellet was further digested with 0.1% trypsin (15 min) and 167 $\mu\text{g/ml}$ DNase (10 min) at 37° C in the same solution. Cells were pelleted by centrifugation (2 min at 2000 rpm) and resuspended in Dulbecco's Modified Eagle Medium (high glucose) containing 100 ng/ml NGF (Harlan), 10% fetal bovine serum, 1x Pen/Strep, 1x L-glutamine and the mitotic inhibitors: 7.5 $\mu\text{g/ml}$ uridine and 17.5 mg/ml 5-fluoro-2'-deoxyuridine. After trituration to disrupt tissue, the cell suspension was seeded on polylysine-coated 48-well or 10 cm plates. Cells were pooled from 3 rats were to seed 48-well plates and cells pooled from 6 rats were used to seed 10 cm plates. Media was changed 24 h and 48 h after plating. On the 5th day of culture, cells were re-fed with serum-free DMEM without NGF. Cells were used on the 6th day of culture.

Co-immunoprecipitation: The ability of DOR to co-immunoprecipitate with KOR was determined using the Thermo Scientific Pierce Crosslink IP kit according to manufacturers directions followed by Western blot analysis. Briefly, 4 x 10 cm plates of cells were subjected to cell surface cross-linking with membrane insoluble bis[sulfosuccinimidyl]suberate (1 mM, Pierce) for 30 min at room temperature followed by lysis with 0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4. Lysed material was applied to a Pierce spin column containing anti-KOR antibody (10 μg , H70, Santa Cruz Biotechnology) covalently immobilized to Protein A/G agarose beads and incubated overnight at 4°C. Following centrifugation, samples (20 μl , 500 μg) were resolved on NuPage 4%-12% SDS-polyacrylamide gradient gels (Invitrogen), and transferred to polyvinyl difluoride (PVDF) membrane using the iBlot transfer system (Invitrogen). Western blots were blocked in LiCOR Odyssey blocking buffer (1 h 23° C) and incubated overnight with anti-DOR (1:200, RA19072, Neuromics) or anti-KOR (1:500, Santa Cruz Biotechnology) antibody, followed by the Goat anti-rabbit IR 800 secondary antibody (1:10,000; IRDye 800CW, Licor). Samples from rat liver (500 μg), which doesn't

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contain KOR, and elution buffer only were applied to Pierce spin columns containing anti-KOR antibody as controls. In addition, samples from TG (500 μ g) were applied to spin columns without the anti-KOR antibody. Images were obtained and analyzed with an Odyssey Infrared Western blot imager (LiCor).

Measurement of cellular cAMP levels: Opioid receptor-mediated inhibition of adenylyl cyclase activity was determined by measuring the amount of cAMP accumulated in the presence of the phosphodiesterase inhibitor, rolipram, and the adenylyl cyclase activator, PGE₂. Cultures in 48-well plates were washed twice with HBSS containing 20 mM HEPES, pH 7.4 (wash buffer). Cells were pre-incubated in 250 μ l wash buffer per well for 15 min at 37°C (room air) with or without BK (10 μ M). To assess opioid agonist-mediated responses, cells were incubated with rolipram (0.1 mM) along with opioid receptor ligands in triplicate for 15 min at 37°C, followed by addition of a maximal concentration of PGE₂ (1 μ M) and incubation for a further 15 min. Incubations were terminated by aspiration of the buffer and addition of 500 μ l ice cold absolute ethanol. The ethanol extracts from individual wells were dried under a gentle air stream and reconstituted in 100 μ l 50 mM sodium acetate, pH 6.2. The cAMP content of each well was determined by radioimmunoassay.

Behavior assay: Opioid agonist-mediated changes in paw withdrawal latency (PWL) to a thermal stimulus were measured with a plantar test apparatus (Hargreaves et al., 1988) as described previously (Rowan et al., 2009). Briefly, rats were placed in plastic boxes with a glass floor maintained at 30° C. After a 30 min habituation period, the plantar surface of the hindpaw was exposed to a narrow beam of radiant heat through the glass floor. The intensity of the thermal stimulus was adjusted so that baseline PWL values were close to 10 s; cut-off time was 25 s. All drugs were dissolved in phosphate buffered saline and administered via intraplantar

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(i.pl.) injection (50 μ l) into the rat hindpaw. To induce functional competence of the opioid receptors (Berg et al., 2007a; Patwardhan et al., 2005; Rowan et al., 2009), BK (25 μ g, or Veh) was injected i.pl. 15 min before injection of PGE₂ with or without a DOR agonist. When indicated, KOR antagonists were administered 15 min before agonist (with BK). The PWL measurements were taken in duplicate at least 30 s apart at 5 min intervals continuing until 20 min after the last injection and the average was considered for statistical analysis. Observers were blinded to the treatment allocation.

Data analysis: For TG cell culture experiments, concentration-response data were fit to a logistic equation (Equation #1) using non-linear regression analysis to provide estimates of maximal response (R_{\max}), potency (EC_{50}) and slope factor (n).

$$R = R_o - \frac{R_o - R_i}{1 + \left(\frac{[A]}{EC_{50}}\right)^n} \quad \text{Equation \#1}$$

where R is the measured response at a given agonist concentration (A), R_o is the response in the absence of agonist, R_i is the response after maximal inhibition by the agonist, EC_{50} is the concentration of agonist that produces half-maximal response, and n is the slope factor. R_{\max} (the maximal inhibition produced by the agonist) was calculated as $R_o - R_i$. Experiments were repeated at least 4 times, in triplicate, using cells obtained from different groups of rats. Statistical differences in concentration-response curve parameters between groups were analyzed with Student's paired t-test. When only a single concentration was used, statistical significance was assessed using one-way analysis of variance followed by Dunnet's post-hoc or Student t-test (paired) using Prism software (Graphpad Software, Inc., San Diego, CA). $p < 0.05$ was considered statistically significant.

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For behavioral experiments, time-course data were analyzed with two-way analysis of variance, followed by Bonferroni's post-hoc test. $p < 0.05$ was considered statistically significant and data are presented as mean \pm SEM.

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Results

DOR co-immunoprecipitates with KOR from primary sensory neurons.

Co-immunoprecipitation experiments were done with primary cultures of rat sensory neurons. Following cell surface crosslinking and immunoprecipitation with anti-KOR antibody, a single, 120 kDa immunoreactive band for DOR was visualized via western blot (Figure 1). Similarly, a 120 kDa immunoreactive band for KOR was also visualized along with a lower molecular weight band at 55 kDa. These data suggest that DOR and KOR form heteromeric complexes in primary sensory neurons in culture.

Responses to the putative, DOR-KOR heteromer agonist 6'-GNTI in peripheral sensory neurons are blocked by DOR or KOR antagonists *in vitro* and *in vivo*.

In accord with previous observations that opioid receptors expressed in primary sensory neuronal cultures derived from adult rat TG do not inhibit adenylyl cyclase activity unless cells are pre-treated with an inflammatory mediator, such as BK (Berg et al., 2007a; Berg et al., 2011; Berg et al., 2007b; Patwardhan et al., 2005; Patwardhan et al., 2006), the DOR-KOR ligand, 6'-GNTI, did not alter PGE₂-stimulated cAMP levels unless cells were pre-treated for 15 min with BK (Figure 2A). In cells pre-treated with BK (10 μM, 15 min), 6'-GNTI inhibited PGE₂-stimulated adenylyl cyclase activity with an EC₅₀ of 2 nM (pEC₅₀ 8.72 ± 0.14, n=4) and a maximal inhibition of 76% ± 8. In the absence of BK, 6'-GNTI, at concentrations up to 1 μM, did not alter PGE₂-stimulated cAMP levels. The response to 6'-GNTI in BK-pre-treated cells was

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blocked completely by either the selective KOR antagonist, nor-BNI (3 nM, 100xKi) or the selective DOR antagonist, naltrindole (NTI, 20 nM, 100x Ki) (Figure 2B).

6'-GNTI was also effective in completely blocking PGE₂-induced thermal allodynia when administered to BK pre-treated hindpaws. As shown in Figure 3, intraplantar injection of PGE₂ (0.3 μg), following vehicle pre-treatment, produced a prolonged thermal allodynia (open squares). The injection of 6'-GNTI (1 μg, i.pl.) alone did not alter the PGE₂-induced thermal allodynia (open circles). However, when administered 15 min after a pre-injection of BK (25 μg, i.pl.), 6'-GNTI produced a profound anti-nociceptive response (upward solid triangles) which was blocked completely by pre-treatment (i.pl.) with either NTI (400 μg; downward solid triangles) or nor-BNI (100 μg; solid diamonds).

KOR antagonists regulate DOR agonist responses in a ligand-dependent manner *in vitro* and *in vivo*.

In BK pre-treated TG cultures, the DOR agonist DPDPE inhibited PGE₂-stimulated cAMP accumulation with a maximal inhibition of 64% ± 6% and an EC₅₀ of 0.5 nM (pEC₅₀ = 9.31 ± 0.09; mean ± SEM, n=6; Figure 4A). The KOR antagonist, nor-BNI (3 nM 100 x Ki), shifted the concentration response curve to DPDPE to the left by 10-fold, with no change in the maximal response. In the presence of nor-BNI, the maximal inhibition of PGE₂-stimulated cAMP accumulation produced by DPDPE was 63% ± 3% and the EC₅₀ was 0.06 nM (pEC₅₀ 10.20 ± 0.06, mean ± SEM, n=6; p < 0.05 compared to control, paired t-test). Neither basal nor PGE₂-stimulated cAMP levels were altered by nor-BNI. Basal levels were 0.75 pmol/well ± 0.08 for vehicle treated cells and 0.82 pmol/well ± 0.14 in the presence of nor-BNI (mean ± SEM

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n=6, p= 0.67 paired t-test). PGE₂ stimulated cAMP levels were 166% above basal ± 10% for vehicle and 129% above basal ± 9 % with nor-BNI (mean ± SEM, n=6; p= 0.357 paired t-test).

In contrast to the enhanced sensitivity to DPDPE, nor-BNI decreased the sensitivity to the DOR agonist DADLE to inhibit PGE₂-stimulated cAMP accumulation (Figure 4B). In the presence of nor-BNI, the concentration-response curve to DADLE was shifted to the right by 30-fold, without a change in the maximal inhibition. The pEC₅₀ (mean ± SEM) values for DADLE in the absence and presence of nor-BNI was 10.24 ± 0.05 (0.06 nM) versus 8.74 ± 0.32 (1.8 nM), respectively (n=6, p< 0.05 paired t-test). The maximal inhibition (mean ± SEM) by DADLE in the absence and presence of nor-BNI was 62% ± 3.0 versus 61% ± 11, respectively (n=6).

Both the potency and the efficacy of the DOR agonist SNC80 were reduced by nor-BNI (Figure 4C). The pEC₅₀ (mean ± SEM) values for SNC80 in the absence and presence of nor-BNI were 8.86 ± 0.31 (1.3 nM) versus 7.33 ± 0.32 (47 nM), respectively (n=4, p< 0.05 paired t test). The maximal inhibition (mean ± SEM) by SNC80 in the absence and presence of nor-BNI were 63% ± 7.0 versus 30% ± 7.0, respectively (n=4, < 0.01 paired t-test).

nor-BNI did not alter the concentration-response curve to a μ opioid receptor (MOR) agonist, DAMGO (Figure 4D). The maximal inhibition of PGE₂-stimulated cAMP accumulation by DAMGO was 72% ± 6.0 versus 66% ± 3.0 in the absence and presence of nor-BNI, respectively (n=4, p=0.461 paired t-test). The mean pEC₅₀ ± SEM for DAMGO in the absence and presence of nor-BNI was 7.85 ± 0.14 (10 nM) versus 8.09 ± 0.06 (7 nM), respectively, (n=4, p=0.70 paired t-test).

Similar results with nor-BNI were seen in the behavioral experiments. Figure 5A shows that DPDPE (0.2 μg) injected i.pl. into the BK pre-treated rat hindpaw did not alter PGE₂-

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induced thermal allodynia. This is consistent with our previous study where the ED₅₀ for DPDPE to inhibit PGE₂-induced thermal allodynia was approximately 2.0 μg (Rowan et al., 2009). However, nor-BNI (10 μg) enhanced the effect of this sub-threshold dose of DPDPE to completely eliminate the allodynia produced by PGE₂. In contrast, nor-BNI eliminated the anti-allodynic effect of SNC80 (5 μg, i.pl; Figure 5B).

The experiments above demonstrate that the nature of effect of nor-BNI was dependent upon the DOR agonist both in primary culture of sensory neurons and *in vivo*. Figure 6 shows that the effect on DOR agonist-mediated responses also depends upon the KOR ligand used. In contrast to the enhanced sensitivity of DPDPE produced by nor-BNI (Figures 4A and 5A), the KOR antagonist, 5'-GNTI, reduced responsiveness to DPDPE *in vitro* and *in vivo*. 5'-GNTI did not alter PGE₂-stimulated cAMP accumulation (p= 0.91 paired t-test) but dramatically reduced the maximal inhibition of PGE₂-stimulated cAMP accumulation produced by DPDPE from 53% ± 3 to 5% ± 4 in the absence and presence of 5'-GNTI, respectively (Figure 6A; n=6, p< 0.01 paired t-test). Similarly, the anti-allodynic effect of an ED₅₀ dose of DPDPE (2.0 μg, i.pl) in BK pre-treated hindpaws was completely reduced by 5'-GNTI (Figure 6B; 2.0 μg, i.pl).

Effects of a DOR-KOR heteromer-selective monoclonal antibody *in vivo*

A subtractive immunization strategy was used to generate an antibody that selectively recognized endogenous DOR-KOR heteromers, as we have done before (see supplemental data and Gupta et al., 2010). Hybridoma clones secreting monoclonal antibodies were screened by ELISA against untransfected HEK-293 membranes and HEK-293 membranes expressing similar levels of KOR, DOR or co-expressing DOR and KOR receptors. A monoclonal antibody was chosen based on its ability to recognize an epitope in cells co-expressing DOR

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and KOR receptors but not in cells expressing either DOR or KOR alone (Supplemental Figure 1). The selectivity of the antibody for DOR-KOR heteromers was tested by screening against cells expressing DOR, KOR or CB1R receptors alone or expressing MOR-DOR, DOR-KOR, CB1-AT1R, CB1R-CB2R, CB1-MOR, CB1-KOR heteromers. The antibody recognized an epitope in cells expressing DOR-KOR heteromers but not the other receptor complexes (See Supplemental Figure 2).

As shown previously (Figure 5A), intraplantar injection of nor-BNI, enhanced the antiallodynic effect of a subthreshold dose (0.2 μg) of DPDPE (Figure 7A). Intraplantar injection of the DOR-KOR antibody (10 μg) also enhanced the antinociceptive effect of DPDPE but to an extent considerably greater than that produced by nor-BNI (Figure 7A). Whereas DPDPE completely blocked the allodynic effect of PGE_2 following nor-BNI administration, the DOR-KOR antibody promoted a profound analgesic response from a subthreshold dose of DPDPE. Injection of an antibody selective for CB1-AT1 receptor heteromers did not alter the effect of DPDPE (Figure 7B). Neither nor-BNI nor the DOR-KOR or CB1-AT1 antibodies alone altered the allodynic effect of PGE_2 .

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Discussion

In this study, we provide evidence for the presence of DOR-KOR heteromers in peripheral sensory neurons and demonstrate that KOR antagonist ligands regulate DOR agonist function likely via allosteric interactions between the DOR-KOR protomers. Thus, nor-BNI enhanced the potency with no change in efficacy of DPDPE, decreased the potency with no change in efficacy of DADLE, and decreased both potency and efficacy of SNC80 in primary cultures of adult rat peripheral sensory neurons. In contrast to nor-BNI, the KOR antagonist, 5'-GNTI, decreased the DPDPE response. The differential effect of KOR antagonists on DOR agonist responses observed in cultured neurons was also observed in a behavioral model of thermal allodynia. nor-BNI enhanced the anti-allodynic response produced by DPDPE and decreased the anti-allodynic response produced by SNC80. Also consonant with its effect in cultured neurons, 5'-GNTI decreased the anti-allodynic response by DPDPE. Collectively, these parallel studies demonstrate profound, ligand-dependent interactions between KOR and DOR in peripheral sensory neurons, which is a hallmark of allosterism.

Heteromerization between DOR and KOR has been shown in a variety of studies. Epitope-tagged DOR and KOR can be co-immunoprecipitated (Jordan and Devi, 1999; Waldhoer et al., 2005) when co-expressed in HEK cells and DOR-KOR interactions have been observed in bioluminescence resonance energy transfer experiments in live HEK cells (Wang et al., 2005). Although etorphine promotes DOR internalization in HEK cells, it fails to produce substantial internalization in cells that co-express KOR (Jordan and Devi, 1999). Also, co-expression of DOR and KOR changes the affinity values for a variety of ligands (Jordan and Devi, 1999; Bhushan et al., 2004; Waldhoer et al., 2005; Xie et al., 2005). Moreover, 6'-GNTI, previously characterized as a KOR agonist (Sharma et al., 2001), has considerably higher

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efficacy in cells co-expressing DOR and KOR and its effects can be fully antagonized by either naltrindole (DOR antagonist) or nor-BNI (KOR antagonist) (Waldhoer et al., 2005).

In the present study, the presence of functional DOR-KOR heteromers in peripheral sensory neurons is suggested by several lines of evidence. First, DOR co-immunoprecipitated with KOR from primary sensory neuronal cultures. Second, the putative DOR-KOR heteromer-selective agonist, 6'-GNTI (Waldhoer et al., 2005), inhibited PGE₂-stimulated cAMP accumulation with high potency and efficacy in vitro and elicited a strong antinociceptive response in vivo both of which were blocked by either a DOR or KOR antagonist. Third, the antinociceptive response to DPDPE was enhanced by injection of a DOR-KOR heteromer-selective monoclonal antibody into the rat hindpaw. Finally, ligand-dependent effects of KOR antagonists on DOR agonist responses in vitro and in vivo were suggestive of allosteric interactions between DOR-KOR protomers.

Although it is possible that the effects of KOR antagonists on DOR agonist responses occurred as a result of cross-talk between KOR and DOR signaling systems, this mechanism seems unlikely. First, for a cross-talk mechanism to be responsible for the observed effects, both nor-BNI and 5'-GNTI must not be true antagonists but each must regulate some cellular signaling pathway that alters DOR function. This pathway must be independent of the Gi-adenylyl cyclase pathway because the KOR antagonists alone did not alter adenylyl cyclase activity. Although nor-BNI and 5'-GNTI have weak inverse agonist properties for the adenylyl cyclase pathway (Wang et al., 2007), to our knowledge there have been no reports of inverse agonist activity for non-adenylyl cyclase signaling. Both nor-BNI and 5'-GNTI have agonist activity for JNK phosphorylation (Bruchas and Chavkin, 2010), however, nor-BNI-mediated JNK signaling and its action on DOR would have to be qualitatively different from that of 5'-

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GNTI, since the actions of these KOR ligands on DPDPE signaling were opposite in direction. Further, for the effect of KOR ligands to be DOR agonist-dependent, the target of the KOR signaling component must be at DOR itself. Changes in any other component of the DOR signaling system beyond the receptor (e.g. adenylyl cyclase) would be expected to regulate all DOR agonists similarly. Moreover, effects of nor-BNI-mediated alterations in KOR signaling on DOR itself must allow for different (opposite) effects on DOR agonist responses (increased vs. decreased potency of DPDPE and DADLE by nor-BNI).

We suggest the most parsimonious explanation for the mechanism by which KOR antagonists differentially regulate DOR agonist responsiveness is via allosteric interactions between the protomers of DOR-KOR heteromers. One of the hallmarks of allosterism is that effects are dependent upon the nature of both the allosteric modulator and the orthosteric ligand (Kenakin, 2009; Keov et al., 2011; Smith and Milligan, 2010). The same allosteric modulator can either increase, decrease or not alter the activity of an orthosteric agonist, depending upon the agonist (Jakubik et al., 1997). Thus, a conformational change in KOR elicited by occupancy with nor-BNI or 5'-GNTI would lead to a ligand-dependent conformational change in DOR resulting in a change in affinity and/or efficacy of DOR agonists. Our estimation of allosteric constants, α (affinity) and ξ (efficacy), by fitting the concentration-response curve data for DOR agonist inhibition of cAMP accumulation in the absence and presence of nor-BNI or 5'-GNTI to an allosteric model of agonism (see Kenakin, 2005; Supplemental Data) suggests that the differential actions of KOR ligands can be accommodated by models of allosteric mechanisms.

Allosteric interactions between protomers of heteromeric pairs have been previously reported (see Fuxe et al., 2010; Keov et al., 2011; Smith and Milligan, 2010). In heterologous

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expression systems, simply the presence of one receptor can alter the affinity and/or efficacy of a ligand for a second receptor (Jordan and Devi, 1999; Waldhoer et al., 2005). Importantly, the conformation of one receptor of a heteromeric pair can influence the affinity and/or efficacy of a ligand at the other protomer. Xie et al (2005) reported that the DOR antagonist, naltrindole, increased the affinity of nor-BNI, and vice versa. Further, the conformational state of one protomer of a dopamine D2 receptor heteromer differentially altered the efficacy of quinpirole at the second protomer (Han et al., 2009). Here we found that nor-BNI differentially altered the potency and/or efficacy of DOR agonism, depending upon the specific DOR agonist used. In addition, the effect on one DOR agonist, DPDPE, was dependent upon the nature of the KOR antagonist used. Consequently, the most likely mechanism for the ligand-dependent effects of KOR antagonists in peripheral sensory neurons is allosteric modulation between the protomers of a DOR-KOR heteromer.

It has been reported that nor-BNI antagonized the analgesic effect of DPDPE in the mouse tail-flick assay when applied intracerebroventricularly or intrathecally (Lunzer and Portoghese, 2007; Portoghese and Lunzer, 2003). Here we found that intraplantar injection of nor-BNI to rats and direct application of nor-BNI to cultures of rat sensory neurons enhanced DPDPE effects. Whether the differences here relate to differences in route of administration (Lunzer and Portoghese, 2007) or differences in phenotype between peripheral sensory neurons versus CNS neurons or species differences remains to be determined.

Interestingly, a monoclonal antibody that selectively binds to DOR-KOR heteromers potentiated the antinociceptive effects of DPDPE in the rat hindpaw, similar to, but greater than, the effect of nor-BNI. In the presence of this antibody, a sub-threshold, ineffective dose of DPDPE became capable of not only inhibiting the thermal allodynia produced by PGE₂, but

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produced close to the maximal possible antinociceptive response in this system. Characterization of this antibody as selective for the DOR-KOR heteromer is presented in Supplemental Data. Injection of this antibody alone did not alter the transient thermal allodynia produced by BK (not shown) or that produced by PGE₂. These data suggest that the binding of the antibody alters the conformation of the DOR-KOR heteromer such that the affinity and/or efficacy of DPDPE is enhanced.

Although there is evidence for MOR-KOR heteromers (Chakrabarti et al., 2010; Wang et al., 2005; Yekkirala et al., 2011) we did not observe an effect of nor-BNI on the potency or efficacy of the MOR agonist, DAMGO. It was recently shown that expression of MOR-KOR heteromers in spinal cord was very low in male rats, but high in females and regulated by female sex hormones (Chakrabarti et al., 2010). Thus the lack of interaction between nor-BNI and DAMGO could be due to low prevalence of MOR-KOR heteromers in the male rats in this study. It is also possible that MOR and KOR are expressed in different cells of the trigeminal ganglion, and therefore would not interact allosterically. Alternatively, given the ligand-dependent nature of allosterism, it is possible that visualization of interactions between MOR-KOR heteromers requires the use of different ligands.

In summary, our data suggest that DOR-KOR heteromers exist in rat primary sensory neurons and that KOR antagonists can modulate DOR agonist responses most likely through allosteric interactions between the protomers of the DOR-KOR heteromer. Allosteric regulation of opioid agonist responses may provide opportunities for development of analgesic drugs with greater selectivity as effects will occur only where both protomers are co-expressed in the same cells. Given the ligand dependency of allosteric interactions, it is important to identify optimal

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pairs of ligands such that low doses of the analgesic agonist can be used, thereby reducing the incidence and severity of dose-related adverse effects.

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Authorship contributions:

Participated in research design: Berg, Devi and Clarke.

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Performed data analysis: Berg, Devi, Gupta, Gomes, Rowan and Clarke.

Wrote or contributed to the writing of the manuscript: Berg, Devi, Portoghese, Hargreaves and Clarke.

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Legends for Figures

Figure 1: DOR co-immunoprecipitates with KOR in peripheral sensory neurons. **A)** TG primary cultures in 10 cm plates were treated with membrane insoluble bis[sulfosuccinimidyl] suberate (1 mM) for 30 min at room temperature to cross-link cells surface accessible proteins. Cell lysates were applied to Pierce spin columns containing anti-KOR antibody covalently bound to Protein A/G agarose beads. Samples were eluted, resolved with SDS-PAGE, transferred to PVDF membranes, blotted with anti-DOR or anti-KOR antibody and bands visualized with an Odyssey infrared Western Blot Imager (Licor). Following cell surface crosslinking and immunoprecipitation with KOR antibody, a single, 120 kd immunoreactive band for DOR was visualized via western blot analysis. The image shown is representative of 3 independent experiments. **B, C, D)** Negative control immunoblots with anti-KOR antibody. Lysate from rat liver (**B**), which does not express KOR, or elution buffer only (**C**) was applied to spin columns containing anti-KOR antibody. **D)** TG cell lysate was applied to spin columns without anti-KOR antibody. After elution, SDS-PAGE and transfer to PVDF membranes, blots were probed with anti-KOR and anti-DOR antibodies and visualized with the Odyssey Imager.

Figure 2: Effect of 6'-GNTI on PGE₂-stimulated adenylyl cyclase activity in primary cultures of adult sensory neurons. **A)** TG primary cultures from adult rats were pre-treated with BK (10 μM) or Veh for 15 min. After pre-treatment, cells were incubated with various concentrations of 6'-GNTI for 15 min followed by addition of PGE₂ (1 μM) and further incubation for 15 min. Cellular levels of cAMP were determined by RIA. Data are expressed as the percentage of PGE₂-stimulated cAMP levels and are the mean ± SEM, n=4. Basal (non-stimulated) cAMP levels were 2.76 ± 0.20 pmol/well and PGE₂-stimulated cAMP levels were

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67% above basal \pm 3% (mean \pm SEM, n=4). Neither basal nor PGE₂-stimulated cAMP levels were altered by BK pretreatment (p= 0.29 and p=0.86 for basal and PGE₂ cAMP levels, respectively, paired t-test). **(B)** The inhibition of PGE₂-stimulated cAMP accumulation by 6'-GNTI in BK pre-treated sensory neurons was blocked by either the DOR antagonist, NTI or the KOR antagonist nor-BNI. TG primary cultures were pretreated with BK (10 μ M) in the absence or presence of NTI (20 nM, 100 x Ki) or nor-BNI (3 nM, 100 x Ki) for 15 min. After pretreatment, cells were incubated with a maximal concentration of 6'-GNTI (100 nM) for 15 min followed by addition of PGE₂ (1 μ M) and further incubation for 15 min. Cellular levels of cAMP were determined by RIA. Data are expressed as the percentage of PGE₂-stimulated cAMP levels and are the mean \pm SEM, n=4. ***p< 0.01 compared to Veh, one-way ANOVA with Dunnett's post hoc.

Figure 3: Effect of 6'-GNTI on PGE₂-induced thermal allodynia in the rat hindpaw.

Animals were pre-injected (i.pl.) with vehicle, BK (25 μ g), BK (25 μ g) with nor-BNI (100 μ g), or BK (25 μ g) with NTI (400 μ g) 15 min before co-injection (i.pl.) with PGE₂ (0.3 μ g) and either vehicle or 6'-GNTI (1 μ g). Paw withdrawal latency (PWL) was measured in duplicate at 5 min intervals until 20 min following the last injection. Data are expressed as the change (sec) from individual baseline values (9.66 \pm 0.21 sec) and represent mean \pm SEM of 6-12 animals per group. ***p< 0.001; **p < 0.01 vs. other groups by two-way ANOVA with Bonferroni's multiple comparison test post-hoc.

Figure 4: The effect of the KOR antagonist, nor-BNI, on the response to DOR and MOR agonists. TG primary cultures from adult rats were pre-treated with BK (10 μ M) for 15 min

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followed by incubation with various concentrations of the DOR agonists DPDPE (**A**), DADLE (**B**) or SNC80 (**C**) or the MOR agonist, DAMGO (**D**) in the absence or presence of the KOR antagonist, nor-BNI (3 nM), for 15 min. After this incubation, cells were incubated with PGE₂ (1 μM) for 15 min. Cellular levels of cAMP were determined by RIA. Data represent the mean ± SEM of 4-6 experiments each. Basal cAMP levels were 0.85 ± 0.15 pmol/well and PGE₂-stimulated cAMP accumulation was 132% above basal ± 9%, mean ± SEM, n= 20.

Figure 5: The effect of the KOR antagonist, nor-BNI, on DOR agonist mediated inhibition of PGE₂-induced thermal allodynia. Animals were pre-injected (i.pl.) with BK (25 μg) with or without nor-BNI (10 μg) 15 min before co-injection (i.pl.) with (**A**) DPDPE (0.2 μg, subthreshold) or (**B**) SNC80 (5 μg) and PGE₂ (0.3 μg). Paw withdrawal latency (PWL) was measured in duplicate at 5 min intervals until 20 min following the last injection. Data are expressed as the change (sec) from individual baseline (A: 9.44 ± 0.23 sec, B: 10.80 ± 0.26 sec) values and represent mean ± SEM of 6-12 animals per group. ***p< 0.001; **p< 0.01, *p< 0.05 vs. other groups by two-way ANOVA with Bonferroni's multiple comparison test post-hoc.

Figure 6: The effect of the KOR antagonist, 5'-GNTI, on DOR agonist mediated inhibition of PGE₂-stimulated cAMP accumulation (A) and PGE₂-induced thermal allodynia (B). (A) TG primary cultures from adult rats were pre-treated with BK (10 μM) for 15 min followed by incubation with various concentrations of DPDPE in the absence or presence of the KOR antagonist, 5'-GNTI (4 nM), for 15 min. After this incubation, cells were incubated with PGE₂ (1 μM) for 15 min. Cellular levels of cAMP were determined by RIA. Data represent the mean ± SEM of 8 experiments. In the absence of 5'-GNTI, the pEC₅₀ (± SEM) for DPDPE was

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9.77 ± 0.067 . Basal cAMP levels were 0.82 ± 0.16 pmol/well and PGE₂-stimulated activity was 113% above basal $\pm 21\%$, mean \pm SEM, n= 6. Neither basal nor PGE₂-stimulated cAMP levels were altered by 5'-GNTI treatment (p= 0.075 and p=0.913 for basal and PGE₂ cAMP levels, respectively, paired t-test). **(B)** Animals were pre-injected (i.pl.) with BK (25 μ g) with or without 5'-GNTI (2 μ g) 15 min before co-injection (i.pl.) with DPDPE (2 μ g) and PGE₂ (0.3 μ g). Paw withdrawal latency (PWL) was measured in duplicate at 5 min intervals until 20 min following the last injection. Data are expressed as the change (sec) from individual baseline values (10.84 ± 0.31 sec) and represent mean \pm SEM of 6-9 animals per group. **p< 0.01, *p< 0.05 vs. other groups by two-way ANOVA with Bonferroni's multiple comparison test post-hoc.

Figure 7. The effect of a DOR-KOR heteromer selective antibody on DPDPE-mediated thermal anti-allodynia. Animals were pre-injected (i.pl.) with BK (25 μ g) without or with nor-BNI (10 μ g) and/or **(A)** a monoclonal, DOR-KOR heteromer-selective antibody (DOR-KOR Ab, 10 μ g) or **(B)** a monoclonal, AT1-CB1 heteromer-selective antibody (AT1-CB1 Ab, 10 μ g). 15 min later, rats received (i.pl.) DPDPE (0.2 μ g, subthreshold) or vehicle (Veh) and PGE₂ (0.3 μ g). Paw withdrawal latency (PWL) was measured in duplicate at 5 min intervals until 20 min following the last injection. Data are expressed as the change (sec) from individual baseline (A: 10.85 ± 0.21 sec; B: 10.53 ± 0.12 sec) values and represent mean \pm SEM of 4 animals per group. ***p< 0.001; **p< 0.01, *p< 0.05 vs. the vehicle-treated group by two-way ANOVA with Bonferroni's multiple comparison test post-hoc.

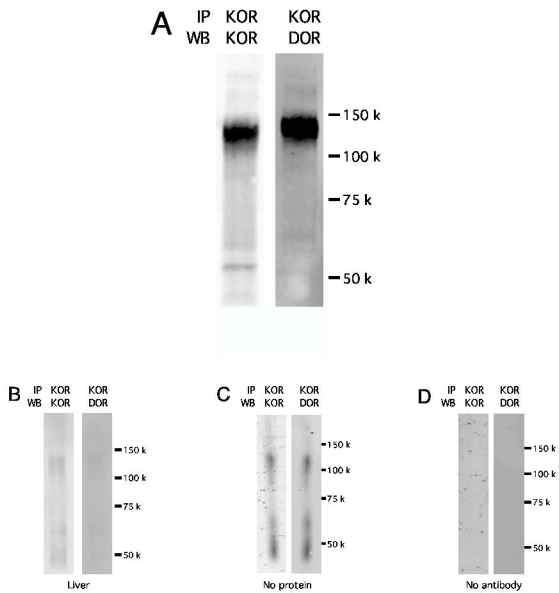


Figure 1

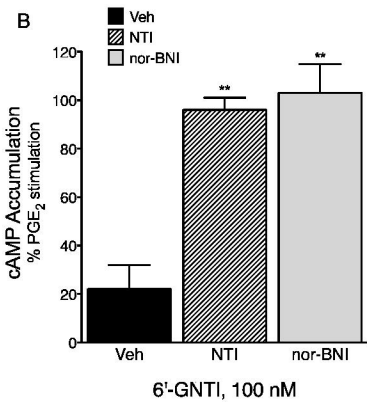
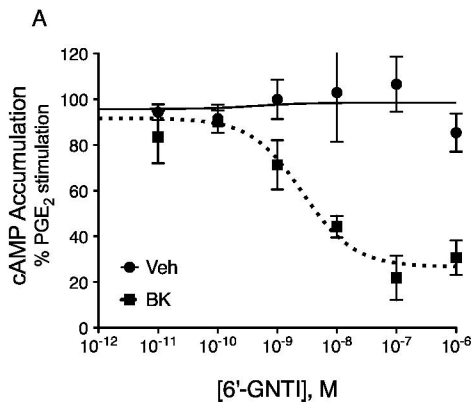


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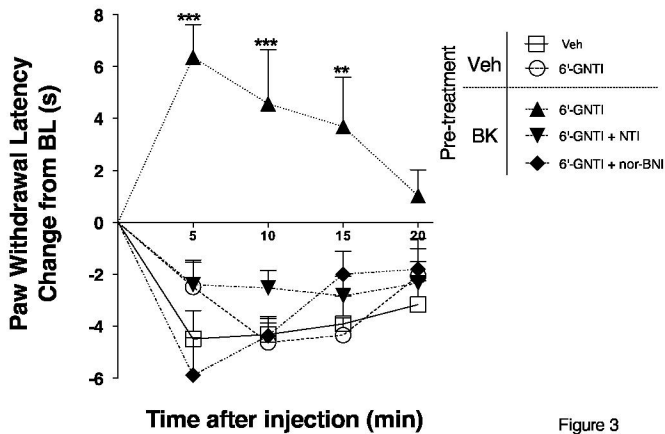


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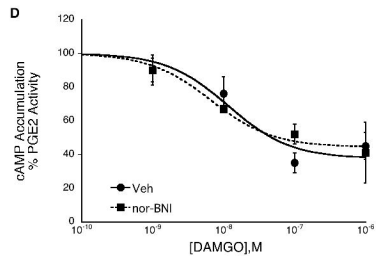
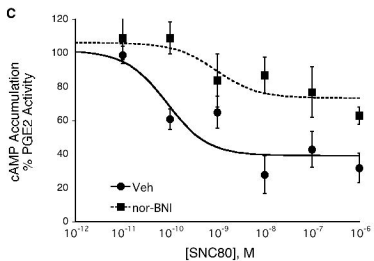
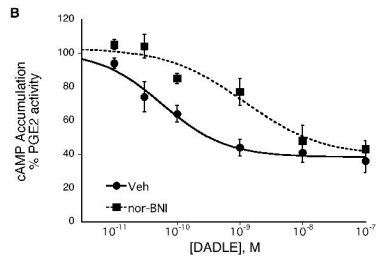
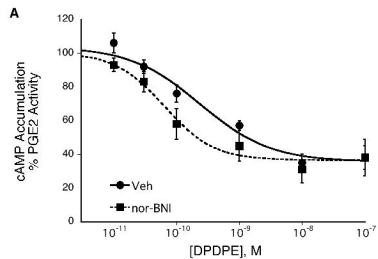


Figure 4

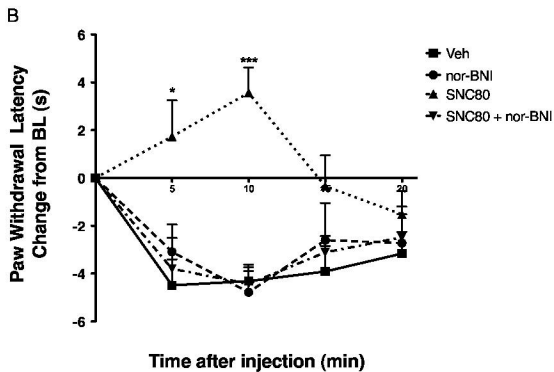
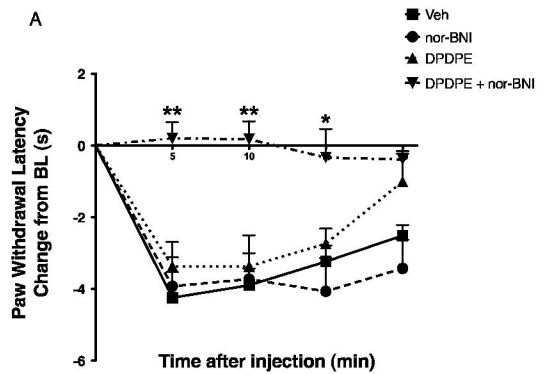


Figure 5

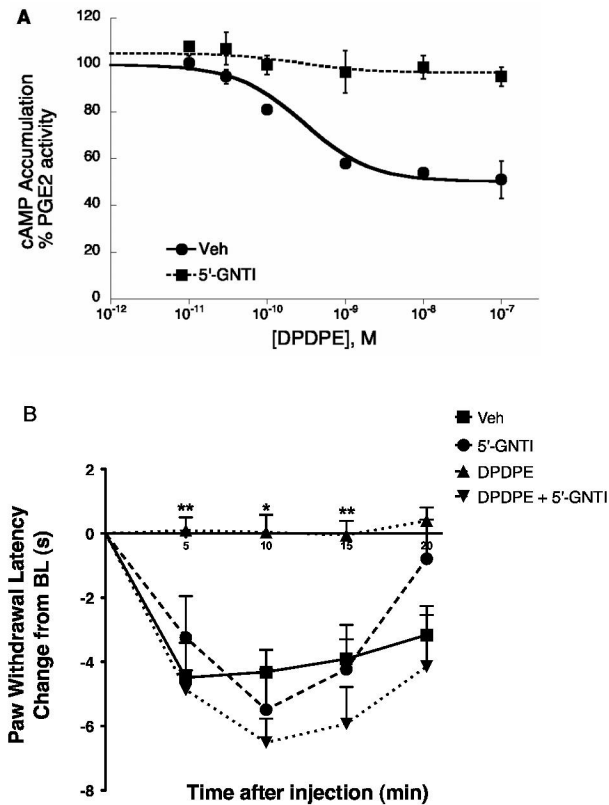


Figure 6

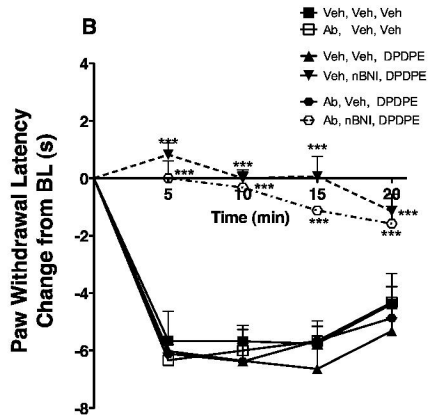
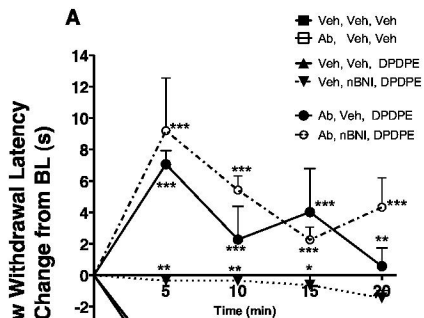


Figure 7