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

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Supplementary Materials

Methods for generation of DOR-KOR heteromer antibody

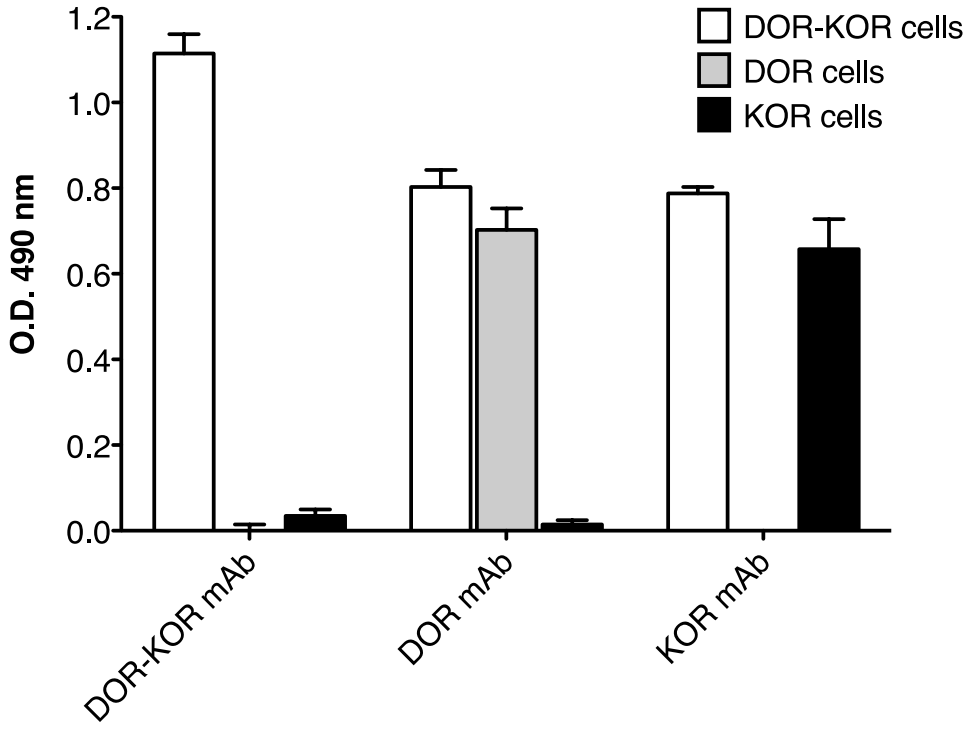
Subtractive immunization: The DOR-KOR heteromer antibody was generated using the subtractive immunization strategy as described (Gomes et al., 1999). Briefly, for induction of tolerance to immunogenic epitopes in HEK-293 membranes, female balb/c mice (6-8 weeks old, 25-35 g body weight) were injected intraperitoneally (i.p.) with 5 mg HEK-293 membranes and 15 min later with cyclophosphamide (100 mg/kg body weight, i.p.). The cyclophosphamide injection was repeated after 24 and 48 h respectively. Mice were bled every 15 days and antibody titers checked by ELISA against HEK-293 membranes. This protocol was repeated at 2 week intervals until stable background titers were obtained with HEK-293 membranes. Mice were then given an i.p. injection of membranes from HEK-293 cells co-expressing DOR-KOR receptors (5 mg) in complete Freund's adjuvant. Booster i.p. injections of HEK-293 membranes co-expressing DOR-KOR receptors were administered every 15 days. Antibody titers were checked by ELISA against HEK-293 membranes from untransfected cells and from cells co-expressing DOR-KOR receptors. Spleens from animals giving a high titer with HEK-293 membranes co-expressing DOR-KOR receptors were fused with SP-20 myeloma cells to generate monoclonal antibodies as

described (Gomes et al., 1999). Clones secreting monoclonal antibodies were screened by ELISA against untransfected HEK-293 membranes, and HEK-293 membranes expressing KOR, DOR or co-expressing DOR-KOR receptors as described (Gupta et al., 2007) using 1:10 hybridoma supernatant and 1:500 horse radish peroxidase labeled anti-mouse IgG. Hybridoma supernatant from positive clones was concentrated using Centricon 10 and stored at a concentration of 10 μg protein/ μl.

Cell culture and transfection: HEK-293 expressing KOR, DOR, CB1R, DOR-KOR, MOR-DOR were generated using Lipofectamine as per manufacturer's protocol (Invitrogen, Carlsbad, CA). Neuro 2A cells which endogenously express CB1 cannabinoid receptors (CB1R) were transfected with either Flag tagged AT1 angiotensin receptors (AT1R), HA-tagged CB2 cannabinoid receptors (CB2R), myc tagged DOR, Flag tagged MORs or Flag tagged KORs as described (Gupta et al., 2010; Rozenfeld et al., 2011).

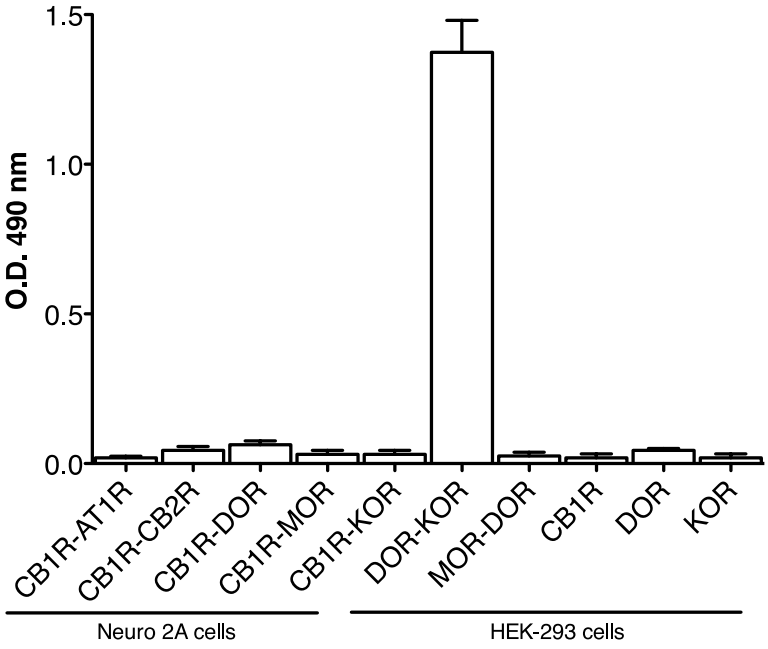
Enzyme-linked immunosorbent assay (ELISA): ELISA was carried out as described previously (Gupta et al., 2007; Gupta et al., 2010; Rozenfeld et al., 2011) using cells (2 x 10^5 cells /well) expressing individual receptors or cells co-expressing KOR and DOR, CB1R and AT1R, CB1R and CB2R, CB1R and DOR, CB1R and MOR, CB1R and KOR, and MOR and DOR. Cells were probed with KOR, DOR, and/or DOR-KOR monoclonal antibodies (1 μg/ml) and horseradish peroxidase labeled anti-mouse IgG (1:500).

Supplemental Figure 1



Supplemental Figure 1: DOR-KOR monoclonal antibody selectively detects an epitope in cells co-expressing DOR-KOR receptors. HEK-293 cells expressing KOR, DOR or co-expressing DOR-KOR receptors were subjected to ELISA using monoclonal antibodies against KOR (2), DOR (2) or DOR-KOR. Results represent Mean ± SEM (n=3).

Supplemental Figure 2



Supplemental Figure 2: Heteromer selectivity of DOR-KOR monoclonal antibody. HEK-293 cells expressing KOR, DOR, CB1R or co-expressing MOR-DOR and DOR-KOR receptors and Neuro 2A cells (that endogenously express CB1R) stably expressing AT1R, CB2R, KOR, DOR, MOR receptors were subjected to ELISA using DOR-KOR heteromer-selective monoclonal antibodies as described (Rozenfeld et al., 2011). Results represent Mean ± SEM (n=3)

Estimation of allosteric constants α and ξ.

Estimates of the allosteric constant parameters (α and ξ) were obtained by fitting concentration-response data to the Equation 2 (see Kenakin, 2005).

$$R = Basal - \frac{{}^{A}\!/_{K_A} \bullet \tau \left(1 + \alpha \bullet \xi \bullet {}^{B}\!/_{K_B}\right) E_{\max}}{{}^{A}\!/_{K_A}\left(1 + \alpha \bullet {}^{B}\!/_{K_B} + \tau\left(1 + \alpha \bullet \xi \bullet {}^{B}\!/_{K_B}\right)\right) + {}^{B}\!/_{K_B} + 1} \qquad \text{Eq. 2}$$

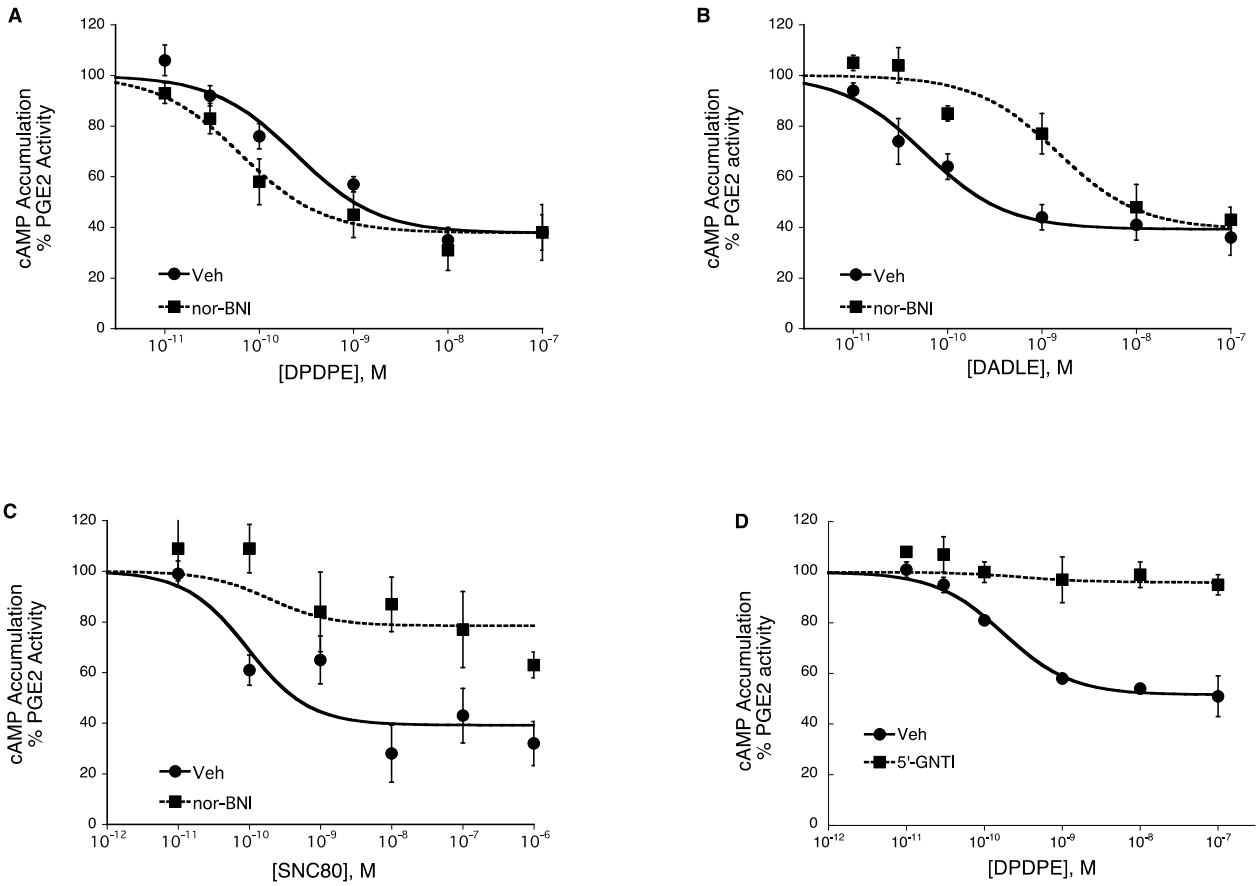
where, R is the measured response for a given agonist concentration (A), Basal is the level of PGE_2-stimulated cAMP accumulation in the absence of agonist, E_{max} is the maximal response of the system, τ is a composite term that describes the sensitivity of the system and the efficacy of the agonist to produce the response, B is the concentration of the allosteric regulator, K_A and K_B are the equilibrium dissociation constants of the agonist and the allosteric regulator for the receptor, respectively, α (cooperativity factor) is the ratio of the affinity of the agonist for the receptor in the presence and absence of the allosteric regulator, and ξ is the ratio of efficacy values (τ) of the agonist in the presence and absence of the allosteric regulator. α and ξ describe the effect of the allosteric modulator on the affinity or efficacy of the agonist, respectively.

To obtain estimates of α and ξ, concentration-response data for each agonist (DPDPE, DADLE, or SNC80) obtained in the absence of the allosteric modulator (nor-BNI or 5'-GNTI) were first fit to Equation 2 where α and ξ were constrained to 1 and basal and E_{max} were constrained to 100 to obtain estimates of τ and K_A for each agonist. These values were then used to constrain τ and K_A when fitting the concentration-response data for each agonist in the presence of nor-BNI or 5'-GNTI. For this fit, the K_B values used for nor-BNI and 5'-GNTI were 0.03 nM and 0.04 nM, respectively. To assess the effect of nor-BNI on the shift in potency of DPDPE and DADLE (where there was no change in the maximal response produced by nor-BNI, see Figures 4A and 4B), ξ was constrained to 1 to determine the effect of nor-BNI on α. For the effects of nor-BNI on SNC80 and the effect of

5'-GNTI on DPDPE where changes in efficacy were apparent (see Figures 4C and 6B), the value of α was constained to 1 to determine the value of ξ.

Supplementary Table 1: K_A, τ, and allosteric constants α and ξ calculated by fitting data shown in Figures 4 (A, B and C) and 6B to Equation 2.

Agonist	Antagonist	K_A (nM)	τ	α	ξ
DPDPE	nor-BNI	0.64	1.65	3.84	
DADLE	nor-BNI	0.14	1.55	0.029	
SNC80	nor-BNI	0.24	1.55		0.17
DPDPE	5'-GNTI	0.34	0.94		0.035



Supplementary Figure 3: Fit of DOR agonist-mediated inhibition of PGE_2-stimulated cAMP accumulation data to a mathematical model of allosterism (Equation 2). Data points shown are from Figures 4 (A, B and C) and 6B. Solid and dotted lines represent the fit of the data to the model. **A)** Values of τ and K_A for DPDPE in the absence of nor-BNI were 1.65 and 6.4 x 10^{-10} M, respectively. In the presence of nor-BNI, the value of the affinity term, α, was 3.84. **B)** Values of τ and K_A for DADLE in the absence of nor-BNI were 1.55 and 1.4 x 10^{-10} M, respectively. In the presence of nor-BNI, the value of the affinity term, α, was 0.029. **C)** Values of τ and K_A for SNC80 in the absence of nor-BNI were 1.55 and 2.4 x 10^{-10} M, respectively. In the presence of nor-BNI, the value of the efficacy term, ξ, was 0.17. **D)** Values of τ and K_A for DPDPE in the absence of 5'-GNTI were 0.94 and 3.4 x 10^{-10} M, respectively. In the presence of nor-BNI, the value of the efficacy term, ξ, was 0.035.

Supplemental References

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