Supplemental Material

A novel G protein-biased agonist at the δ opioid receptor with analgesic efficacy in models of chronic pain

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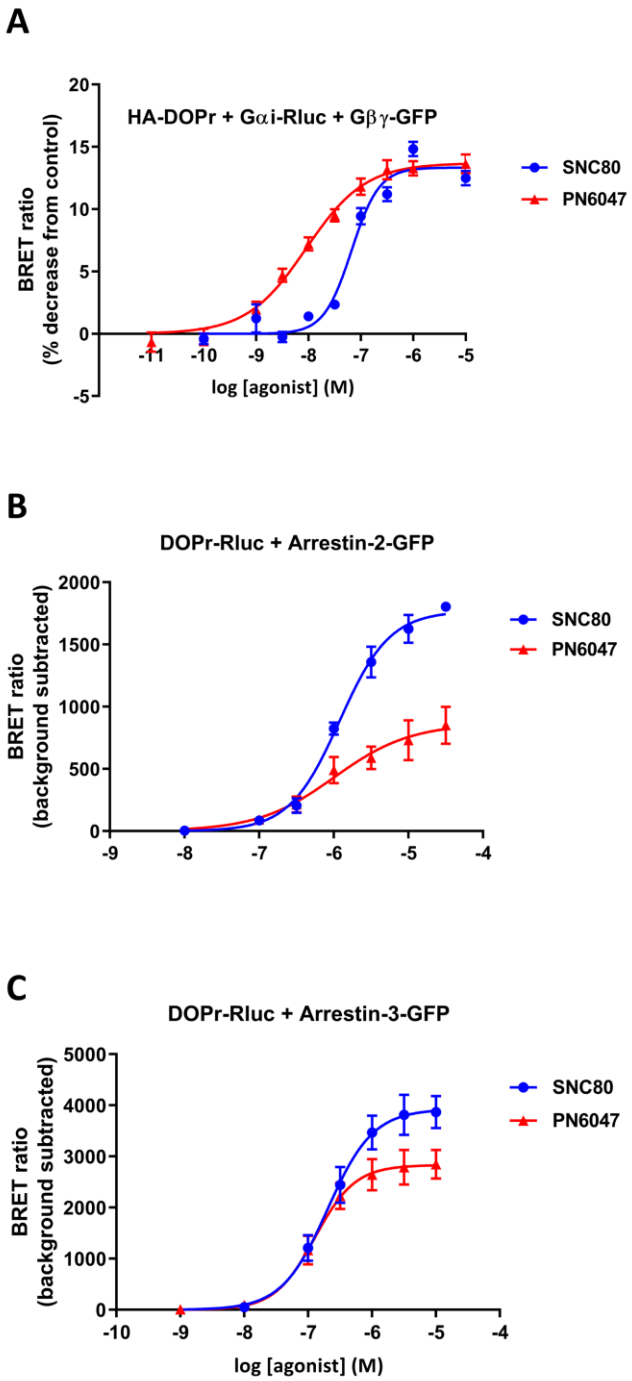
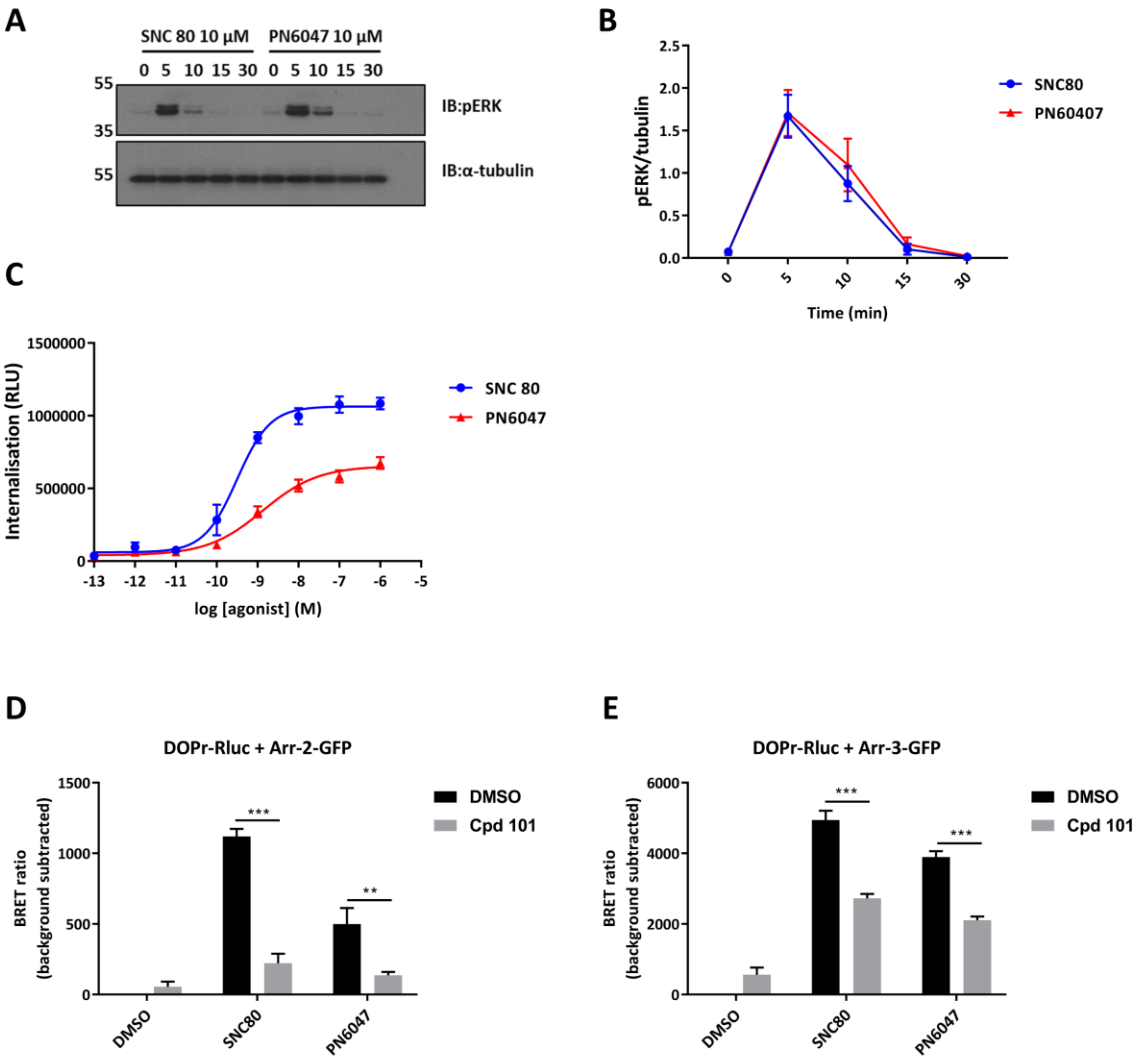
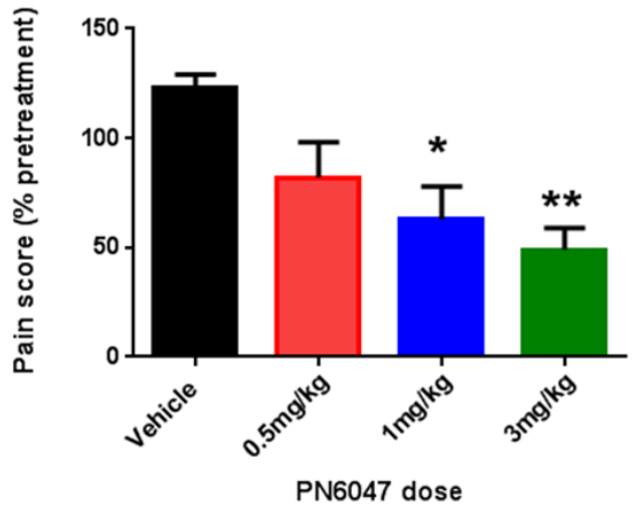


Supplemental figure 1. Comparison of SNC80 and PN6047 *in vitro* signalling profiles. Here data from figure 1 are presented showing only results for SNC80 and PN6047 to highlight the G protein bias profile of PN6047. Note the position of the PN6047 curve relative to the SNC80 curve for G protein activation *v* arrestin recruitment.

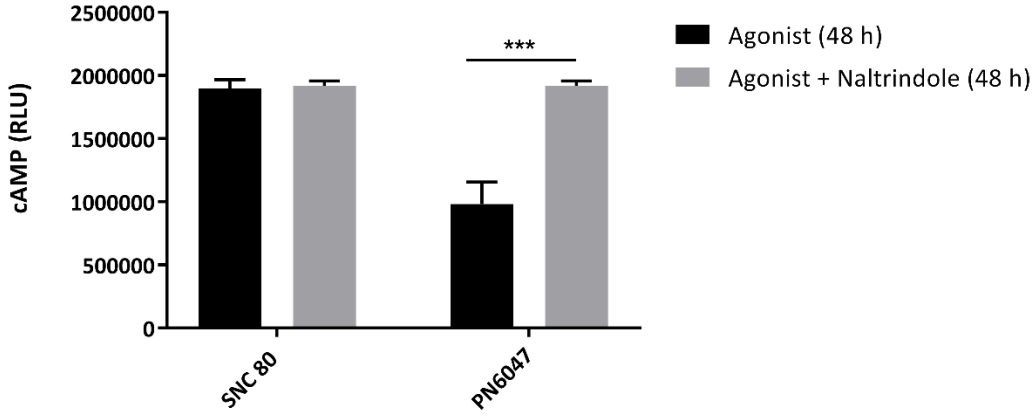


Supplemental figure 2. PN6047 is a full agonist for ERK activation but a partial agonist for internalisation. A and B) Western blot analysis of phospho-ERK signalling induced by 10 µM SNC80 or PN6047 (0-30 min) in HEK-DOR cells. A) Example Western blot B) densitometric analysis of 5 independent experiments. Data represent mean ± SEM and were analysed by two-way ANOVA with Bonferroni's *post-hoc* test. C) Concentration-dependent internalisation of δ receptor in U2OS-OPRD1 cells in response of SNC80 and PN6047. Internalisation was assessed using the PathHunter™ (DiscoverX) assay, data represent mean ± SEM (*n=6-8*). D and E) Agonist-induced arrestin recruitment is reduced by preincubation of cells with compound 101 (100 µM; 30 min), the GRK

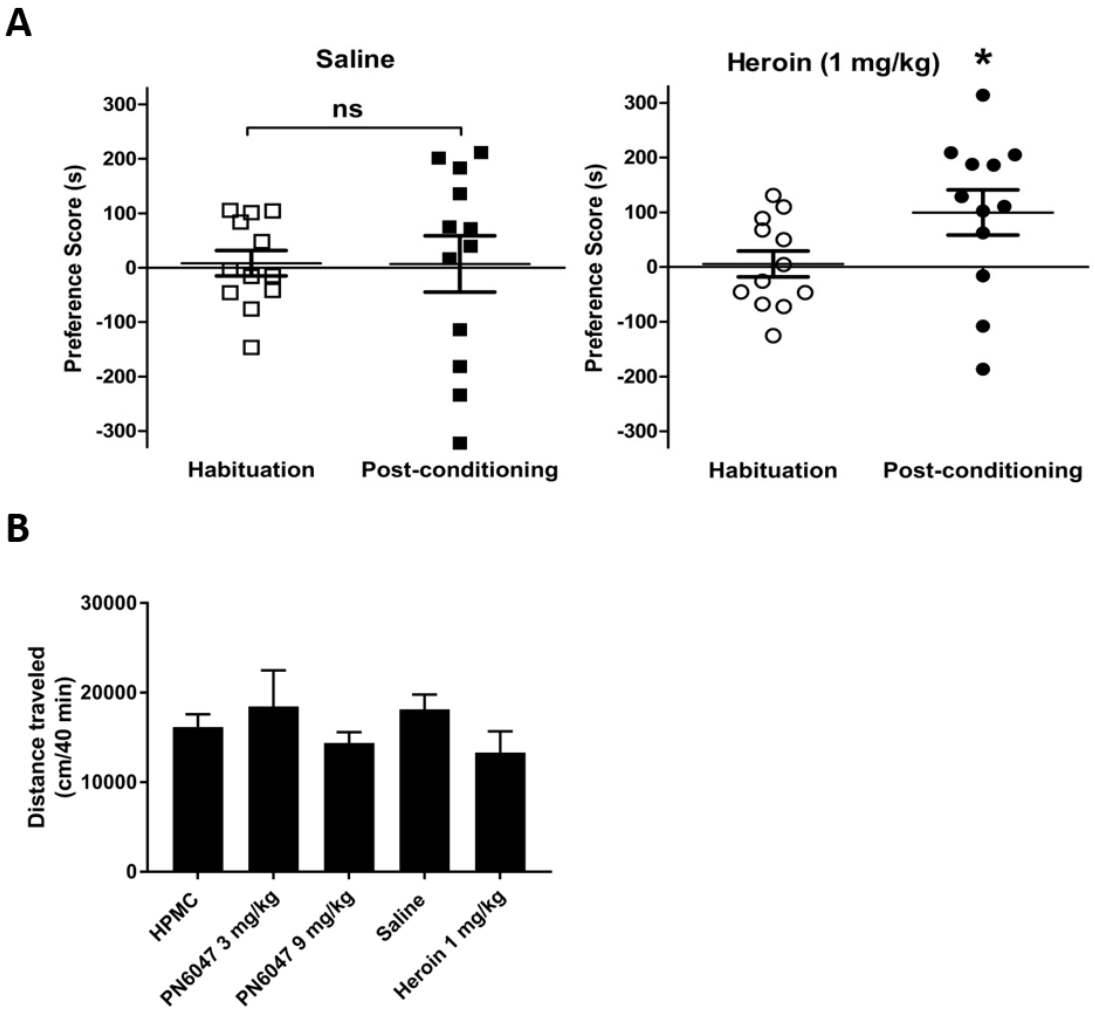
inhibitor. Arrestin-2 (D) and arrestin-3 (E) recruitment was assessed using BRET technology in HEK 293 cells transiently expressing DOR-Rluc and either arrestin-2-GFP or Arrestin-3-GFP. Cells were stimulated with either SNC80 or PN6047 (10 μM; 10 min). Data represent mean ± SEM (*n=3*) and were analysed by two-way ANOVA followed by Bonferroni's *post-hoc* test (***P<0.01* and ****P<0.0001*).



Supplemental figure 3. Dose-dependent anti-hyperalgesic action of PN6047 in the rat SNL model of neuropathic pain. Dose response of PN6047 (0.5, 1, 3 mg/kg; p.o.) induced mechanical anti-hyperalgesia in male Wistar rats (weight 237-310 g) determined 60 min post drug administration, 7 days post-surgery. Data represent mean ± SEM (*n=6-7* rats per dose) and were analysed by one-way ANOVA with Bonferroni's *post-hoc* test (**P<0.05; **P<0.01*).



Supplemental figure 4. PN6047 has no effect in an *in vitro* model of desensitisation when administered to cells for 48 hours. Agonist-induced desensitization of cAMP inhibition in CHO-K1-hDOR cells, cAMP formation was measured using the HitHunter™ (DiscoverX) assay kit. Cells were pre-treated with 1 µM SNC80 or PN6047 for 48 hrs at 37°C. After a 30 min washout period, cells were stimulated with forskolin (1 µM) and re-challenged with either 1 µM SNC80 or PN6047 ± naltrindole (1 µM) for 30 min. Data shown are the means ± SEM (*n=3*). Data were analysed by one-way ANOVA with Sidak's multiple comparison test between selected pairs (****P< 0.0001*).



Supplemental figure 5. Opioid-induced conditioned place preference and locomotion. A) Heroin-induced conditioned place preference. Preference scores for male Wistar rats conditioned with saline (s.c.) or 1 mg/kg heroin (s.c.). Data points are individual rat responses with mean ± SEM overlaid. (*n=12* rats/group) and were analysed by Student's t-test for paired comparison between post-conditioning vs habituation scores (**P<0.05*). B) Locomotor activity of vehicle (HPMC (*n=48; i.p.*) or saline (*n=36; s.c.*)), PN6047 (3 mg/kg (*n=12*) or 9 mg/kg (*n=12*); i.p.) or heroin (1 mg/kg; s.c.) treated rats. Locomotor activity is given as distance travelled in cm over a 40 min test period, during the first two days of conditioning sessions. Data represent mean ± SEM and were analysed by one-way ANOVA with Bonferroni's *post-hoc* test.