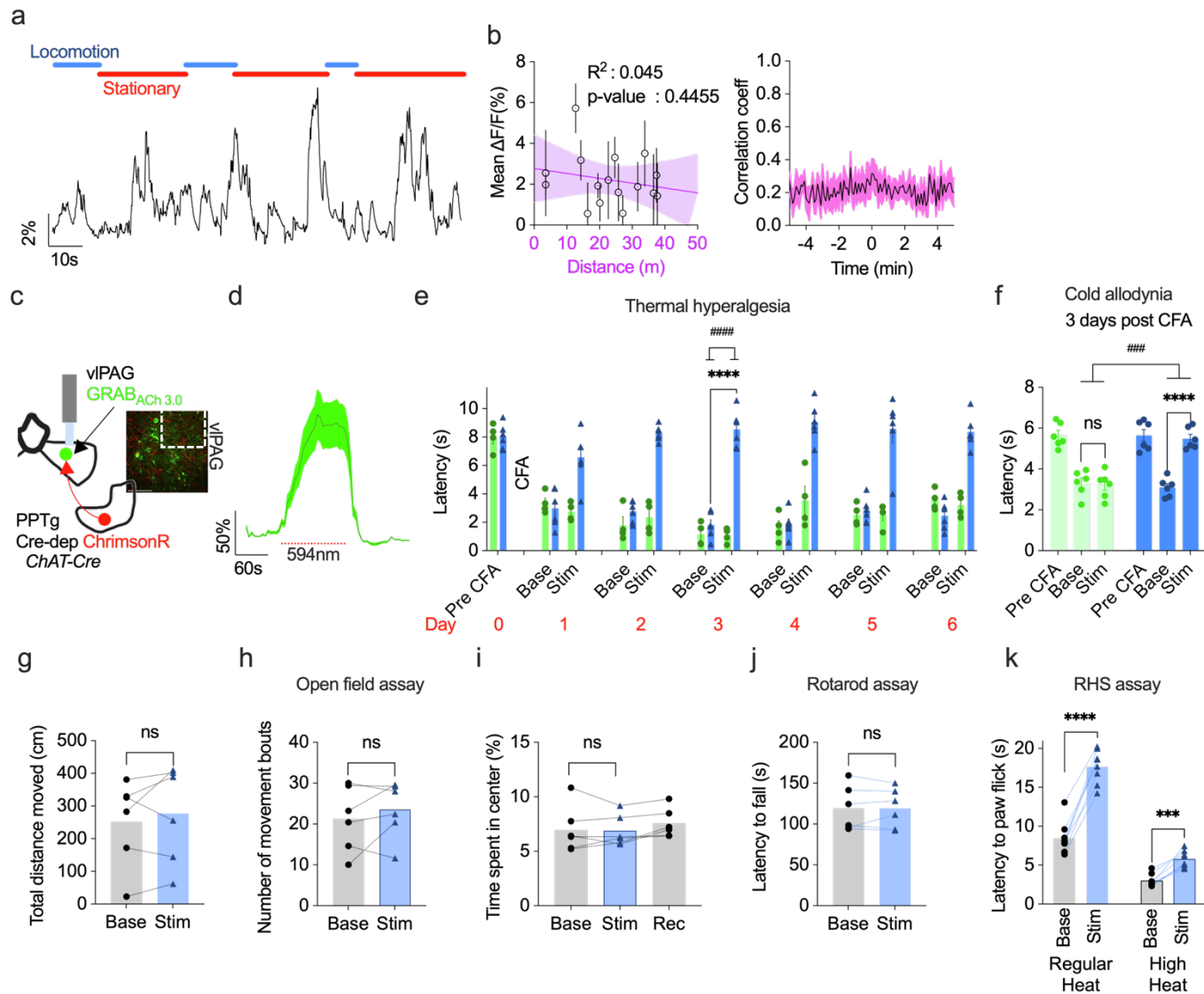


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Supplemental information

**A cholinergic circuit that relieves pain
despite opioid tolerance**

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Supplemental Fig 1: ACh levels in vIPAG are not correlated with movement. Related to Figures 1 and 2.

a) Representative recording of vIPAG GRAB_{ACh 3.0} fluorescence recorded using fiber photometry during open field behavior. Behavior was captured using 2 orthogonally positioned cameras and analyzed manually and using Ethovision (see Methods). Locomotion and stationary phases are annotated at the top in blue and red, respectively.

b) Left: Mean vIPAG GRAB_{ACh 3.0} fluorescence in 5 min time bins during open field behavior is plotted on the y-axis and the corresponding distance travelled during the same 5 min time bin is plotted on the x-axis. Each point represents data from a single mouse. n=3 mice. Right: Correlogram between distance moved in 10s-time bin and mean fluorescence during the same time bin. n=3 mice.

c) Left: Schematic of viral ChrimsonR-tdTomato expression in PPTg^{ChAT+} neurons and pan-neuronal viral GRAB_{ACh 3.0} expression in the vIPAG. Right: Fluorescence image of the vIPAG showing expression of ChrimsonR-tdTomato in PPTg^{ChAT+} terminals (red) and GRAB_{ACh 3.0} (green, scale bar 50 μ m).

d) Mean vIPAG GRAB_{ACh 3.0} fluorescence (green) time locked to optogenetic activation of PPTg^{ChAT+} terminals (red dotted line) collected using simultaneous optogenetics and fiber photometry (2 traces per animal, n=2).

e) Latency to paw withdrawal in RHS assay conducted before (Pre CFA) and after intraplantar CFA injection and optogenetic activation of PPTg^{ChAT+} terminals in the vIPAG. Latencies before and after optogenetic activation are represented as 'Base' and 'Stim', respectively, in Chr2-expressing (blue) and GFP control (green) mice. n=4 in GFP group and n=6 in Chr2 group.

****p<0.0001 paired t-test, #####p<0.0001 interaction RM 2-way ANOVA.

f) Latency to paw withdrawal in cold allodynia assay before (Pre CFA) and after injection of CFA in ChR2 expressing (blue) and GFP control groups (green) during baseline (Base) and after optogenetic activation (Stim) of PPTg^{ChAT+} terminals in the vIPAG. n=6 per group.

****p<0.0001 paired t-test, ###p<0.001 interaction RM 2-way ANOVA.

g) Total distance moved in an open field assay during no-stimulation baseline (Base) and during optogenetic activation of PPTg^{ChAT+} terminals in the vIPAG (Stim). n=6 per group.

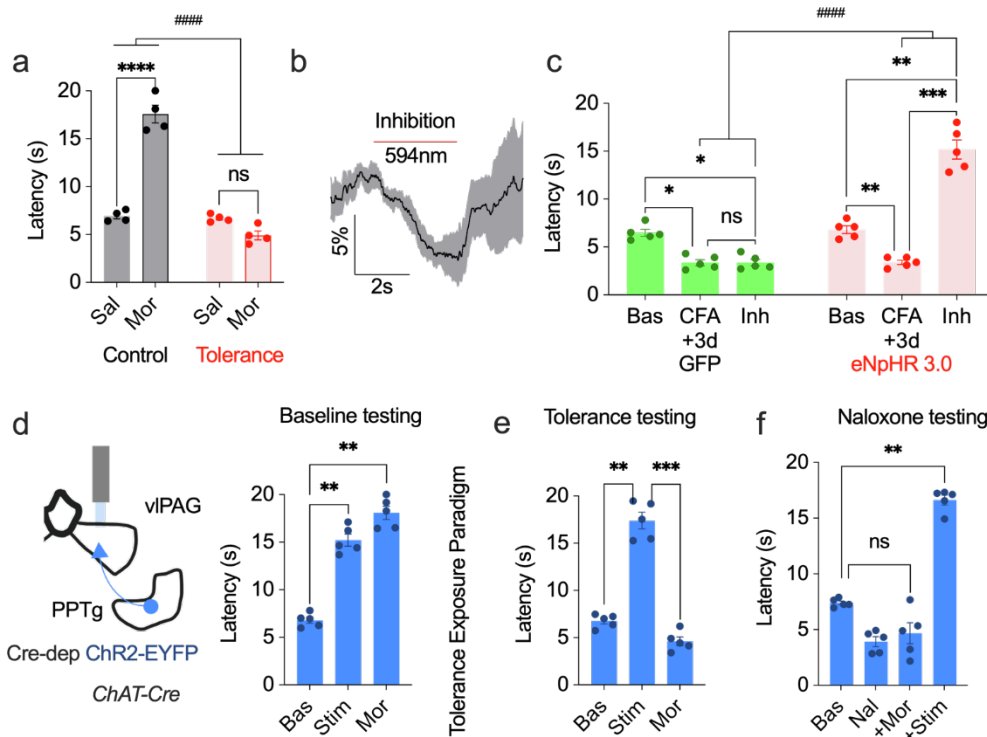
h) Number of movement bouts in an open field assay during no-stimulation baseline (Base) and during optogenetic activation of PPTg^{ChAT+} terminals in the vIPAG (Stim). n=6 per group.

i) Time spent in the center in an open field assay during no-stimulation baseline (Base), during optogenetic activation of PPTg^{ChAT+} terminals in the vIPAG (Stim) and post-activation recovery (Rec). n=6 per group.

j) Latency to fall in a rotarod assay during no-stimulation baseline (Base) and after optogenetic activation of PPTg^{ChAT+} terminals in the vIPAG (Stim). n=6 per group.

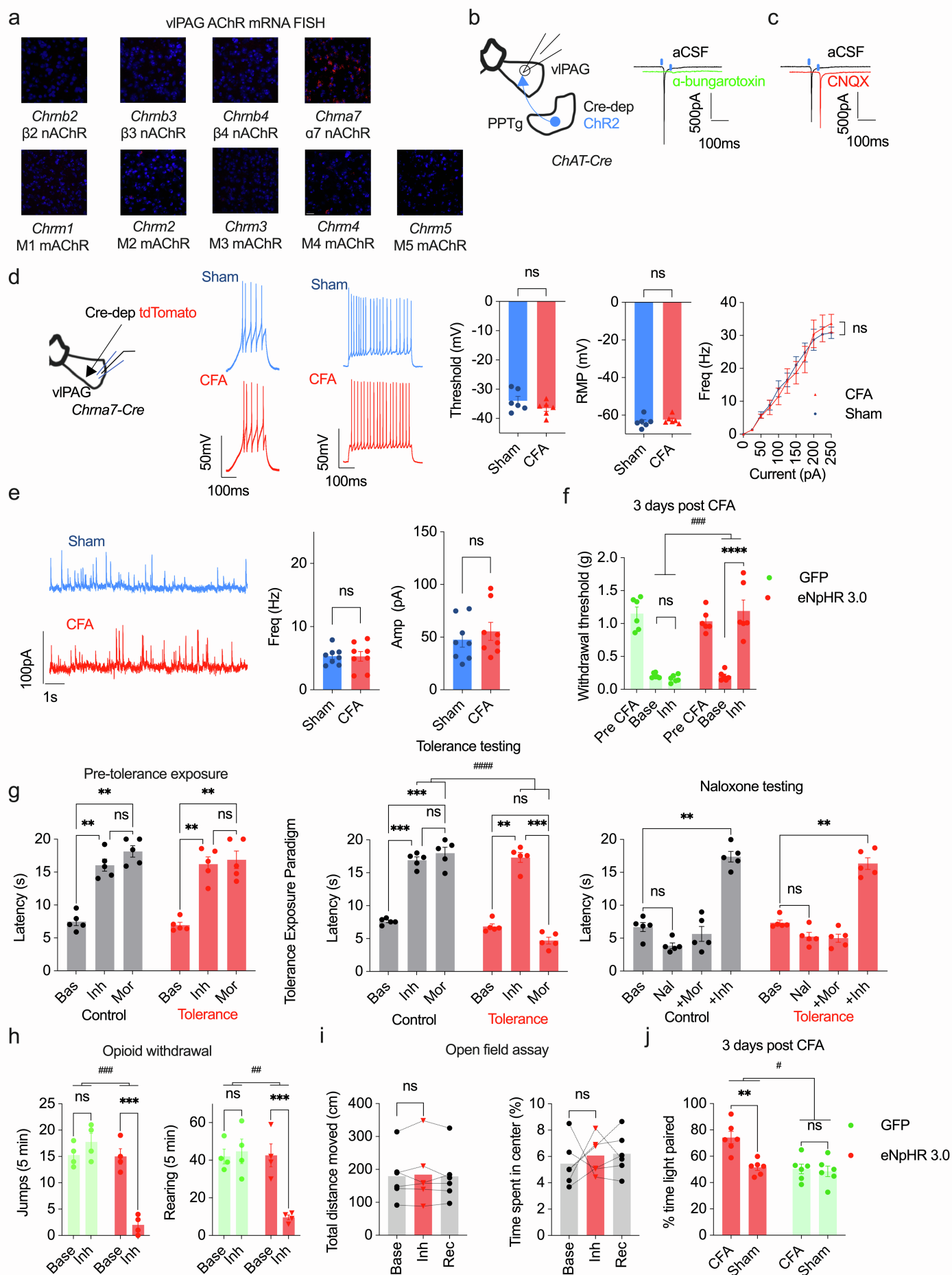
k) Latency to paw withdrawal in RHS assay in regular heat source intensity (Regular Heat) and high heat source intensity (High Heat) in ChR2 expressing groups during baseline (Base) and optogenetic activation (Stim) of PPTg^{ChAT+} terminals in the vIPAG. n=6 per group.

****p<0.0001, ***p<0.001 paired t-test.



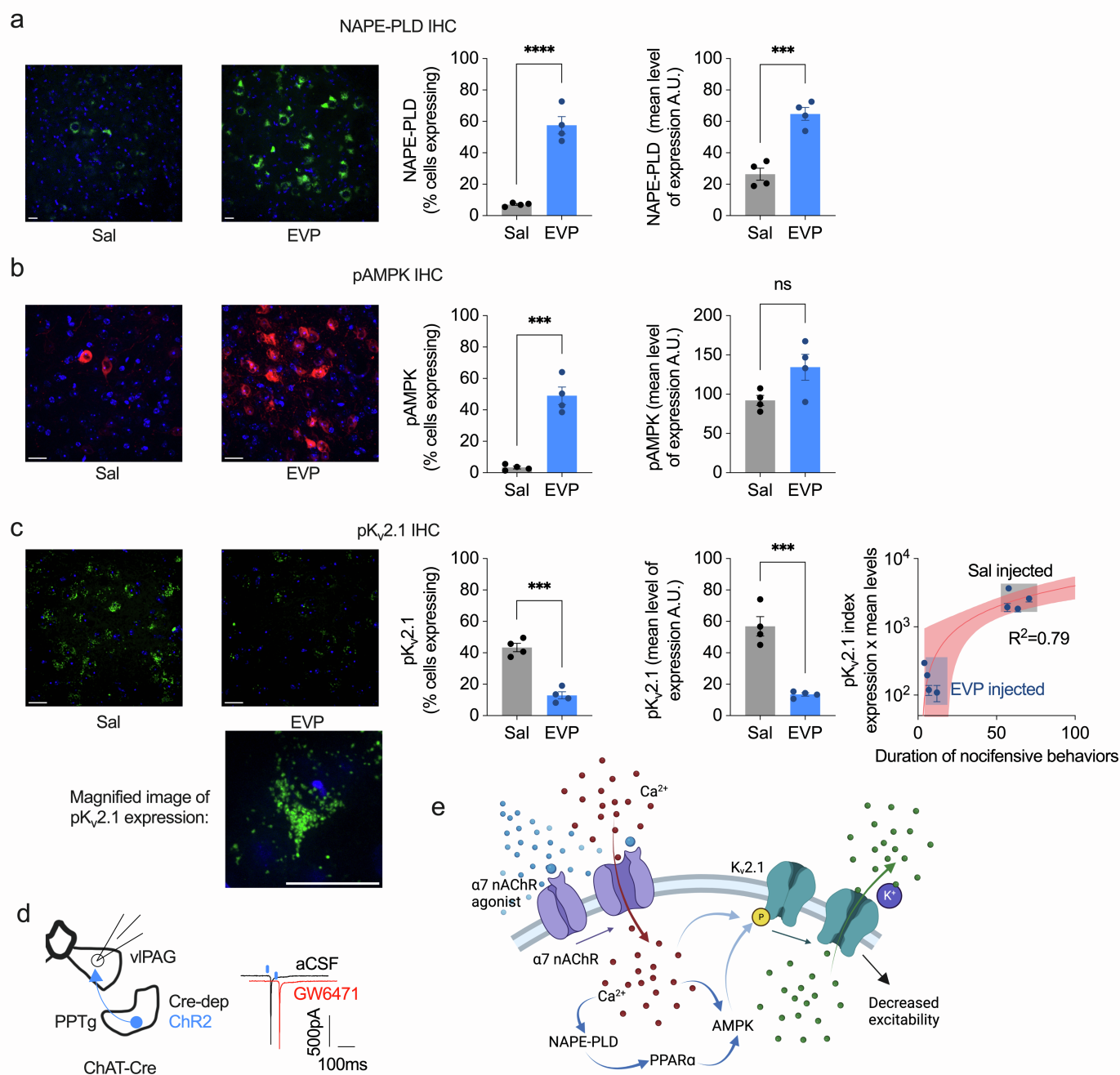
Supplemental Fig 2: Inhibiting vIPAG^{Oprm1+} or activating PPTg^{ChAT+}→vIPAG projections is antinociceptive under baseline and opioid-tolerant conditions. Related to Figures 2 and 3.

- a) Latency to paw withdrawal in RHS assay after opioid tolerance post saline (Sal) and morphine (10mg/kg, Mor) administration in control (grey) and tolerance (red) groups. n=4 per group. ****p<0.0001 paired t-test, #####p<0.0001 interaction RM 2-way ANOVA.
- b) Mean vIPAG^{Oprm1+} GCaMP6 fluorescence (black trace) collected using simultaneous optogenetics and fiber photometry time locked to optogenetic inhibition of vIPAG^{Oprm1+} neurons (1 trace per animal, n=8).
- c) Latency to paw withdrawal before (Bas) and after intraplantar CFA injection (CFA and Inh) in mice expressing GFP (green) or eNpHR 3.0 (red) in vIPAG^{Oprm1+} neurons. CFA+3d refers to 3-days post intraplantar CFA injection and Inh refers to optogenetic inhibition of vIPAG^{Oprm1+} neurons. n=4 per group. *p<0.05, **p<0.01, ***p<0.001 paired t-test, ###p<0.001 interaction RM 2-way ANOVA.
- d) Left: Schematic showing Cre-dependent ChR2 expression in PPTg of *ChAT-Cre* animals and cannula placement in the vIPAG. Right: Latency to paw withdrawal in RHS assay before opioid tolerance under baseline conditions (Bas), during optogenetic stimulation (Stim) and after morphine injection (Mor). n=5 per group.
- e) Latency to paw withdrawal in RHS assay after opioid tolerance under baseline conditions (Bas), during optogenetic stimulation (Stim) and after morphine injection (Mor). n=5 per group.
- f) Latency to paw withdrawal in RHS assay after opioid tolerance and systemic naloxone injections under baseline conditions (Bas), after naloxone (Nal) and morphine administration (Mor), and during optogenetic stimulation (Stim). n=5 per group. **p<0.01, ***p<0.001 paired t-test.



Supplemental Fig 3: Functional cholinergic synaptic transmission between PPTg^{ChAT+} and vIPAG is mediated through $\alpha 7$ nAChRs and exogenous inhibition of vIPAG^{Chrna7+} neurons is antinociceptive. Related to Figures 4 and 5.

- a) Representative fluorescence images of AChR mRNA expression (red) in the vIPAG using fluorescence in situ hybridization with nuclear DAPI stain (blue, common scale bar: 20 μ m).
- b) Left: Slice electrophysiology schematic. ChR2-expressing PPTg^{ChAT+} terminals were activated during voltage clamp recordings (-70mV) from vIPAG neurons to monitor optogenetically-evoked synaptic currents. Right: Representative traces demonstrating PPTg^{ChAT+} terminal activation-evoked inward current is blocked by bath application of $\alpha 7$ nAChR antagonist α -bungarotoxin (100nM, +20 min).
- c) Representative traces demonstrating PPTg^{ChAT+} terminal activation-evoked inward current that is not altered by bath application of AMPAR antagonist CNQX (20 μ M).
- d) Left to right: Slice electrophysiology schematic. vIPAG^{Chrna7+} neurons were fluorescently labeled with tdTomato using viral surgeries. Representative voltage traces in response to ramp and step current injections 3-days after intraplantar sham (blue) or CFA (red) injections. Action potential threshold and resting membrane potential (RMP) after Sham or CFA injections. Current-firing rate relationship after Sham or CFA injections. n= 6 neurons recorded from 4 mice for Sham group and 6 neurons recorded from 5 mice for CFA group.
- e) Left to right: Representative spontaneous IPSC traces recorded from vIPAG^{Chrna7+} neurons in voltage clamp (0mV, Cl⁻ reversal \sim -70mV) 3-days after intraplantar sham (blue) or CFA (red) injections. Mean sIPSC amplitude and frequency monitored from vIPAG^{Chrna7+} neurons. n= 8 neurons recorded from 4 mice for Sham group and 8 neurons recorded from 3 mice for CFA group.
- f) Paw withdrawal threshold in von Frey assay before (Pre CFA) and after injection of CFA in GFP control (green) and eNpHR 3.0 expressing groups (red) during baseline (Base) and after optogenetic inhibition (Inh) of vIPAG^{Chrna7+} neurons. n=6 per group.
****p<0.0001 paired t-test, ###p<0.001 interaction RM 2-way ANOVA.
- g) Left: latency to paw withdrawal in RHS assay before opioid tolerance under baseline conditions (Bas), during optogenetic inhibition (Inh) and after morphine injection (Mor) in control (gray) and tolerance group (red).
Middle: Latency to paw withdrawal in RHS assay after opioid tolerance under baseline conditions (Bas), during optogenetic inhibition (Inh) and after morphine injection (Mor) in control (gray) and tolerance group (red).
Right: Latency to paw withdrawal in RHS assay after opioid tolerance and systemic naloxone injections under baseline conditions (Bas), after naloxone (Nal) and morphine administration (Mor), and during optogenetic Inhibition (Inh) in control (gray) and tolerance group (red). n=5 per group.
p<0.01, *p<0.001 paired t-test, #####p<0.0001 interaction RM 2-way ANOVA.
- h) Opioid exposed animals (6 days, 2x/day 10 \rightarrow 30mg/kg) were administered naloxone and subsequent jumping (left) and rearing (right) behaviors were monitored in GFP (green) and eNpHR 3.0 (red) expressing mice before (Base) and after optogenetic inhibition (Inh) of vIPAG^{Chrna7+} neurons.
***p<0.001 paired t-test, ##p<0.01, ###p<0.001 interaction RM 2-way ANOVA.
- i) Total distance moved (left) and time spent in center (right) in an open field assay during no-stimulation baseline (Base), during optogenetic inhibition of vIPAG^{Chrna7+} neurons (Inh), and during post inhibition recovery. n=6 per group.
- j) Time spent by mice in optogenetic inhibition-paired chamber. eNpHR 3.0 expressing mice are shown in red, GFP expressing mice in green. n=6 per group.
**p<0.01 paired t-test, #p<0.05 interaction RM 2-way ANOVA.



Supplemental Fig 4: $\alpha 7$ nAChR activation inhibits vIPAG^{ChRNA7+} neurons to produce antinociceptive effects. Related to Figure 6.

a) Left: Representative fluorescence images showing NAPE-PLD expression in the vIPAG after saline or EVP-6124 (0.3mg/kg) administration (scale bar 10 μ m). Right: Mean percent of cells showing NAPE-PLD expression and mean level of expression per cell after saline and EVP-6124 administration. n=4 per group.

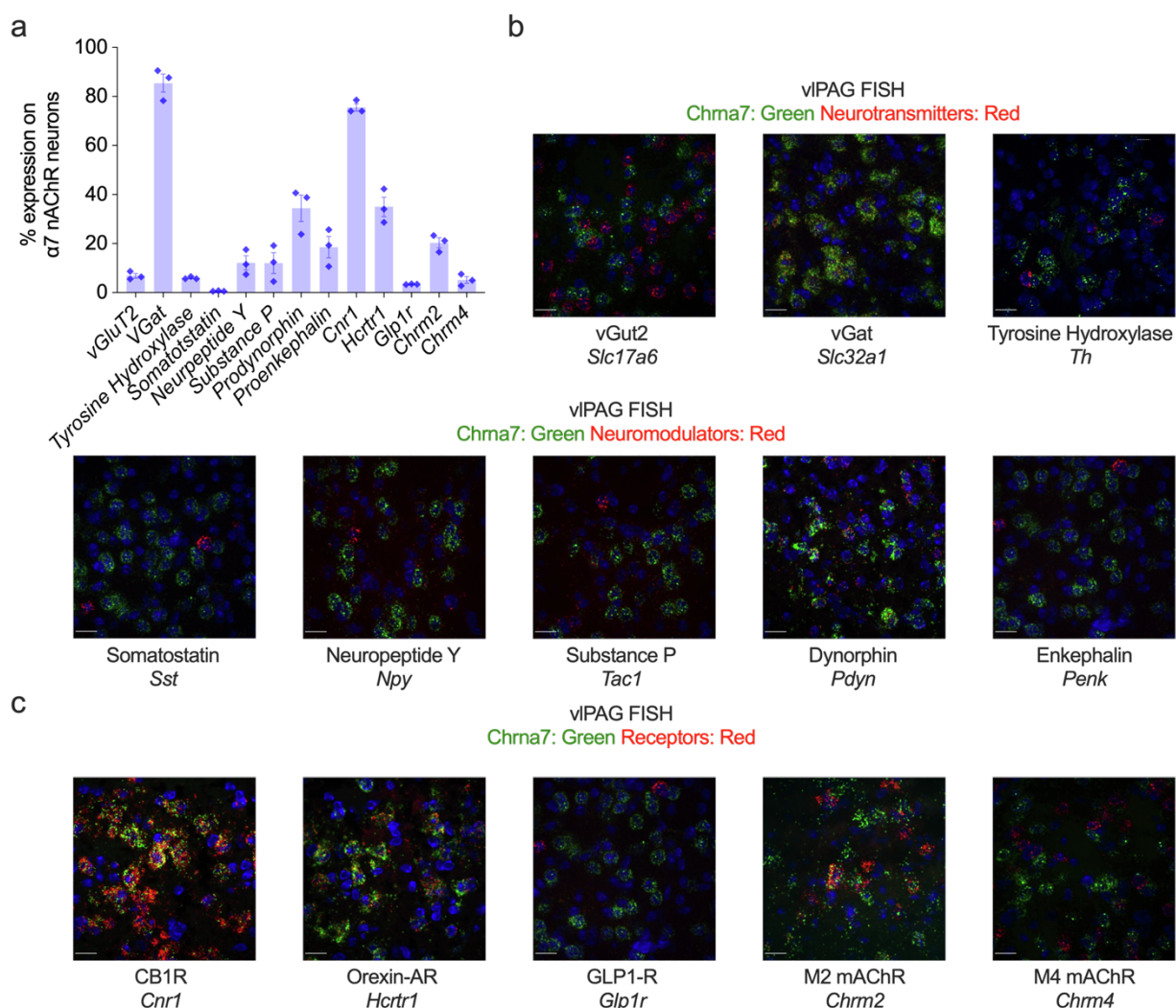
b) Left: Representative fluorescence images showing phosphorylated AMPK expression in the vIPAG after saline or EVP-6124 (0.3mg/kg) administration (scale bar 10 μ m). Right: Mean percent of cells showing pAMPK expression and mean level of expression per cell after saline and EVP-6124 administration. n=4 per group.

c) Left: Representative fluorescence images showing phosphorylated K_v2.1 expression in the vIPAG after saline or EVP-6124 (0.3mg/kg) administration (scale bar 10 μ m). Right: Mean percent of cells showing pK_v2.1 expression and mean level of expression per cell after saline and EVP-6124 administration. Correlation curve between pK_v2.1 index and duration of nocifensive behaviors in the formalin assay. n=4 per group.

d) Left: Slice electrophysiology schematic: ChR2-expressing PPTg^{ChAT+} terminals were activated during voltage clamp recordings (-70mV) from vIPAG neurons to monitor optogenetically evoked synaptic currents. Right: Representative traces demonstrating PPTg^{ChAT+} terminal activation-evoked inward current is unaltered by bath application of PPAR α antagonist GW 6471 (100nM).

e) Schematic depicting the proposed intracellular signaling mediating the observed decrease in neuronal excitability post $\alpha 7$ nAChR activation.

p<0.001, *p<0.0001 unpaired t-test.

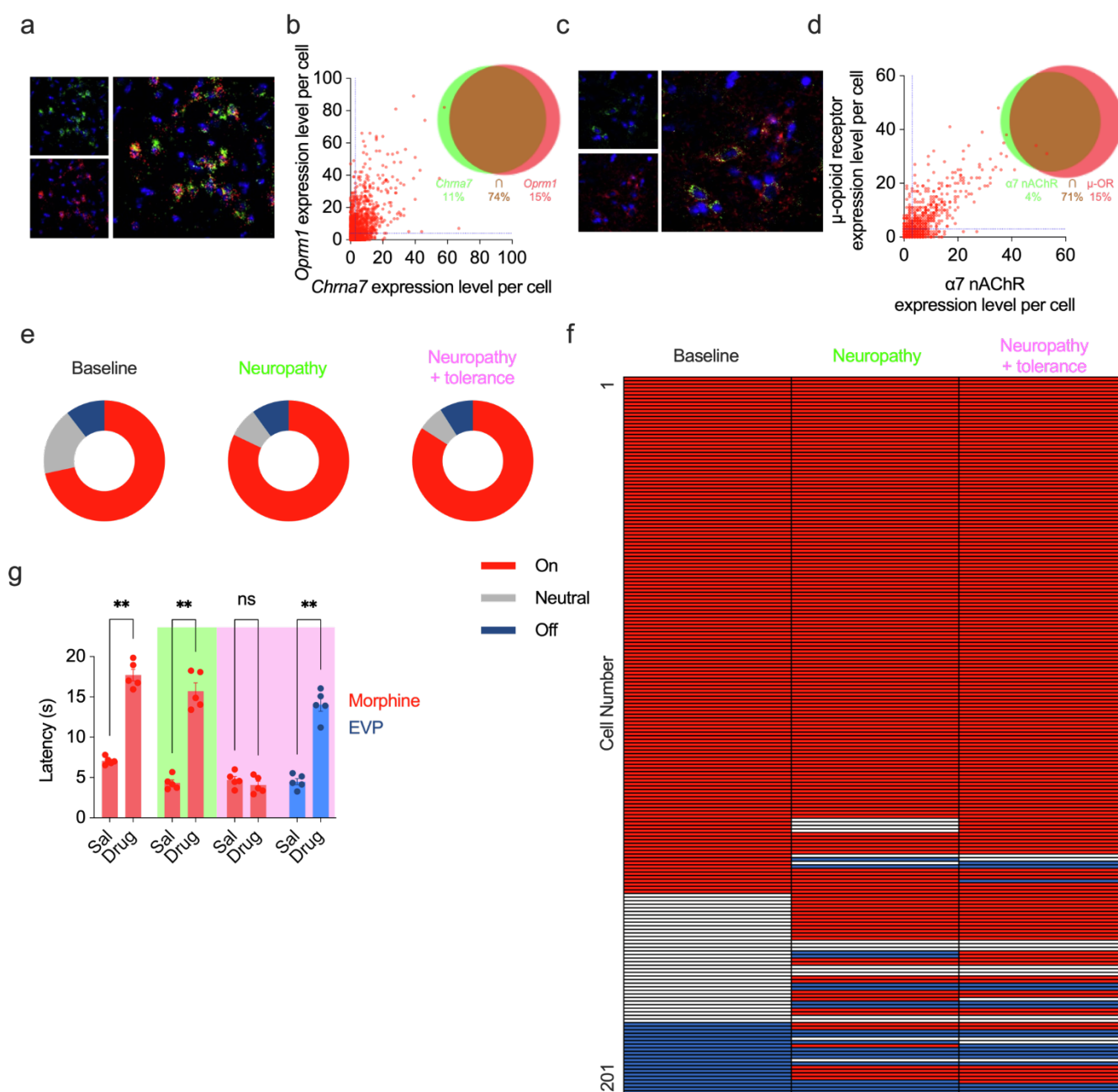


Supplemental Fig 5: α7 nAChRs are expressed on GABAergic vIPAG neurons. Related to Figure 7.

a) Percent co-expression of α7 nAChR mRNA on vIPAG neurons expressing common neurotransmitters, modulators and receptors explored using fluorescence *in situ* hybridization (FISH). Six representative vIPAG images were taken per mouse. From each image, the percent of cells positive for both α7 nAChR and the other mRNA were calculated and then averaged across all 6 images. n=3.

b) Representative images showing co-expression of α7 nAChR mRNA (green) with neurotransmitters (top, red) and neuromodulators (bottom, red) with nuclear DAPI stain (blue, scale bar 20μm).

c) Representative images showing co-expression of α7 nAChR mRNA (green) with receptors (red) with nuclear DAPI stain (blue, scale bar 20μm).



Supplemental Fig 6: $\alpha 7$ nAChR agonist inhibit opioid-responsive and pain-encoding ensembles to relieve pain. Related to Figure 8.

a) Representative fluorescence *in situ* hybridization images showing co-expression of *Chrna7* (green) and *Oprm1* (red) mRNA with nuclear DAPI stain (blue).

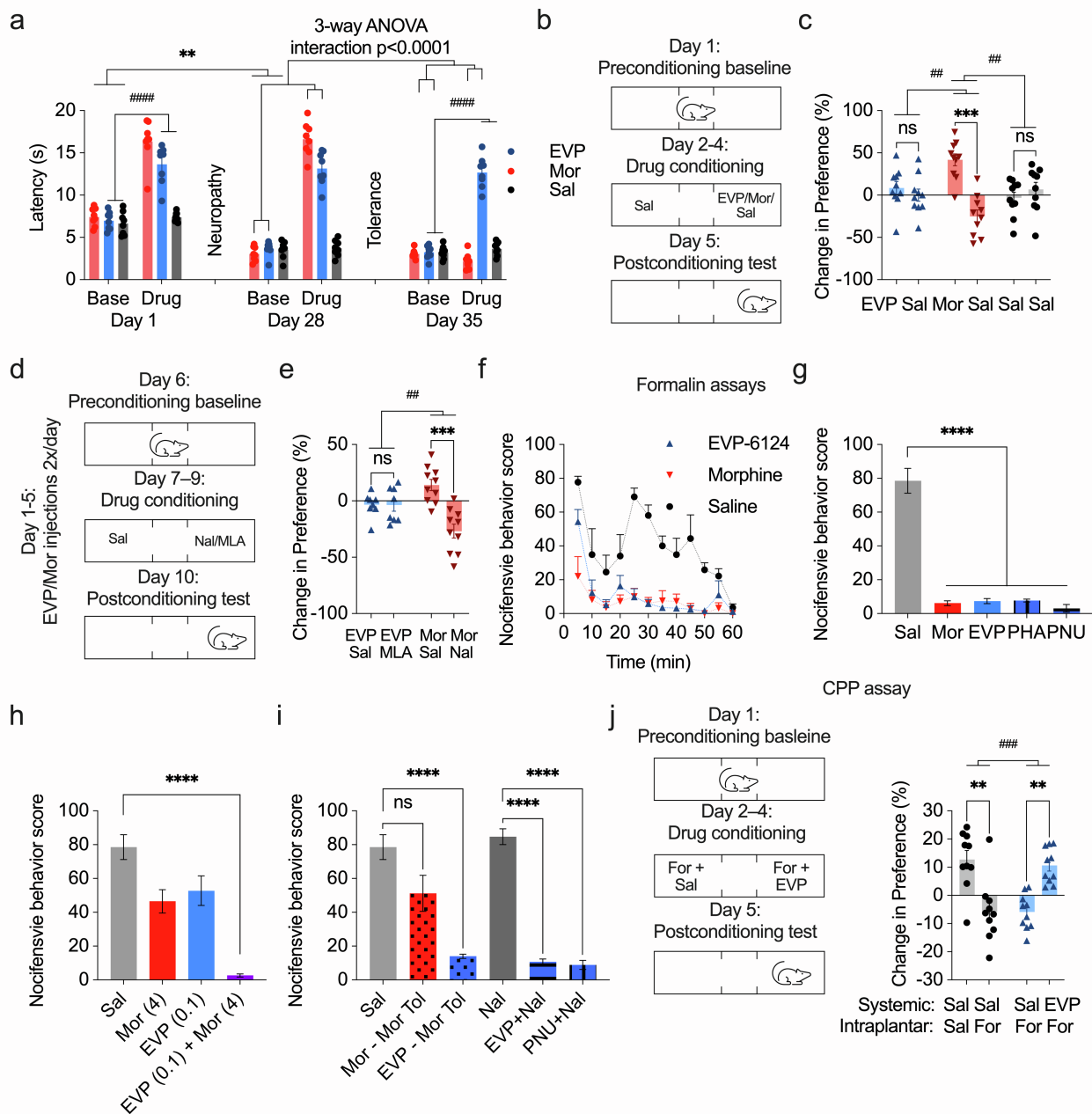
b) Expression levels per cell for *Oprm1* mRNA plotted against *Chrna7* expression levels. Inset shows a Venn diagram demonstrating 74% overlap in mRNA expression on cells. n=1562 cells from 3 animals.

c) Representative fluorescence image with antibody staining for μ -opioid receptors (red) and fluorescently conjugated α -bungarotoxin stain for $\alpha 7$ nAChRs (green) with nuclear DAPI stain (blue).

d) Expression levels per cell for μ -opioid receptors and $\alpha 7$ nAChRs. Inset shows a Venn diagram demonstrating 71% overlap in protein expression on cells. n=1481 cells from 3 animals.

e) Pie charts demonstrating progression of neuronal responses to noxious stimuli across baseline, neuropathic pain and tolerance. Neurons with a statistically significant increase in activity (bootstrap method) in response to a noxious stimuli are termed 'On' cells (red). Neurons with a statistically significant decrease in activity are termed 'Off' cells (blue). Remaining neurons are termed 'Neutral' (gray).

- f) Progression of individual cellular identity across different phases of testing. Similar labeling used as panel Supplemental Fig 6e.
- g) Latency to paw withdrawal for the mice undergoing *in vivo* imaging experiments after drug injections (morphine: red, EVP-6124: blue) and across different pathological states of neuropathy and opioid tolerance. n=5.
- **p<0.01 paired t-test.



Supplemental Fig 7: Targeting $\alpha 7$ nAChRs relieves the somatic and affective components of pain, without tolerance, reward, or withdrawal phenotype. Related to Figure 8.

a) Latency to paw withdrawal in RHS assay is plotted before (Base) and after drug administration (Drug) of morphine (red, 10mg/kg), EVP-6124 (blue, 0.3mg/kg), or saline (gray). Tests were conducted before (Day 1) and after chronic neuropathic pain state (Day 28), and after the induction of opioid tolerance. The baseline, neuropathic pain, and tolerance assays were conducted on Days 1, 28 and 35 respectively. $n=8$ per group.

** $p < 0.01$ paired t-test, **** $p < 0.0001$, ##### $p < 0.0001$ interaction RM 2-way ANOVA.

b) Schematic showing conditioned place preference paradigm for reward phenotype. On Day 1, preconditioning baseline preference was tested. On Days 2-4, mice were conditioned to EVP-6124 (blue), morphine (red) or saline (gray) in the drug-paired chamber and saline was administered in the other chamber. On Day 5, post-conditioning preference for the drug- and saline-paired chamber was evaluated.

c) Percent change in preference on post-conditioning day as compared to preconditioning baseline for the drug-paired or the saline-paired chambers. Saline control group is in gray, morphine group in red and EVP-6124 group in blue. n=10 per group.

*** $p < 0.001$ paired t-test, ### $p < 0.01$ interaction RM 2-way ANOVA.

d) Schematic showing conditioned place preference paradigm for withdrawal phenotype. On Days 1-5, EVP-6124 (0.3mg/kg) or morphine (10mg/kg) was administered 2x/day. On Day 6, preconditioning baseline preference was established. On Days 7-8, mice were conditioned to MLA (blue), or naloxone (red) in the drug-paired chamber and saline was administered in the other chamber. Mice received EVP-6124 and morphine in both chambers. On Day 10, post-conditioning preference for the drug- and saline-paired chamber was evaluated.

e) Percent change in preference on post-conditioning day as compared to preconditioning baseline for the drug-paired or the saline-paired chambers. EVP-6124 and MLA group is in blue and morphine and naloxone group is in red. n=8 in EVP-MLA group, 10 in Mor-Nal group.

*** $p < 0.001$ paired t-test, ### $p < 0.01$ interaction RM 2-way ANOVA.

f) Nocifensive behavior score (% time spent in 5 min time bin licking or lifting paw) during formalin assay after EVP-6124 (blue, 0.3mg/kg), morphine (red, 10mg/kg) and saline injections. n=5 per group.

g) Mean nocifensive behavior score during the tonic inflammatory phase (Phase 2) of the formalin assay after saline (gray), morphine (red, 10mg/kg), EVP-6124 (blue, 0.3mg/kg), PHA (blue with vertical stripes, 10mg/kg), PNU (blue horizontal stripes, 10mg/kg) administration. n=4 per group. Common saline group used in subsequent figures and analysis.

**** $p < 0.0001$ unpaired t-test.

h) Mean nocifensive behavior score during the tonic inflammatory phase (Phase 2) of the formalin assay after low-dose morphine (red, 4mg/kg), low-dose EVP-6124 (blue, 0.1mg/kg), and the combination dose (purple). n=4 per group.

**** $p < 0.0001$ unpaired t-test.

i) Mean nocifensive behavior score during the tonic inflammatory phase (Phase 2) of the formalin assay after morphine administration in morphine tolerant mice (red with dots), EVP-6124 administration in morphine tolerant mice (blue with dots), mice injected with naloxone (6mg/kg grey with horizontal stripes), EVP-6124 administration after naloxone (blue with horizontal stripes) and PNU-120596 after naloxone (blue with vertical stripes). n=4 per group.

**** $p < 0.0001$ unpaired t-test.

j) Left: Schematic showing conditioned place preference paradigm for affective pain testing. On Day 1, preconditioning baseline preference was established. On Days 2-4, test mice (blue) were conditioned to intraplantar formalin injections in both chambers, but in one chamber they received systemic EVP-6124 (0.3mg/kg) and in saline in the other. The control mice (gray) received intraplantar formalin in one chamber and intraplantar saline in the other but received systemic saline in both chambers. On Day 5, post-conditioning preference for the drug- and saline-paired chamber was evaluated. Right: Percent change in preference on post-conditioning day as compared to preconditioning baseline for the EVP-6124 and saline-paired chambers. Test group is in blue and control group is in gray. n=10 per group.

** $p < 0.01$ paired t-test, ### $p < 0.001$ interaction RM 2-way ANOVA.