Supplementary Information

Loss of μ-opioid receptor signaling in nociceptors, and not spinal microglia, abrogates morphine tolerance without disrupting analgesic efficacy

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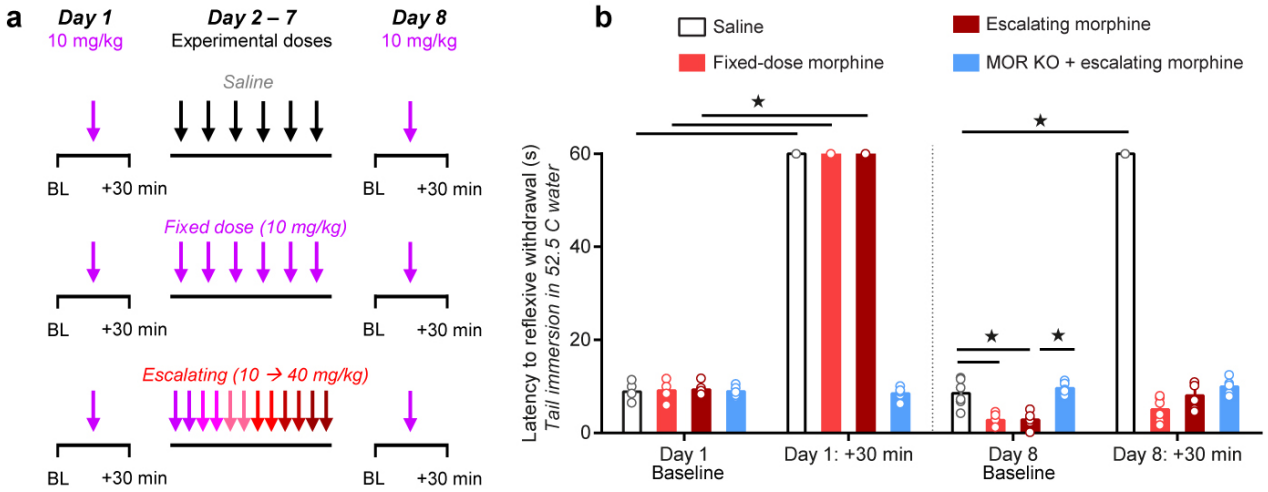
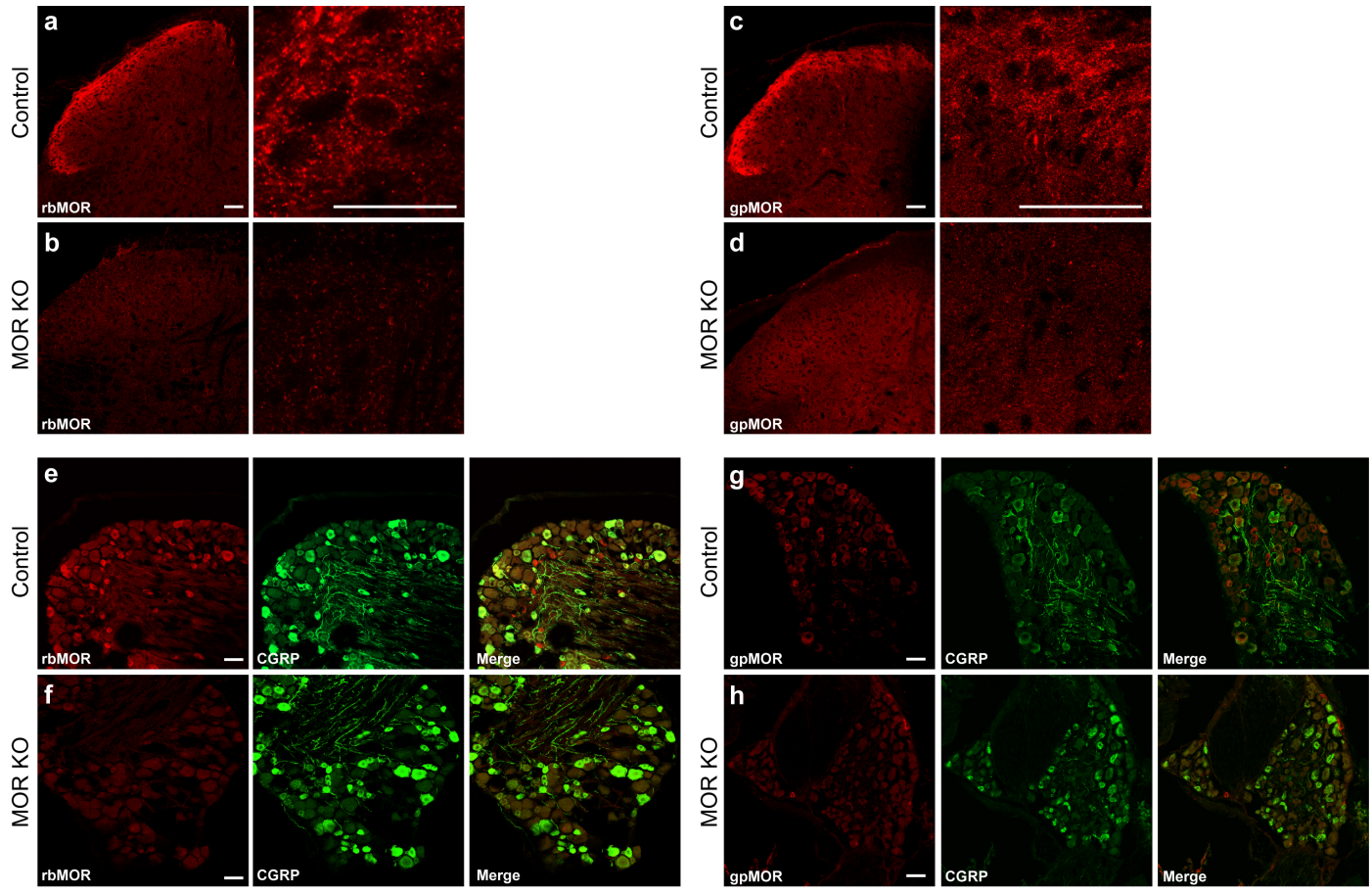
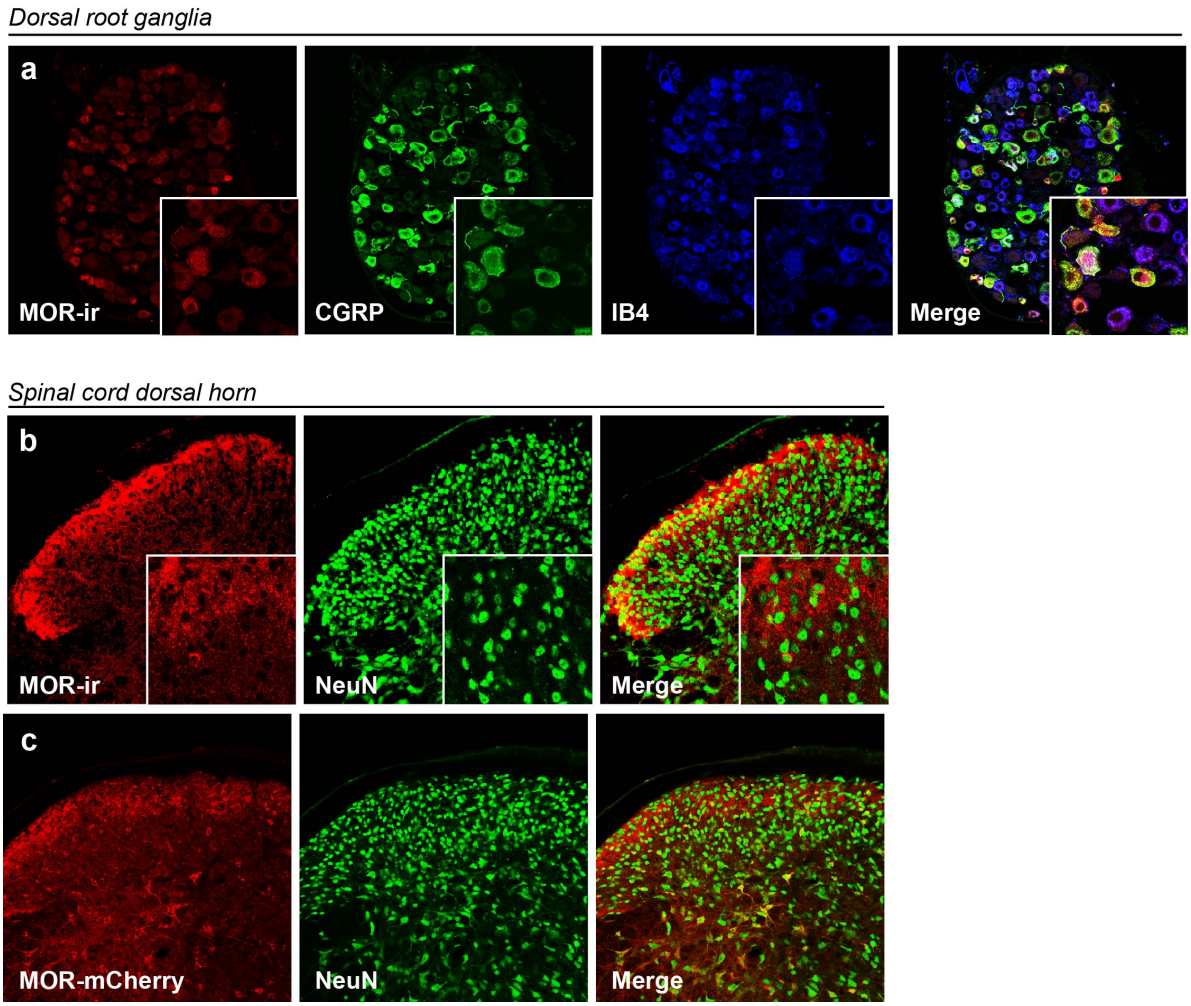
Supplementary Figures



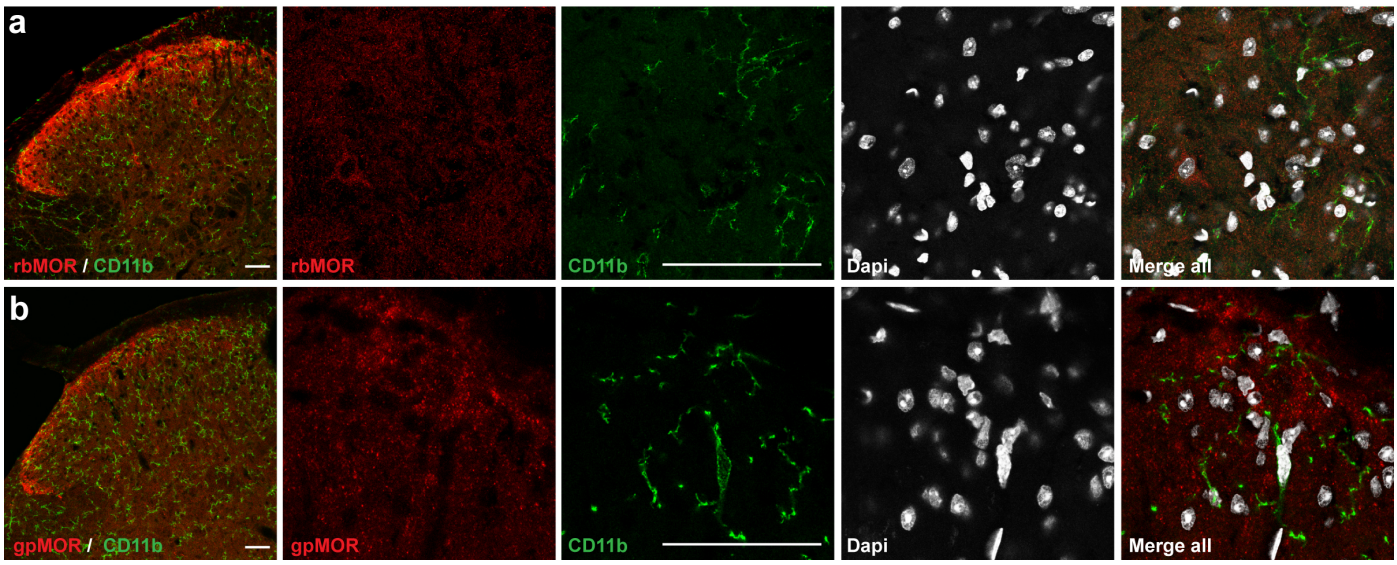
Supplementary Figure 1. Morphine dosing schedules and behavioral testing timecourse. (**a**) Drug dosing schedules for repeated saline or morphine administrations. On Day 1 all mice are given an acute morphine injection (10 mg/kg, subcutaneous) to establish a baseline analgesic response in the thermal tail immersion assay. Mice are then divided into treatment groups for saline, fixed-dose morphine (10 mg/kg, once daily, subcutaneous), or escalating morphine (10, 20, 30, 40, 40, 40 mg/kg, twice daily, subcutaneous). These treatments are given for 6 days (Day 2 -7). On Day 8 sensory levels are assessed prior to morphine for the presence of OIH, and then all mice are given an acute morphine injection (10 mg/kg, subcutaneous) to test for the presence of analgesic tolerance. (**b**) Untransformed, raw sensory threshold scores for same data presented in Figure 1, panels a and b. n = 6 for all groups. ★ $P < 0.05$. Error bars are ± SEM.



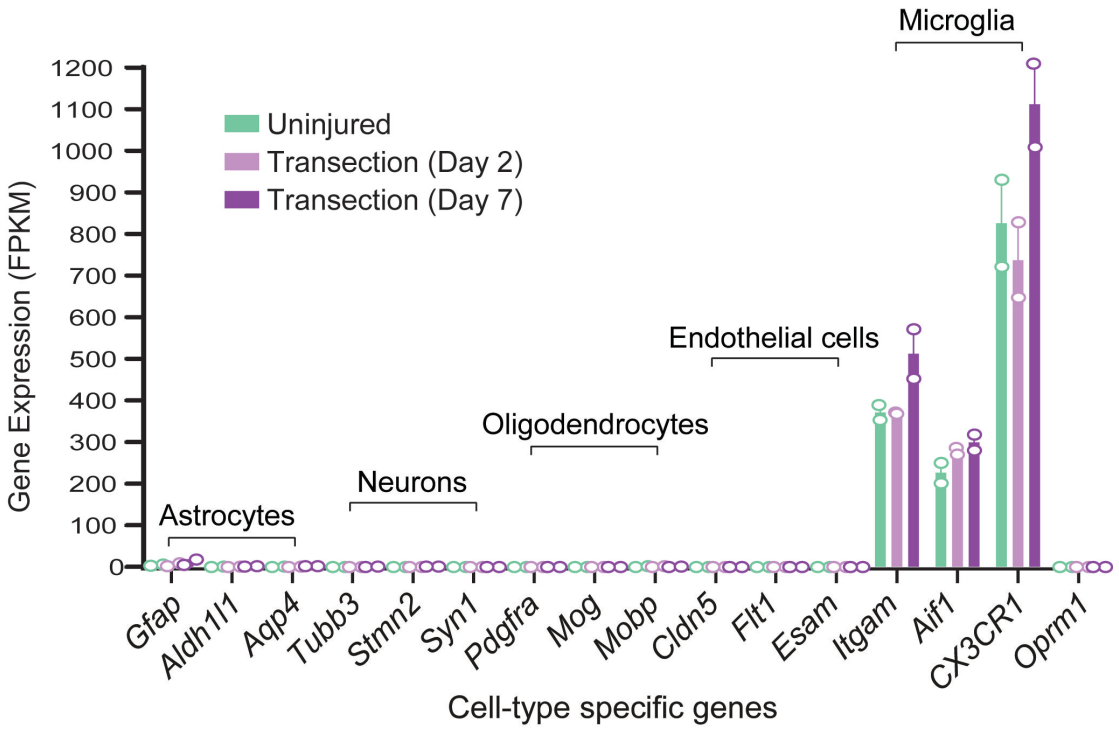
Supplementary Figure 2. **Anti-MOR antibody validation.** (**a**) In wild-type C57Bl/6 mice, rabbit anti-MOR antibody (1:100; Abcam) labels primary afferent terminals in the spinal cord dorsal horn, as well as spinal neurons in the dorsal horn. (**b**) In global MOR KO mice, immunostaining is lost in the afferent terminals and dorsal horn neurons. (**c**) In C57Bl/6 mice, guinea pig anti-MOR (1:1,000; Neuromics) produces a similar immunostaining pattern in the dorsal horn. (**d**) In global MOR KO mice, this MOR-immunoreactivity is lost, demonstrating its specificity. (**e,g**) Both the Abcam rabbit and Neuromics guinea pig anti-MOR antibodies label DRG neurons, including peptidergic CGRP-expressing nociceptors, consistent with the known MOR expression pattern. (**f,h**) Immunostaining of MOR in DRG with both antibodies is lost in global MOR KO mice, indicating staining specificity. Scale bars = 50 μm



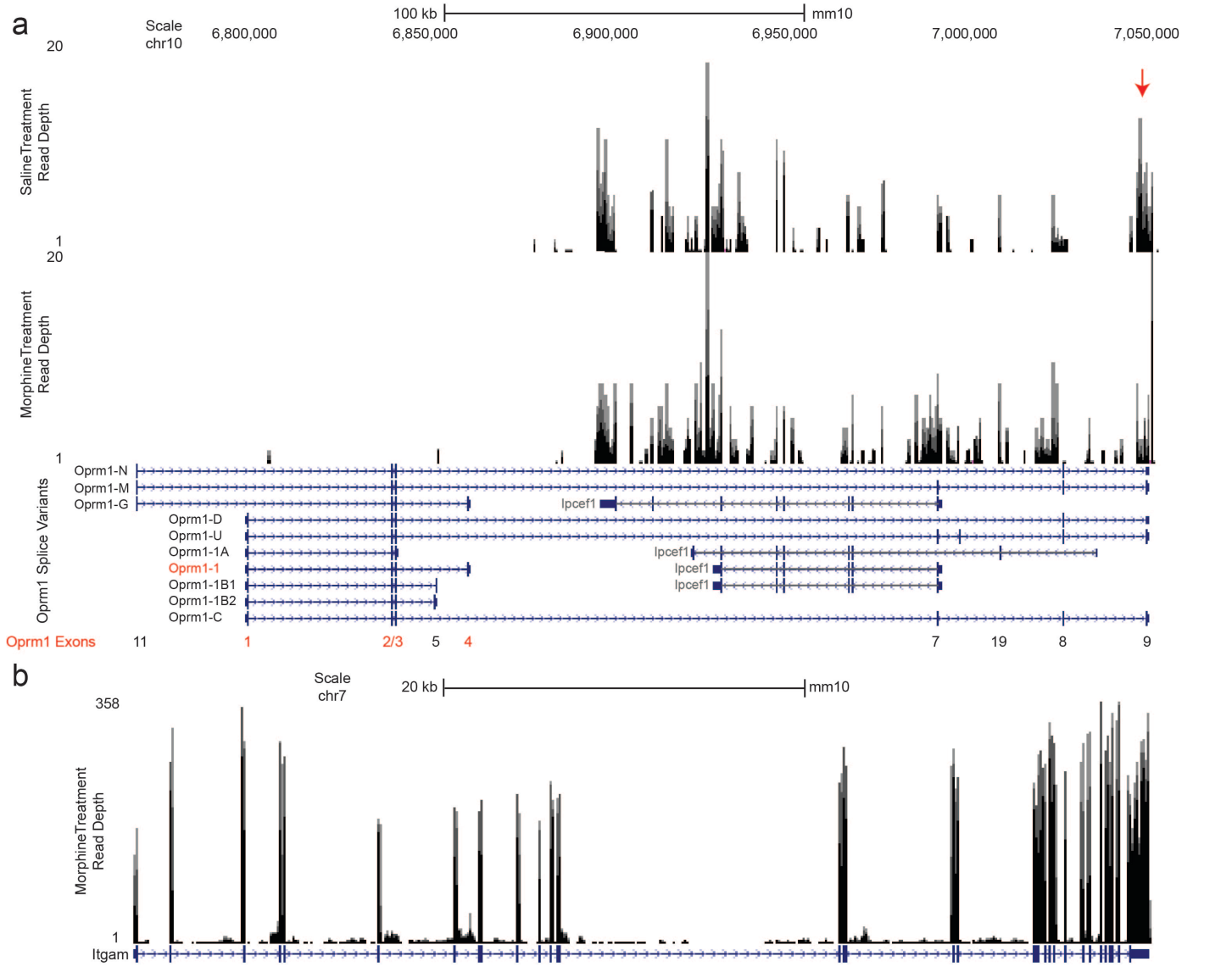
Supplementary Figure 3. MOR is expressed by DRG nociceptors and spinal neurons in the dorsal horn. (**a**) Immunohistochemistry for MOR (Abcam anti-MOR) in dorsal root ganglion (DRG) shows that MOR is predominantly expressed by CGRP+ nociceptors. (**b,c**) MOR expression is also observed in NeuN+ neurons in the spinal cord dorsal horn with (b) immunohistochemistry for MOR and in (c) MOR-mCherrry reporter mice.



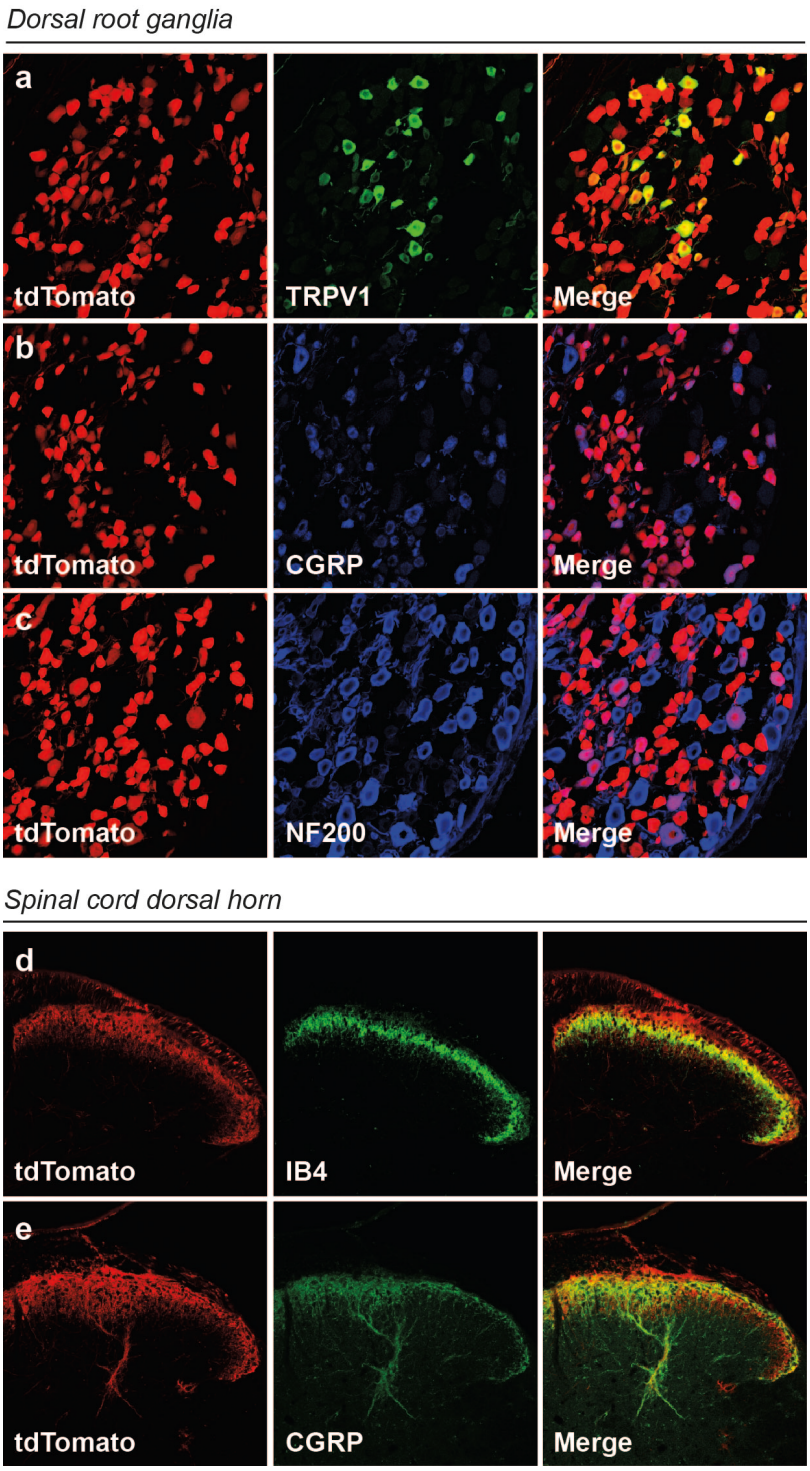
Supplementary Figure 4. **Additional immunohistochemical evidence that MOR is not expressed by spinal microglia.** Absence of clear histological evidence for MOR expression by CD11b+ microglia using (**a**) Abcam rabbit anti-MOR and (**b**) Neuromics guinea pig anti-MOR antibodies. Scale bars = 50 μm



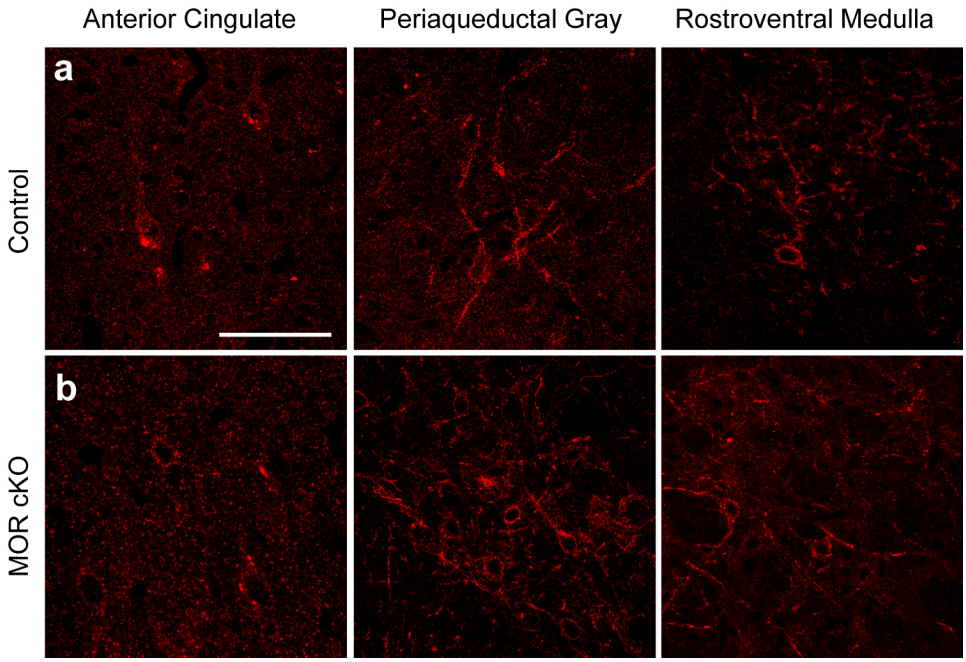
Supplementary Figure 5. Mapped reads from RNA-seq transcriptome profiling of acutely purified spinal microglia from wild-type mice with complete transection of the sciatic nerve. We find little to no contamination from other cell-types based on sub-type specific gene markers, and importantly no evidence of *Oprm1* expression in microglia. FPKM = Fragments Per Kilobase of transcript per Million mapped reads.



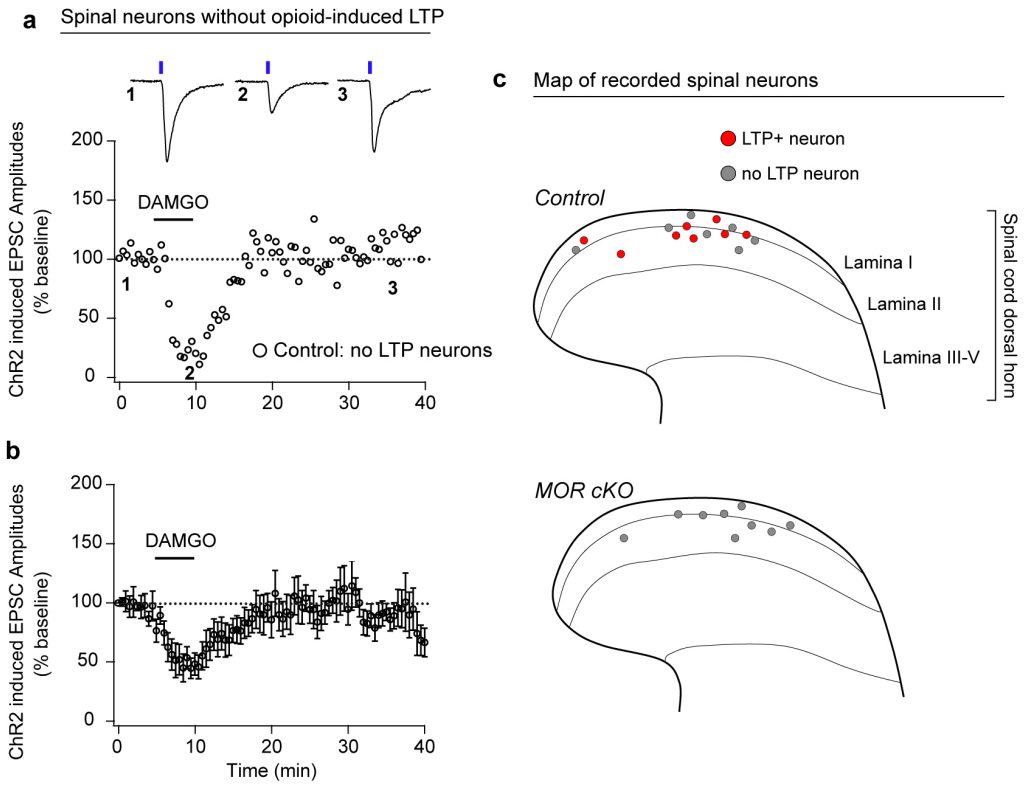
Supplementary Figure 6. RNA-seq of microglia after morphine treatment. (a) Example wiggle plots showing microglial RNA-seq reads mapped to the genomic locus containing *Oprm1*. Sequencing reads of microglia mRNA isolated from morphine- (bottom) or saline-injected (top) animals corresponded to FPKM values of 0.00 and 0.86, respectively. No reads were associated with *Oprm1* exons 1 - 4, indicated by red numbers. The reads (black bars) were mapped either to introns of the *Oprm1* gene, in particular to the region overlapping with Ipcef1 (a gene known to be expressed by microglia), or to the alternative exon 9 of the *Oprm1* gene (red arrow). These plots are representative of 3 morphine replicates (all with *Oprm1* FPKM values of 0.00) and 4 saline replicates (2 with FPKM values 0.00 and the other two with values of 1.22 and 0.86). (b) Example wiggle plot for the microglial marker Itgam (CD11b) expression from the morphine treated replicate in (a). Consistent reads across all exons indicates no three-prime bias in our sequencing results.



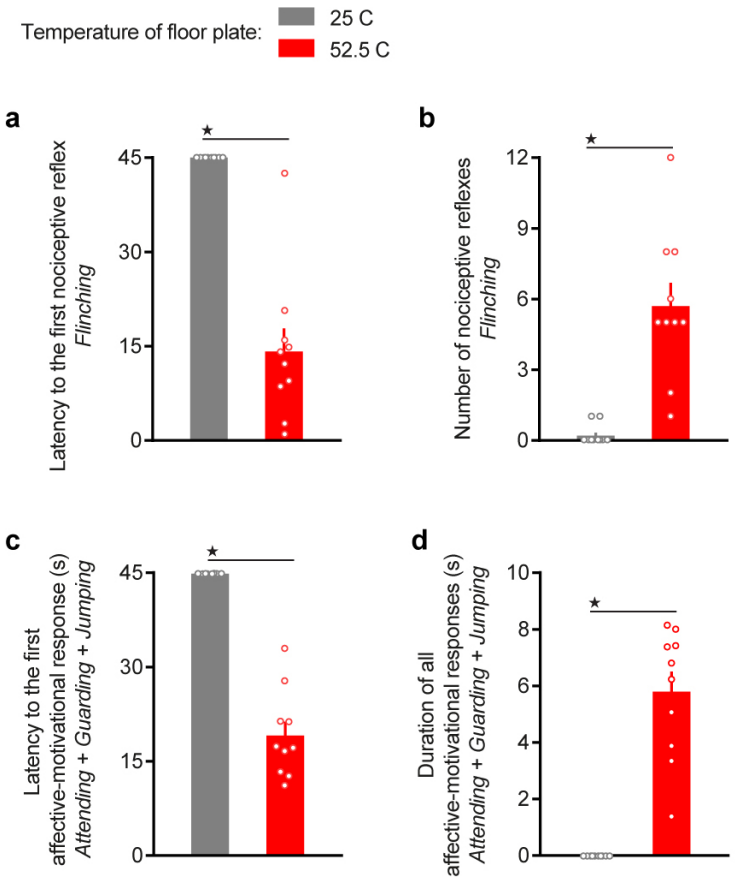
Supplementary Figure 7. Cre recombinase activity in DRG and spinal cord of *Trpv1*^{Cre} mice. *Trpv1*^{Cre} mice were crossed with the Ai14 Cre-dependent tdTomato reporter mouse line that generates the expression of a red-fluorescent protein in all cells that express, or have expressed, Cre recombinase throughout development. (**a - c**) Lineage map of tdTomato expression in dorsal root ganglia populations, determined by co-immunohistochemistry: (a) TRPV1, (b) CGRP, and (c) NF200. (**d,e**) tdTomato expression in the central terminals of DRG neurons, but not in spinal cord neurons.



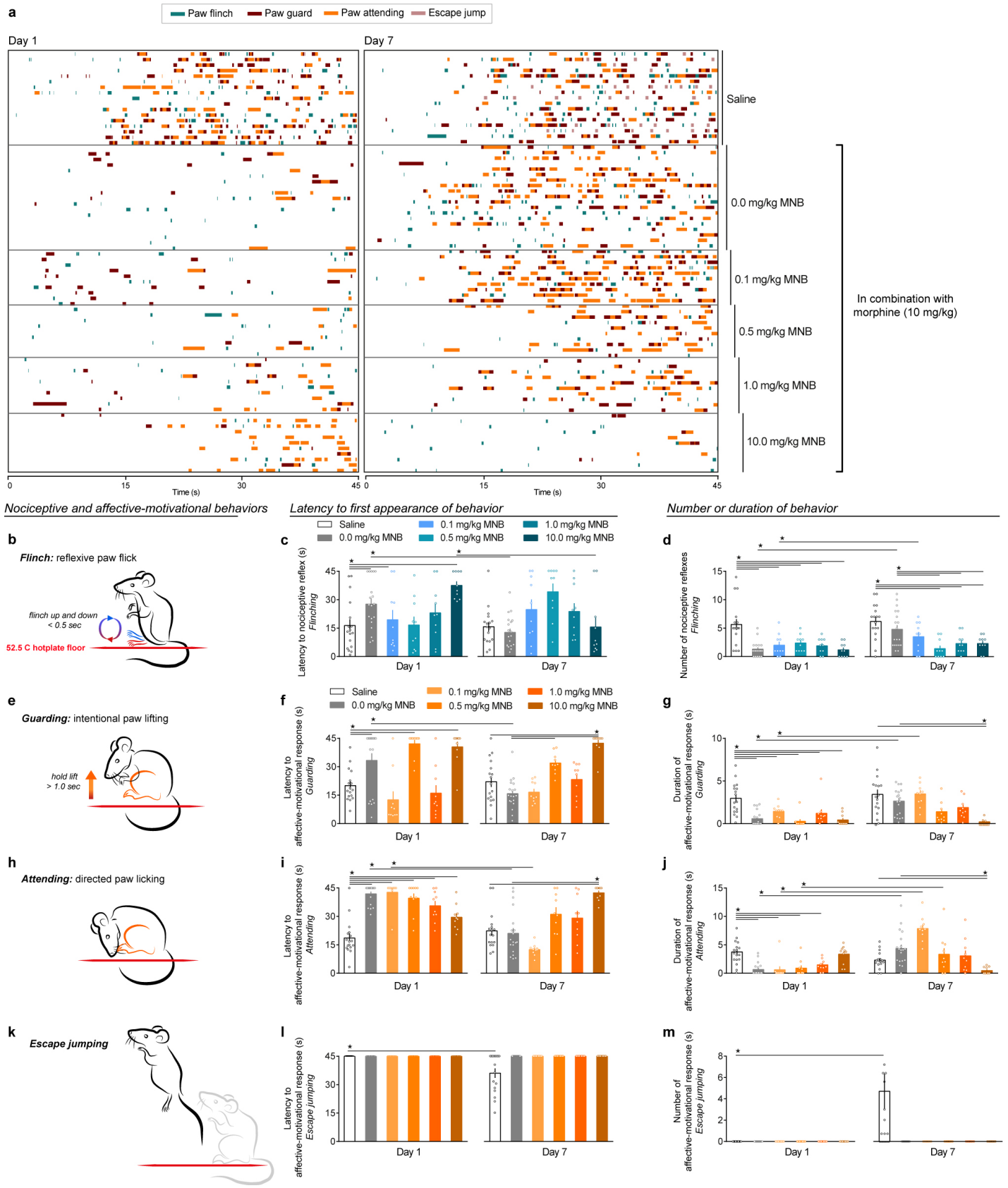
Supplementary Figure 8. MOR expression in brain of cKO mice. MOR immunoreactivity is similar between (**a**) control and (**b**) MOR cKO mice in pain-related brain regions. Scale bar = 50 μm



Supplementary Figure 9. Spinal neurons that do not exhibit opioid-induced LTP. (a) Representative spinal cord neuron from control mice showing DAMGO-induced depression of blue light-evoked (473 nm, 1.0 mW/cm^2, 0.2 ms, 0.05 Hz) EPSCs, but without DAMGO-washout rebound LTP. Numbered inset traces correspond to individual EPSCs before, during, and after wash-out of bath applied DAMGO (500 nM; 5 min duration). **(b)** Summary of 7 out of 15 recorded spinal neurons that did not show opioid-induced LTP. (c) Illustrative map of recorded neurons throughout the spinal cord dorsal horn for control and MOR cKO mice. Red cells are neurons that showed a rebound LTP. Gray cells are neurons that did not show a rebound LTP.

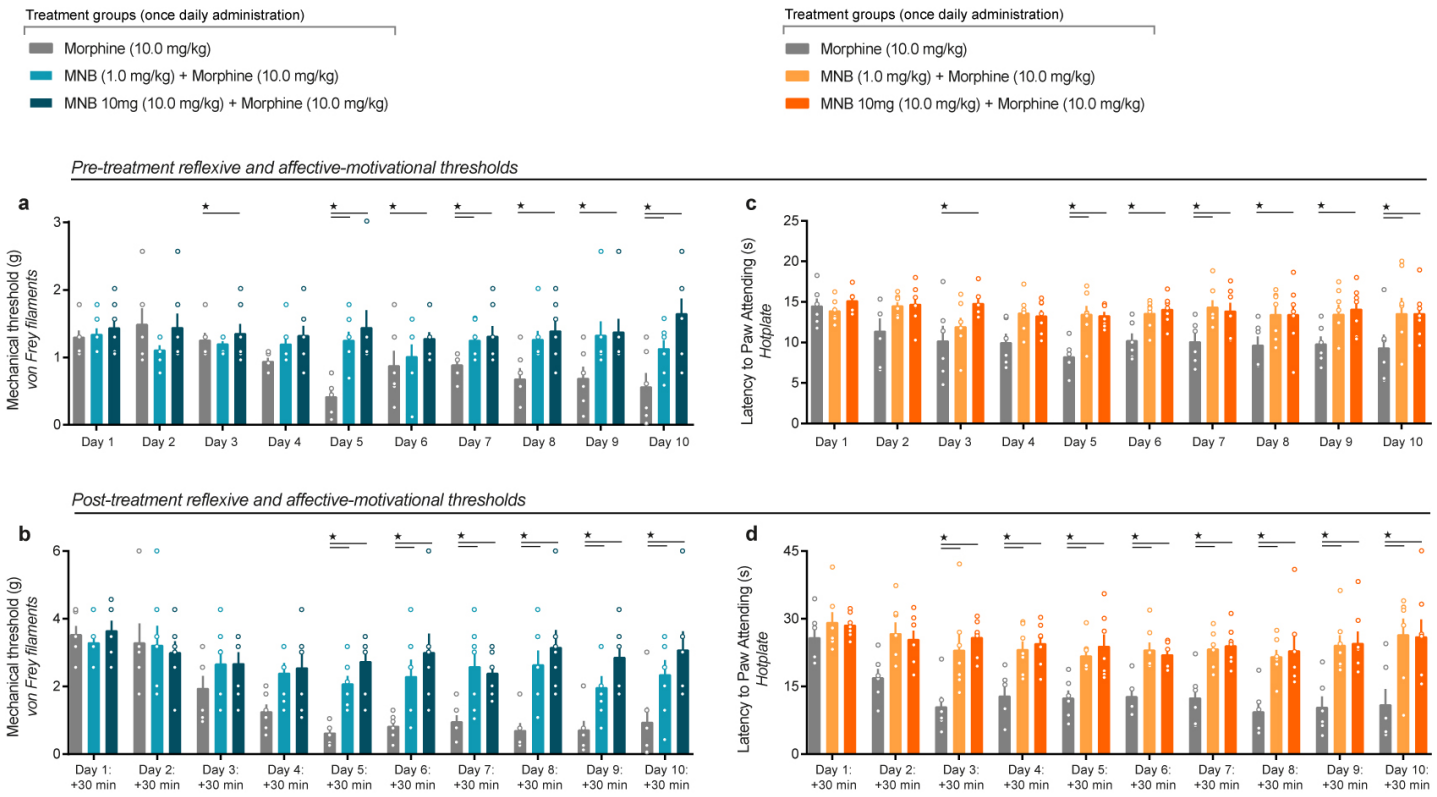


Supplementary Figure 10. Behavioral characterization in an inescapable thermal environment. (**a,b**) Nociceptive reflexes and (**c,d**) affective-motivational behaviors on a temperature controlled floor plate set at an innocuous 25 °C or a noxious 52.5 °C. Behaviors were recorded during a 45 second exposure trial.

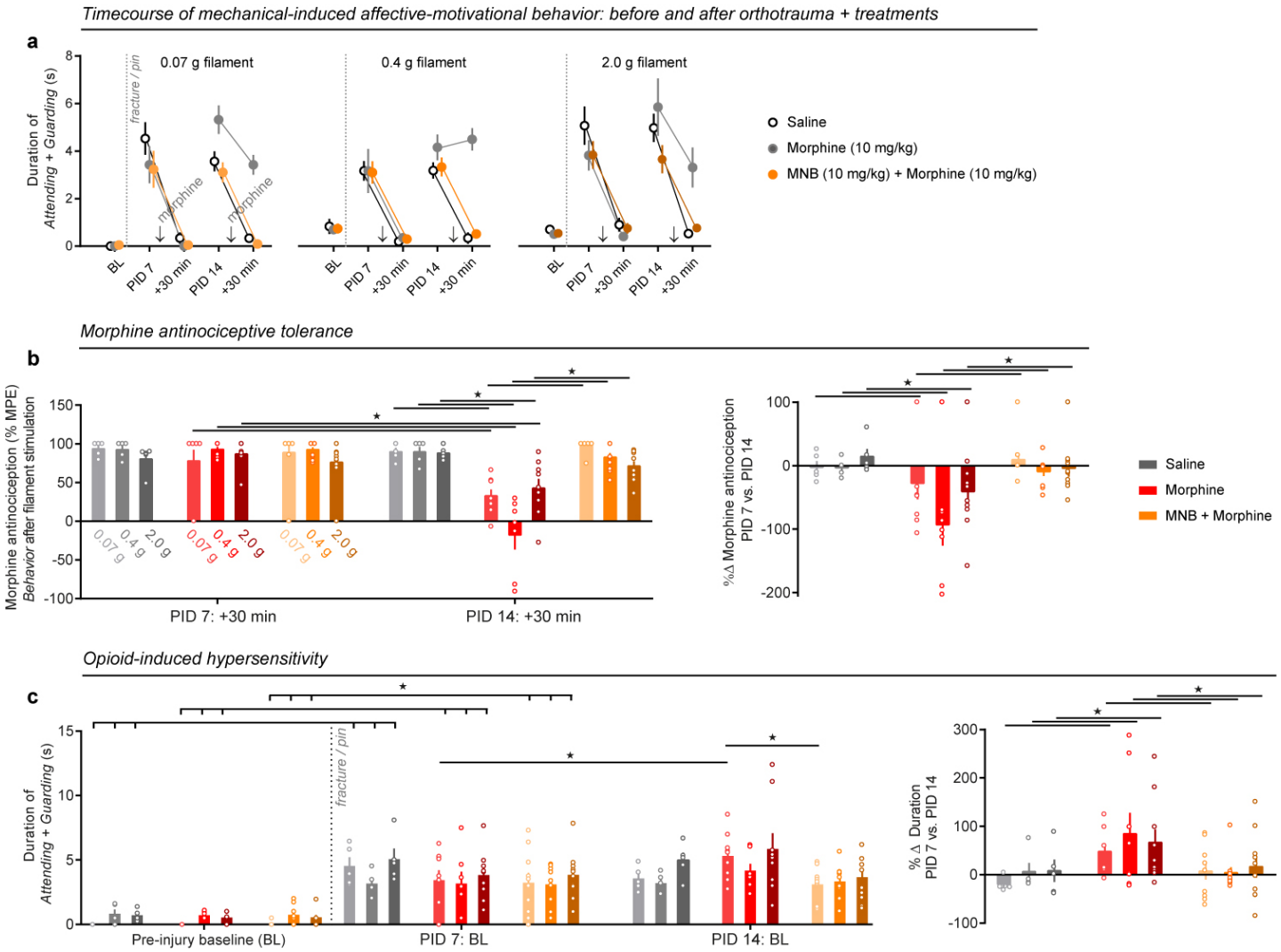


Supplementary Figure 11. Chronic morphine antinociceptive tolerance and the combinatorial dose-response effect of methyl naltrexone bromide (MNB). (a) Raster plots depicting multiple behaviors displayed during the 45 second hot plate test for all dose groups. Raster data for saline, morphine + 0.0 mg/kg MNB, and morphine + 10.0 mg/kg MNB is the same presented in Figure 4a. **(b)** Visual depiction of reflexive

paw flinching behavior. **(c)** Latencies to the first paw flinch. **(d)** Number of paw flinches (**Supplemental Note 4**). **(e)** Visual depiction of hindpaw guarding behavior. **(f)** Latency to the first onset of hindpaw guarding. **(g)** Total duration of time spent engaged in hindpaw guarding. **(h)** Visual depiction of hindpaw attending. **(i)** Latency to the first onset of hindpaw attending. **(j)** Total duration of time spent engaging in hindpaw attending. **(k)** Visual depiction of escape jumping behavior. **(l)** Latency to the first jump. For mice that did not jump, 45 s was used as the cutoff value. **(m)** Number of escape jumps. Control saline, n = 17; 10 mg/kg morphine + 0.0 mg/kg MNB, n = 19; morphine + 0.1 mg/kg MNB, n = 10; morphine + 0.5 mg/kg MNB, n = 10; morphine + 1.0 mg/kg MNB, n = 10; morphine + 10.0 mg/kg MNB, n = 10. ★ $P < 0.05$. Error bars are ± SEM. *Note: These are the same mice presented in Figure 5a-e*.



Supplementary Figure 12. 10-day pharmacological blockade of peripheral MOR by methylnaltrexone bromide (MNB) prevents the onset of morphine antinociceptive tolerance and OIH. (**a,b**) Mechanical von Frey filament induced reflex thresholds (a) before ($F_{2, 17}$ = 31.35, P < 0.0001) and (b) after ($F_{2, 17}$ = 20.01, P < 0.0001) treatment. (**c,d**) Thermal 52.5 °C hotplate-induced affective-motivational thresholds (c) before ($F_{2, 17}$ = 13.38, P = 0.0003) and (d) after ($F_{2, 17}$ = 24.34, P < 0.0001) treatment. For all panels, Morphine, n = 6; MNB 1mg/kg + morphine, n = 7; MNB 10 mg/kg + morphine, n = 7. Repeated Measures 2-way ANOVA + Bonferonni. ★P < 0.05. Error bars are mean ± SEM.



Supplementary Figure 13. MNB combination treatment with morphine prevents the development of antinociceptive tolerance and OIH for mechanically-induced affective-motivational behaviors in tibia fracture and bone pinning model of orthotrauma chronic pain. (a) Timecourse of affective-motivational behaviors (paw attending and guarding) in response to a one-second von Frey filament stimulation (0.07, 0.4, 2.0 g) following leg fracture and bone pinning, and the effect of chronic saline (n = 10), morphine (10 mg/kg) (n = 10), or morphine + MNB (10 mg/kg) (n = 10) treatments. Treatments are first given on PID 7 and re-administered once daily until PID 14, with the exception that the saline group is administered acute morphine only on PID 7 and PID 14. **(b)** Morphine antinociceptive tolerance and **(c)** OIH are significantly reduced when given in combination with MNB. Student's *t* Test, two-tailed (right panels of b and c). Two-way ANOVA + Bonferonni (left panels of b and c). ★ $P < 0.05$. Error bars are mean ± SEM. Overlaid points are individual animal scores. PID = post-injury day. *Note: These are the same mice presented in Figure 6a-f.*

Supplementary Notes

Supplementary Note 1: The properties of several cell-types and circuits in which MOR is present are altered following chronic opioid treatments[1], including at the level of DRG nociceptors[2,3], spinal neurons[4–6], spinal microglia[7–10], brainstem nuclei (i.e., periaqueductal grey and rostral ventromedial medulla)[11–14], and sub-cortical and cortical brain regions (i.e. ventral tegmental area and anterior cingulate cortex)[15,16]. Thus, while an extensive literature indicates that MOR also contributes to tolerance and OIH in a variety of CNS regions, MOR function in DRG nociceptors was of particular interest[17,18]. Chronic opioids recruit multiple pathways in nociceptors, many of which are also implicated in nociceptor sensitization during inflammation and transition to chronic pain[2,19]. Thus, while acute opioid action on MOR in nociceptors inhibits voltage-gated calcium channels [20] and reduces the release of pronociceptive glutamate and peptides (i.e., substance P, CGRP)[21–26], prolonged opioid treatment and opioid withdrawal cause excitatory effects. For example, chronic morphine upregulates substance P and CGRP, which could then facilitate nociception and cause OIH and tolerance[27–32]. In agreement with this idea, we and others showed that mice null for substance P expression show very limited morphine tolerance[27,33]. Antagonists for substance P receptor (NK1R) and CGRP receptor[29,30], and ablation of spinal neurons expressing NK1R[6], also strongly diminish opioid tolerance. Acute and chronic opioids upregulate components of the adenylyl cyclase–PKA pathway[34,35], such that opioid withdrawal induces AC superactivation, which increases cAMP and PKA activities that in turn modify AMPA, NMDA, and TRPV1 receptor function[36–40]. MOR signaling in nociceptors appears to be particularly complex, as PKCε[2,19], nitric oxide[41,42], PDGFR-β[43], presynaptic NMDA receptors[44], among many other receptor and intracellular signaling pathways, have also been shown to contribute to tolerance and OIH. Additionally excitatory MOR splice variants exist[45,46], but whether they are induced by opioids in MOR+ nociceptors to cause tolerance and OIH is not clear. Specific ablation of TRPV1-expressing DRG neurons causes a profound reduction in morphine analgesic tolerance[47]. In contrast, a previous study using MOR floxed mice found no change in tolerance following Cre expression in neurons expressing the sodium channel Nav1.8[48]. Note, however, that numerous DRG neurons, including the nociceptors that express TRPV1, but not Nav1.8[49] still express MOR with this strategy, and that the short 4-day chronic morphine treatment might have led to incomplete tolerance.

Supplementary Note 2: In our RNA-seq analysis we find similar numbers of transcripts for *Aif1* (encoding Iba1) and *Itgam* (encoding CD11b) in mice with nerve injury and in uninjured mice. While this result can be surprising at first, it is consistent with recent studies on microglia response to nerve injury, given that each of our replicates utilize a constant amount of RNA from purified microglia. Indeed, these recent studies showed that nerve injury-induced microgliosis is predominantly the consequence of intense microglia proliferation, rather than strong amplification of gene expression in a stable population of resident spinal microglia[50–52].

Supplementary Note 3: We previously published an article describing the differential expression of DOR and MOR in functionally distinct primary afferents (predominantly in IB4-binding C nociceptors and myelinated mechanosensory afferents versus TRPV1 heat-sensitive C nociceptors, respectively)[33,53]. A number of studies have now confirmed this model of segregated expression of DOR and MOR in DRG neurons, including unbiased, single-cell RNA-seq analyses[54]. Note that the previous behavioral studies used selective MOR and DOR agonists (DAMGO and SNC80, respectively), administered spinally, at low and gradually-increased doses, to determine against which pain modality, heat or mechanical, each agonist was more efficient [33]. DAMGO blocked heat pain at low doses at which it did not alter mechanical pain thresholds, conversely, SNC80 blocked mechanical pain at low doses at which it did not alter heat pain thresholds. The authors concluded that in these conditions, at the spinal level, DOR predominantly regulates mechanical pain, in particular injury-induced mechanical allodynia, while MOR predominantly regulates activity in TRPV1 nociceptors to decrease heat pain acutely. Thus, the experimental goals and conditions are drastically different in the present study, where morphine, a MOR agonist with distinct pharmacological and pharmacokinetic properties from DAMGO, is administered systemically. It is clear that in these conditions morphine is able to

reduce pain across modalities, including mechanical pain, both in rodents and in humans, presumably in part through an action on brain circuits mediating the affective component of pain, or descending pathways influencing spinal sensory-reflexive behaviors.

Supplementary Note 4: As shown in Supplemental Figure 11c, the effect of MNB on morphine tolerance followed a bell-shaped dose-response curve only for the latency to nociceptive reflexes when scoring the number of flinches. There is clinical evidence of a similar ceiling effect of MNB on laxation responses, with a higher percentage of responders after 5 or 12.5 mg subcutaneous MNB compared to a 20 mg dose [55]. Translation of our results therefore requires clear dose-finding studies to ensure that the dosing regimen selected is optimized to the most relevant outcomes.

Supplementary Note 5: Previous studies support the idea that maladaptations leading to tolerance and OIH occur in TRPV1 nociceptors following chronic opioid treatment. In the peripheral nervous system, MOR is predominantly expressed by TRPV1 nociceptors, which co-express substance P and CGRP [33,53,54,56,57]. Interestingly, chronic morphine treatment induces the upregulation and increased release of substance P and CGRP by these nociceptors, which could then act on their receptors on spinal neurons to facilitate nociception and cause OIH and tolerance[24,31]. In agreement with this idea, we and others showed that genetic knockout of substance P[27,33] or pharmacological antagonists for the substance P receptor (NK1R) and CGRP receptor [29,30], are sufficient to diminish opioid tolerance. Ablation of spinal neurons expressing NK1R with a substance P-saporin conjugate[6], also strongly reduces opioid tolerance. Other studies have shown that sensitization of nociceptors contributes to the onset of opioid antinociceptive tolerance, physical dependence, and pronociceptive effects of opioids[2,4,19,58–62]. There are similarities between the molecular mechanisms underlying opioid tolerance and OIH, and those underlying injury-induced nociceptor sensitization during chronic pain development[2,63–65]. In particular, adaptive molecular events occurring within nociceptors, in response to opioids or injury, can influence activity in second-order spinal neurons in the spinal cord, including via changes in synaptic strength, which we and others have demonstrated in synaptic long-term potentiation (LTP) electrophysiology studies (Figure 4; for review see [66]). Thus, opioids not only depress synaptic transmission acutely in the spinal cord, but can increase excitatory plasticity, via LTP mechanisms[44,67–69]. Importantly, LTP between primary afferent nociceptors and superficial dorsal horn neurons is thought to be a synaptic substrate for injury-induced hyperalgesia, as well as OIH and tolerance[40,67,69]. Resiniferatoxin-mediated ablation of TRPV1 nociceptors is thus sufficient to block opioid-induced LTP in the spinal cord[44], and to attenuate the development of morphine antinociceptive tolerance[47,70,71]. Altogether, these studies and our findings suggest that chronic opioids cause maladaptive plasticity in peripheral TRPV1 nociceptors, which in turn alters activity in downstream nociceptive networks in the CNS, leading to tolerance and OIH.

Supplementary references

1. Christie, M. J. Cellular neuoadaptations to chronic opioids: tolerance withdrawal and addiction. (2008).
2. Joseph, E. K., Reichling, D. B. & Levine, J. D. Shared mechanisms for opioid tolerance and a transition to chronic pain. *J Neurosci* **30,** 4660–4666 (2010).
3. Zeng, J., Thomson, L. M., Aicher, S. a & Terman, G. W. Primary afferent NMDA receptors increase dorsal horn excitation and mediate opiate tolerance in neonatal rats. *J. Neurosci.* **26,** 12033–12042 (2006).
4. Gardell, L. R. *et al.* Sustained morphine exposure induces a spinal dynorphin-dependent enhancement of excitatory transmitter release from primary afferent fibers. *J. Neurosci.* **22,** 6747–6755 (2002).
5. Mao, J. & Mayer, D. J. Spinal cord neuroplasticity following repeated opioid exposure and its relation to pathological pain. *Ann. N. Y. Acad. Sci.* **933,** 175–184 (2001).
6. Vera-Portocarrero, L. P. *et al.* Spinal NK-1 receptor expressing neurons mediate opioid-induced hyperalgesia and antinociceptive tolerance via activation of descending pathways. *Pain* **129,** 35–45 (2007).
7. Cui, Y. *et al.* Activation of p38 mitogen-activated protein kinase in spinal microglia mediates morphine antinociceptive tolerance. *Brain Res.* **1069,** 235–243 (2006).
8. Ferrini, F., Trang, T. & Mattioli, T. Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl-homeostasis. *Nat. Neurosci.* **16,** 183–92 (2013).
9. Horvath, R. J. & DeLeo, J. A. Morphine enhances microglial migration through modulation of P2X4 receptor signaling. *J. Neurosci.* **29,** 998–1005 (2009).
10. Wang, Z., Ma, W., Chabot, J.-G. & Quirion, R. Cell-type specific activation of p38 and ERK mediates calcitonin gene-related peptide involvement in tolerance to morphine-induced analgesia. *FASEB J.* **23,** 2576–2586 (2009).
11. Eidson, L. N. & Murphy, A. Z. Blockade of Toll-Like Receptor 4 Attenuates Morphine Tolerance and Facilitates the Pain Relieving Properties of Morphine. *J. Neurosci.* **33,** 15952–15963 (2013).
12. Morgan, M. M., Fossum, E. N., Levine, C. S. & Ingram, S. L. Antinociceptive tolerance revealed by cumulative intracranial microinjections of morphine into the periaqueductal gray in the rat. *Pharmacol. Biochem. Behav.* **85,** 214–219 (2006).
13. Vanderah, T. W. *et al.* Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. *J. Neurosci.* **21,** 279–286 (2001).
14. Connor, M., Bagley, E. E., Chieng, B. C. & Christie, M. J. β -Arrestin-2 knockout prevents development of cellular μ -opioid receptor tolerance but does not affect opioid-withdrawal- related adaptations in single PAG neurons. *Br. J. Anaesth.* **172,** 492–500 (2015).
15. Mazei-Robison, M. S. & Nestler, E. J. Opiate-induced molecular and cellular plasticity of ventral tegmental area and locus coeruleus catecholamine neurons. *Cold Spring Harb Perspect Med* **2,** a012070 (2012).
16. Ko, S. W., Wu, L.-J., Shum, F., Quan, J. & Zhuo, M. Cingulate NMDA NR2B receptors contribute to morphine-induced analgesic tolerance. *Mol. Brain* **1,** 2 (2008).
17. Roeckel, L.-A., Le Coz, G.-M., Gaveriaux-Ruff, C. & Simonin, F. Opioid-induced Hyperalgesia: Cellular and Molecular Mechanisms. *Neuroscience* (2016). doi:10.1016/j.neuroscience.2016.06.029
18. Rivat, C. & Ballantyne, J. The dark side of opioids in pain management : basic science explains clinical observation. **1,** 1–9 (2016).
19. Dioneia Araldi, X., Ferrari, L. F. & Jon Levine, X. D. Repeated Mu-Opioid Exposure Induces a Novel Form of the Hyperalgesic Priming Model for Transition to Chronic Pain. **35,** 12502–12517 (2015).
20. Taddese, A., Nah, S. & Mccleskeyt, E. W. Selective Opioid Inhibition of Small Nociceptive Neurons. *Science (80-.).* **270,** (1995).
21. Chang, H. M., Berde, C. B., Holz, G. G. th, Steward, G. F. & Kream, R. M. Sufentanil, morphine, met-enkephalin, and kappa-agonist (U-50,488H) inhibit substance P release from primary sensory neurons:

a model for presynaptic spinal opioid actions. *Anesthesiology* **70,** 672–677 (1989).
22. Kohno, T., Kumamoto, E., Higashi, H., Shimoji, K. & Yoshimura, M. Actions of opioids on excitatory and inhibitory transmission in substantia gelatinosa of adult rat spinal cord. *J Physiol* **518 (Pt 3,** 803–813 (1999).
23. Heinke, B., Gingl, E. & Sandkühler, J. Multiple targets of μ-opioid receptor-mediated presynaptic inhibition at primary afferent Aδ- and C-fibers. *J. Neurosci.* **31,** 1313–1322 (2011).
24. Suarez-Roca, H. & Maixner, W. Morphine produces a multiphasic effect on the release of substance P from rat trigeminal nucleus slices by activating different opioid receptor subtypes. *Brain Res* **579,** 195–203 (1992).
25. Miller, J. & Hammond, D. L. Inhibitory Actions of S , - , a , - , and p-Opioid Receptor Agonists on Excitatory Transmission in Lamina II Neurons of Adult Rat Spinal Cord. **74,** (1994).
26. Zhou, H.-Y., Chen, S.-R., Chen, H. & Pan, H.-L. Sustained inhibition of neurotransmitter release from nontransient receptor potential vanilloid type 1-expressing primary afferents by mu-opioid receptor activation-enkephalin in the spinal cord. *J. Pharmacol. Exp. Ther.* **327,** 375–382 (2008).
27. Guan, J. S. *et al.* Interaction with vesicle luminal protachykinin regulates surface expression of δ-opioid receptors and opioid analgesia. *Cell* **122,** 619–631 (2005).
28. Tumati, S., Roeske, W. R., Largent-Milnes, T. M., Vanderah, T. W. & Varga, E. V. Intrathecal PKA-selective siRNA treatment blocks sustained morphine-mediated pain sensitization and antinociceptive tolerance in rats. *J. Neurosci. Methods* **199,** 62–68 (2011).
29. Menard, D. P. *et al.* A calcitonin gene-related peptide receptor antagonist prevents the development of tolerance to spinal morphine analgesia. *J. Neurosci.* **16,** 2342–2351 (1996).
30. Powell, K. J., Quirion, R. & Jhamandas, K. Inhibition of neurokinin-1-substance P receptor and prostanoid activity prevents and reverses the development of morphine tolerance in vivo and the morphine-induced increase in CGRP expression in cultured dorsal root ganglion neurons. *Eur. J. Neurosci.* **18,** 1572–1583 (2003).
31. Belanger, S., Ma, W., Chabot, J. G. & Quirion, R. Expression of calcitonin gene-related peptide, substance P and protein kinase C in cultured dorsal root ganglion neurons following chronic exposure to mu, delta and kappa opiates. *Neuroscience* **115,** 441–453 (2002).
32. Suarezroca, H., Abdullah, L., Zuniga, J., Madison, S. & Maixner, W. Multiphasic effects of morphine on the release of substance P from rat trigeminal nucleus slices. *Brain Res.* **579,** 187–194 (1992).
33. Scherrer, G. *et al.* Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* **137,** 1148–1159 (2009).
34. Zachariou, V. *et al.* Distinct roles of adenylyl cyclases 1 and 8 in opiate dependence: behavioral, electrophysiological, and molecular studies. *Biol Psychiatry* **63,** 1013–1021 (2008).
35. Christie, M. J. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *Br. J. Pharmacol.* **154,** 384–396 (2008).
36. Bao, Y. *et al.* crosstalk in TRPV1 activation involves morphine anti-nociception , tolerance and dependence The mechanism of m -opioid receptor (MOR) - TRPV1 crosstalk in TRPV1 activation involves morphine anti-nociception , tolerance and dependence. **6950,** (2015).
37. Chen, Y., Geis, C. & Sommer, C. Activation of TRPV1 contributes to morphine tolerance: involvement of the mitogen-activated protein kinase signaling pathway. *J. Neurosci.* **28,** 5836–5845 (2008).
38. Spahn, V. *et al.* Opioid withdrawal increases transient receptor potential vanilloid 1 activity in a protein kinase A-dependent manner. *Pain* **154,** 598–608 (2013).
39. Corder, G. *et al.* Constitutive μ-opioid receptor activity leads to long-term endogenous analgesia and dependence. *Science (80-.).* **341,** 1394–1399 (2013).
40. Ruscheweyh, R., Wilder-Smith, O., Drdla, R., Liu, X.-G. & Sandkühler, J. Long-term potentiation in spinal nociceptive pathways as a novel target for pain therapy. *Mol. Pain* **7,** 20 (2011).
41. Aley, K. O. & Levine, J. D. Dissociation of tolerance and dependence for opioid peripheral antinociception in rats. *J. Neurosci.* **17,** 3907–3912 (1997).

42. Aley, K. O. & Levine, J. D. Different Mechanisms Mediate Development and Expression of Tolerance and Dependence for Peripheral □ -Opioid Antinociception in Rat. **17,** 8018–8023 (1997).
43. Wang, Y. *et al.* Blockade of PDGFR-β activation eliminates morphine analgesic tolerance. *Nat. Med.* **18,** 385–387 (2012).
44. Zhou, H. Y., Chen, S. R., Chen, H. & Pan, H. L. Opioid-induced long-term potentiation in the spinal cord is a presynaptic event. *J Neurosci* **30,** 4460–4466 (2010).
45. Pasternak, G. W. Opioids and their receptors: Are we there yet? *Neuropharmacology* **76,** 198–203 (2014).
46. Xu, J. *et al.* Stabilization of morphine tolerance with long-term dosing : Association with selective upregulation of mu-opioid receptor splice variant mRNAs. (2014). doi:10.1073/pnas.1419183112
47. Chen, S. R., Prunean, a., Pan, H. M. L., Welker, K. L. & Pan, H. M. L. Resistance to morphine analgesic tolerance in rats with deleted transient receptor potential vanilloid type 1-expressing sensory neurons. *Neuroscience* **145,** 676–685 (2007).
48. Weibel, R. *et al.* Mu Opioid Receptors on Primary Afferent Nav1.8 Neurons Contribute to Opiate-Induced Analgesia: Insight from Conditional Knockout Mice. *PLoS One* **8,** 1–18 (2013).
49. Shields, S. D. *et al.* Na v1.8 expression is not restricted to nociceptors in mouse peripheral nervous system. *Pain* **153,** 2017–2030 (2012).
50. Guan, Z. *et al.* Injured sensory neuron–derived CSF1 induces microglial proliferation and DAP12-dependent pain. *Nat. Neurosci.* **19,** 1–10 (2015).
51. Denk, F., Crow, M., Didangelos, A., Lopes, D. M. & McMahon, S. B. Persistent Alterations in Microglial Enhancers in a Model of Chronic Pain. *Cell Rep.* **15,** 1–11 (2016).
52. Gu, N. *et al.* Spinal Microgliosis Due to Resident Microglial Proliferation Is Required for Pain Hypersensitivity after Peripheral Nerve Injury Report Spinal Microgliosis Due to Resident Microglial Proliferation Is Required for Pain Hypersensitivity after Peripheral Ner. *CellReports* **16,** 605–614 (2016).
53. Bardoni, R. *et al.* Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn. *Neuron* **81,** 1312–1327 (2014).
54. Usoskin, D. *et al.* Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat. Neurosci.* **18,** 145–153 (2014).
55. Portenoy, R. K. *et al.* Subcutaneous Methylnaltrexone for the Treatment of Opioid-Induced Constipation in Patients with Advanced Illness : A Double-Blind , Randomized , Parallel Group , Dose-Ranging Study. **35,** 458–468 (2008).
56. Li, J. *et al.* Immunocytochemical localization of mu -opioid receptor in primary afferent neurons containing substance P or calcitonin gene-related peptide. A light and electron microscope study in the rat. *Brain Res.* **794,** 347–352 (1998).
57. Minami, M., Maekawa, K., Yabuuchi, K. & Satoh, M. Co-existence of opioid receptor mRNAs with preprotachykinin A mRNA in the rat dorsal root ganglia. *Neurosci. Res. Suppl.* **19,** S58 (1994).
58. Mao, J. Opioid-induced abnormal pain sensitivity: Implications in clinical opioid therapy. *Pain* **100,** 213–217 (2002).
59. Ossipov, M. H., Lai, J., King, T., Vanderah, T. W. & Porreca, F. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolym. - Pept. Sci. Sect.* **80,** 319–324 (2005).
60. Chu, L. F., Angst, M. S. & Clark, D. Opioid-induced hyperalgesia in humans: molecular mechanisms and clinical considerations. *Clin. J. Pain* **24,** 479–496 (2008).
61. Aley, K. O., Green, P. G. & Levine, J. D. Opioid and adenosine peripheral antinociception are subject to tolerance and withdrawal. *J. Neurosci.* **15,** 8031–8038 (1995).
62. Ossipov, M. H., Lai, J., Vanderah, T. W. & Porreca, F. Induction of pain facilitation by sustained opioid exposure: Relationship to opioid antinociceptive tolerance. *Life Sci.* **73,** 783–800 (2003).
63. Reichling, D. B. & Levine, J. D. Critical role of nociceptor plasticity in chronic pain. *Trends Neurosci.* **32,** 611–618 (2009).

64. Latremoliere, A. & Woolf, C. J. Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity. *J. Pain* **10,** 895–926 (2009).
65. Mayer, D. J., Mao, J., Holt, J. & Price, D. D. Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7731–7736 (1999).
66. Sandkühler, J. & Gruber-Schoffnegger, D. Hyperalgesia by synaptic long-term potentiation (LTP): An update. *Curr. Opin. Pharmacol.* **12,** 18–27 (2012).
67. Drdla, R., Gassner, M., Gingl, E., Sandkuhler, J. & Sandkühler, J. Induction of synaptic long-term potentiation after opioid withdrawal. *Science (80-.).* **325,** 207–210 (2009).
68. Zhao, Y. L., Chen, S. R., Chen, H. & Pan, H. L. Chronic opioid potentiates presynaptic but impairs postsynaptic N-methyl-D-aspartic acid receptor activity in spinal cords: Implications for opioid hyperalgesia and tolerance. *J. Biol. Chem.* **287,** 25073–25085 (2012).
69. Heinl, C., Drdla-Schutting, R., Xanthos, D. N. & Sandkuhler, J. Distinct Mechanisms Underlying Pronociceptive Effects of Opioids. *J. Neurosci.* **31,** 16748–16756 (2011).
70. Chen, S.-R. & Pan, H.-L. Loss of TRPV1-expressing sensory neurons reduces spinal mu opioid receptors but paradoxically potentiates opioid analgesia. *J. Neurophysiol.* **95,** 3086–3096 (2006).
71. Chen, S. R. & Pan, H. L. Removing TRPV1-expressing primary afferent neurons potentiates the spinal analgesic effect of δ-opioid agonists on mechano-nociception. *Neuropharmacology* **55,** 215–222 (2008).