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Constitutive μ -Opioid Receptor Activity Leads to Long-term Endogenous Analgesia and Dependence

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Abstract

Opioid receptor antagonists increase hyperalgesia in humans and animals, indicating that endogenous activation of opioid receptors provides relief from acute pain; however, the mechanisms of long-term opioid inhibition of pathological pain have remained elusive. We found that tissue injury produced μ -opioid receptor constitutive activity (MOR_{CA}) that repressed spinal nociceptive signaling for months. Pharmacological blockade during the post-hyperalgesia state with MOR inverse agonists reinstated central pain sensitization, and precipitated hallmarks of opioid withdrawal (including cAMP overshoot and hyperalgesia) that required N-methyl-D-aspartate receptor activation of adenylyl cyclase type 1 (AC1). Thus, MOR_{CA} initiates both analgesic signaling as well as a compensatory opponent process that generates endogenous opioid

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Author Contributions:

G.C. and B.K.T. formulated the hypotheses, designed, analyzed and coordinated all experiments. G.C. performed surgeries, behavioral pharmacology, histology, and fluorescence imaging and analyzed the data. G.C. and B.L.J. carried out the biochemical studies and GC analyzed the data. S.D. and G.C. carried out Ca^{2+} imaging experiments and analyzed the data. M.W., K.E.M., G.C., and B.K.T. designed the GTP γ S³⁵ binding studies, M.K.W. collected the data, and G.C., K.E.M. and B.K.T. analyzed the data. D.R.S. supplied the AC1^{-/-} breeders. Z.J.W., G.C., and B.K.T. designed the conditioned place preference experiments, and Y.H. and X.H. conducted experiments and analyzed the data. J.M., J.W., G.C., and B.K.T. designed the Mouse Grimace Scale experiments, and J.W. conducted the experiments. G.C. and B.K.T. wrote the manuscript.

Supplementary Materials

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Materials and Methods

Figs. S1–S13

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dependence. Tonic MOR_{CA} suppression of withdrawal hyperalgesia may prevent the transition from acute to chronic pain.

Chronic pain is determined by facilitatory mechanisms such as long-term potentiation (LTP) of synaptic strength in dorsal horn neurons (1–3). While exogenously applied opioids prevent (4, 5) and/or erase (6) spinal LTP, and spinal enkephalin release exerts inhibitory control of acute pain intensity soon after tissue injury (7, 8), it remains unclear how the endogenous opioid system might persistently repress pathological pain. Opiate administration provides powerful pain relief, but repeated administration leads to the development of compensatory neuroadaptations underlying opiate tolerance and dependence (9), including the selective upregulation of calcium-sensitive AC isoforms (10, 11). Cessation of opiates leads to cellular and behavioral symptoms of withdrawal (12–16). An intriguing hypothesis of drug addiction suggests that chronic opiates increase MOR constitutive activity (MOR_{CA}) to preserve physical and psychological dependence (17–21), which is enhanced by enkephalins (22). Whether MORs adopt constitutive signaling states in other disease syndromes, such as chronic pain, is unknown. We tested the hypothesis that tissue injury increases MOR_{CA} in the spinal cord. With sufficient time after injury, enhanced basal MOR signaling should produce endogenous cellular and physical dependence in the CNS.

We first discovered that spinal opioid signaling promotes the intrinsic recovery of acute inflammatory pain and orchestrates long-lasting antinociception. In mice, a unilateral intraplantar injection of complete Freund's adjuvant (CFA) produced mechanical hyperalgesia that resolved within 10 d (Fig. 1A). Subcutaneous chronic minipump infusion of naltrexone hydrochloride (NTX), a non-selective opioid receptor antagonist, prolonged hyperalgesia throughout the 14 d infusion period in CFA-injured mice ($F_{3,17} = 25.4$; $P < 0.0001$; Fig. 1B), while having no effect in sham-injured mice. Upon NTX-pump removal, hyperalgesia rapidly declined. NTX did not alter the induction phase of CFA-induced hyperalgesia (fig. S1A–B; Supplementary Note 1); however, when delivered 21d after CFA, in the complete absence of pain, systemic NTX reinstated hyperalgesia ($F_{1,21} = 41$, $P < 0.0001$; Fig. 1C) in a dose-dependent manner with no effect in shams (Fig. 1D). By contrast, systemic injection of naltrexone methobromide (NMB), an opioid receptor antagonist that does not cross the blood brain barrier, failed to alter mechanical thresholds at either the ipsilateral or contralateral paws (both $P > 0.05$; Fig. 1E). Intrathecal administration of either NTX or NMB precipitated robust hyperalgesia in CFA-21d mice at both the injured ipsilateral paw ($P < 0.05$; Fig. 1F) and uninjured contralateral paw ($P < 0.05$; Fig. 1F), with no effect in shams (Fig. 1G). NTX also induced heat hyperalgesia ($P < 0.05$; Fig. 1H) as well as spontaneous pain in males ($P < 0.05$; Fig. 1I) and females (fig S3). Intrathecal NTX reinstated hyperalgesia in a model of post-surgical pain ($P < 0.05$; Fig. 1J) (23), several other models of inflammatory and neuropathic pain, and in multiple mouse strains (not shown).

Whether MOR-G-protein signaling can be maintained for sufficient duration to oppose chronic pain is unknown. First, we found that disruption of $G\alpha_{i/o}$ signaling with intrathecal injection of pertussis toxin precipitated hyperalgesia in CFA-21d but not shams ($P < 0.05$;

Fig. 1K). Second, we assessed guanosine-5'-O-(3-[35S]thio)triphosphate (GTP γ S³⁵) binding in fresh spinal cord slices (Fig. 1L and 1M). In control slices, the MOR-selective agonist DAMGO elicited a stimulation of GTP γ S³⁵ binding with an E_{max} and EC₅₀ of 58.02 ± 0.67% and 0.24 ± 0.01 μM, respectively (Fig. 1M). E_{max} was potentiated in CFA-21d slices, not only in the ipsilateral (79.85 ± 7.35%; *P* < 0.05 compared to sham; Fig. 1M) but also the contralateral (74.05 ± 4.13%; *P* < 0.05 compared to sham; fig. S4) dorsal horns, with no change in the EC₅₀. Third, the antinociceptive effects of intrathecal DAMGO were potentiated in CFA-21d mice (*P* < 0.05; Fig. 1N), reflecting increases in receptor number, receptor affinity, or descending modulatory circuits. Fourth, intrathecal injection of Phe-Cys-Tyr-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), a MOR-selective antagonist, reinstated hyperalgesia in CFA-21d mice but not shams (*P* < 0.05; Fig. 1M).

We next asked whether central sensitization (increased responsiveness of CNS nociceptive neurons to normal or sub-threshold afferent input) persists in the post-hyperalgesia state and remain under the control of endogenous MOR inhibitory mechanisms. Tested 21 days after CFA, innocuous light-touch of the injured hindpaw did not increase the dorsal horn expression of phosphorylated extracellular signal-regulated kinase (pERK) (Fig. 1Q and 1S). However, intrathecal NTX increased touch-evoked pERK in lamina I–II (*P* < 0.05; Fig. 1R and 1S, fig. S4) and III–V (*P* < 0.05; Fig. 1R and 1S). NTX also increased pERK at the contralateral dorsal horn (7.34 ± 1.37 cells per slice, *P* < 0.05; Fig. 1S and 1T). Confocal microscopy revealed that pERK was expressed in neurons (Fig. 1U–W, fig. S5), but not in microglia or astrocytes (fig. S5).

We next tested the hypothesis that N-methyl-D-aspartate receptor (NMDA-R)–Ca²⁺-dependent mechanisms of central sensitization (1, 24) continue to operate after the resolution of inflammatory pain. Using live-cell Fura-2 ratiometric analysis in adult spinal cord slices (25), we found that glutamate-evoked [Ca²⁺]_i in lamina II neurons was potentiated at 3 d after CFA and then resolved by day 21 (*F*_{3,17} = 15, *P* < 0.0001; Fig. 2A); this coincides with the temporal onset and resolution of inflammatory hyperalgesia. Perfusion of either CTOP or NTX increased the peak amplitude of glutamate-evoked [Ca²⁺]_i in CFA-21d but not sham slices (Lamina II: *P* < 0.05; Fig. 2B and 2C and fig. S6; Lamina I: *P* < 0.05, fig. S7) or CFA-24hr slices (fig. S1C). The activity-dependent NMDA-R blocker, MK-801, prevented the NTX-mediated rise in [Ca²⁺]_i (*F*_{1,16} = 4.6, *P* < 0.05; Fig. 2C), hyperalgesia (*F*_{3,22} = 6.5, *P* < 0.005; Fig. 2D) and dorsal horn pERK levels (*P* < 0.05; Fig. 2E, ipsilateral and fig. S8, contralateral).

Opioids produce their acute actions in part through inhibition of adenylyl cyclases (ACs), whereas chronic opiate exposure produces a homeostatic upregulation of ACs (9, 14). In this opioid-dependent state, receptor antagonists produce cellular withdrawal, characterized by an adenosine 3',5'-cyclic monophosphate (cAMP) overshoot response. To determine whether similar homeostatic mechanisms operate in the setting of tonic opioid receptor signaling after injury, we sampled intracellular cAMP content from *ex vivo* lumbar spinal tissue. Basal spinal cAMP levels were comparable in sham and CFA-21d mice, suggestive of a return to baseline AC function (Fig. 2F; Supplementary Note 2). In CFA-21d mice, however, intrathecal CTOP or NTX increased cAMP levels in CFA-21d mice (*P* < 0.05; Fig. 2F), indicative of AC superactivation. Because the Ca²⁺ stimulated isoforms of ACs are

activated by NMDA-Rs (26), we hypothesized that NMDA-R signaling contributes to this cAMP overshoot. Intrathecal MK-801 abolished the NTX-precipitated increases in cAMP ($P < 0.05$ compared to NTX group; Fig. 2G). Moreover, direct activation of spinal NMDA-Rs and ACs by intrathecal NMDA or forskolin, respectively, increased nocifensive behaviors ($P < 0.05$; Fig. 2H and 2J) and spinal cAMP levels ($P < 0.05$; Fig. 2I and 2K) in CFA-21d mice as compared to shams, suggesting latent upregulation, but not occlusion, of NMDA-R–AC1 pathways.

Adenylyl cyclase type 1 (AC1) in the brain is intricately linked to morphine dependence (27, 28) and chronic pain (29), while in the spinal cord it contributes to activity-dependent LTP (30). Baseline mechanical thresholds were similar in wild-type and AC1 knockout mice (AC1^{-/-}) (29) (Fig. 2L). However, AC1 gene deletion reduced inflammatory hyperalgesia (3d vs. baseline: $P < 0.05$, t test; $F_{1,11} = 31.5$, $P < 0.0005$, Genotype X Time; Fig. 2L), without affecting edema (fig. S9). At day 21 after CFA, NTX reinstatement was lost in AC1^{-/-} mice ($F_{1,7} = 20.3$, $P < 0.005$; Fig. 2M). Furthermore, intrathecal NB001, a selective AC1 inhibitor (30), prevented NTX-reinstatement of hyperalgesia ($F_{1,9} = 6.6$, $P < 0.05$; Fig. 2N), and cAMP overshoot and spontaneous pain ($P < 0.05$; Fig. 2O–P). These data suggest that withdrawal from tonic MOR signaling increases pronociceptive neural excitability consequent to AC1 superactivation (Fig. 2Q).

Tonic MOR signaling arises from either continuous agonist stimulation or constitutive (agonist-independent) activity (31–34). MOR_{CA} develops with chronic morphine administration, leading to physical and affective signs of opiate dependence and addiction (17, 19–22). To determine the existence and physiologic significance of MOR_{CA} in pathological pain processing, we utilized the neutral antagonist 6 β -naltrexol, a structural analog of NTX (35). Intrathecal 6 β -naltrexol alone did not change Ca²⁺ levels in sham or CFA-21d spinal slices (Fig. 3A), and failed to precipitate a cAMP overshoot (Fig. 3B) or hyperalgesia (Fig. 3C). 6 β -naltrexol abolished the ability of NTX to produce Ca²⁺ mobilization ($P < 0.05$; Fig. 3A), cAMP overshoot ($P < 0.05$; Fig. 3B), and hyperalgesia in CFA-21d mice ($P < 0.05$; Fig. 3C). 6 β -naltrexol also abolished NTX-induced reinstatement of mechanical hyperalgesia in a postoperative pain model (23) (Fig. 3D). These data suggest that NTX acts as an inverse agonist to inactivate MOR_{CA} in multiple models of inflammatory pain (Supplementary Notes 3 and 4) (36, 37).

Intrathecal administration of an alternative μ -selective inverse agonist, β -funaltrexamine (β -FNA) (38), reinstated hyperalgesia in CFA-21d but not sham mice ($P < 0.05$; Fig. 3E). Because MOR_{CA} results in elevated basal G-protein cycling (19, 38), we determined whether β -FNA could promote the MOR-inactive state and thereby decrease spontaneous basal GDP/GTP γ S³⁵ exchange. β -FNA concentration-dependently reduced basal GTP γ S³⁵ binding in dorsal horn sections from CFA-21d mice and, to a significantly lesser degree, sham-injured mice, in both ipsilateral and contralateral dorsal horns (Fig. 3F and 3G and fig. S11).

Pain comprises sensory (hyperalgesia) and affective (aversiveness) components; the latter can be identified by changes in the rewarding property of analgesics and associated motivational behavior. In a conditioned place preference paradigm (39–41), the negative

reinforcing capacity of intrathecal lidocaine (motivation to seek pain relief) demonstrates the presence of aversive pain 1d after CFA (40). This aversive component was absent at 21d (Fig. 4A; 'CFA-21d+saline' group). CFA-21d but not sham mice responded to systemic naloxone by spending more time in the chamber paired with intrathecal lidocaine (538 ± 39 s) than with intrathecal saline (283 ± 28 s; $P < 0.001$; Fig. 4A). Systemic NTX, but not saline or NMB, precipitated numerous escape and somato-motor behaviors analogous to classical morphine withdrawal (42, 43) in CFA-21d mice, with no effect in shams (Fig. 4B and 4C).

To determine if pain sensitization and endogenous opioid physical dependence persist beyond tissue healing, we gave periodic injections of NTX during and after the course of inflammatory edema, which subsided within 77 d after CFA (Fig. 4D). NTX, but not saline, reinstated hyperalgesia for at least 105d post-CFA (21d: $F_{1,80} = 8.5$, $P < 0.05$; 49d: $F_{1,72} = 59$, $P < 0.0001$; 77d: $F_{1,72} = 76$, $P < 0.0001$; 105d: $F_{1,64} = 33$, $P < 0.0001$; Fig. 4E). This was true at 200d post-CFA (fig. S12; Supplementary Note 5), and after a single intrathecal injection 105d post CFA, without prior exposure of the animal to the testing environment, of NTX or CTOP (fig. S12). NTX-precipitated escape-jump frequency increased with time after the injury ($F_{4,29} = 14$, $P < 0.0001$; Fig. 4F), suggesting that intensifying opioidergic and compensatory neuroadaptations create a physical and psychological dependence that greatly outlasts acute pain and tissue injury (Supplementary Note 6).

These data indicate that blockade of MOR_{CA} unmasks a silent AC1 central sensitization pathway that persists beyond the resolution of pain and inflammation, reflective of hyperalgesic priming (44). The presence of contralateral spinal MOR_{CA} and neural sensitization illustrates the spread of this pathology to areas of the CNS beyond those directly innervated by the injured tissue. Thus MOR_{CA} might tonically repress wide-spread hyperalgesia (Supplemental Note 7). If true, then loss of MOR_{CA} antinociception (e.g. during stress) could lead to the emergence of rampant chronic pain (45, 46).

We have identified an injury-induced MOR_{CA} that promotes both endogenous analgesia and dependence. Our data suggest that long-term MOR_{CA} inhibition of AC1-mediated central sensitization drives a counter-adaptive, homeostatic increase in pronociceptive AC1 signaling cascades (29, 47), thereby paradoxically promoting the maintenance of latent central sensitization. Thus, injury produces a long-lasting dependence on MOR_{CA} that tonically prevents withdrawal hyperalgesia, consistent with proposed mechanisms of dependence to opiate drugs such as morphine (27, 48). We contend that loss of MOR_{CA}, and the ensuing reinstatement of pain reflects a process of spinal cellular withdrawal (NMDA-mediated AC1 superactivation) to enhance pronociceptive synaptic strength (Supplemental Note 8) (49, 50), as observed following NMDA-R-dependent spinal LTP at C-fiber synapses during withdrawal from exogenous opiates (12). Indeed, stress (46) or injury (51) escalates opposing inhibitory and excitatory influences on nociceptive processing, as a pathological consequence of increased endogenous opioid tone. This raises the prospect that opposing homeostatic interactions between MOR_{CA} analgesia and latent NMDA-R-AC1 pain sensitization create a lasting susceptibility to develop chronic pain.

Supplementary Material

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Acknowledgments

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Fig. 1. Injury-induced pain sensitization is tonically opposed by spinal MOR-G-protein signaling (A) Progression of mechanical hyperalgesia following intraplantar CFA (5 μ l) ($n = 10$). (B) Resolution of hyperalgesia during and 14d after infusion of NTX (10 mg/kg/d, s.c.) in Sham and CFA mice ($n = 5-6$). $\star P < 0.05$ compared to CFA+saline, $\diamond P < 0.05$ compared to Sham+NTX. (C) Time course of reinstatement of hyperalgesia following subcutaneous NTX (3 mg/kg) in CFA-21d mice ($n = 6-13$). (D) Dose-response effects of NTX on hyperalgesia ($n = 6$ per dose). MPE: maximal possible effect. (E-F) Effect on hyperalgesia of (E) subcutaneous or (F) intrathecal NTX (3 mg/kg or 1 μ g) or NMB (3 mg/kg or 0.3 μ g) ($n = 5-10$). (G-J) Effect of NTX (1 μ g, i.t.) on reinstatement of (G) mechanical hyperalgesia in Sham and CFA mice ($n = 5-8$), (H) heat hyperalgesia ($n = 5-10$), (I) spontaneous pain ($n = 4-8$), and (J) post-operative pain ($n = 6-11$). (K) Effect of pertussis toxin (0.5 μ g, i.t.) on hyperalgesia ($n = 6$). (L) Representative radiograms and (M) dose-response effects of DAMGO-stimulated GTP γ S³⁵ binding in lumbar spinal cord; inset: binding E_{max} ($n = 7-9$). (N) Effect of DAMGO (i.t.) on hotplate latency ($n = 8$). (O) Effect of CTOP (100 ng, i.t.) on hyperalgesia ($n = 6-7$). (P-R) Representative images and (S) dorsal horn laminar quantification (I-II and III-V) of light touch-evoked pERK after NTX (1 μ g, i.t.) ($n = 5-7$). (T) Confocal image of pERK+ cells. (U-W) From boxed region in panel T: Co-localization of pERK with NeuN. All scale bars = 200 μ m. $\star P < 0.05$ for all panels. All data shown as mean $\pm$ s.e.m. See fig. S1 for full time course data of panels E-J, O.

Fig. 2. Pain reinstatement requires potentiated N-methyl-D-aspartate receptor activation of calcium-sensitive adenylyl cyclase type 1

(A) Time course of glutamate-evoked (0.3 mM) $[Ca^{2+}]_i$ in spinal cord slices from sham and CFA mice ($n = 4-7$ mice). (B) Effect of CTOP (1 μ M), NTX (10 μ M) or NTX+MK-801 (100 μ M) on $[Ca^{2+}]_i$. Values are relative to pre-drug control responses ($n = 3-5$ mice). (C) Representative F_{380} nm image of dorsal horn neurons from a CFA-21d slice responding to glutamate before (top) and after NTX (10 μ M, bottom) (yellow arrows). Decrease in fluorescence intensity corresponds to increase in $[Ca^{2+}]_i$. The red traces illustrate the rise in $[Ca^{2+}]_i$ for the indicated cell (red arrow). Insets depict area in white box. Scale bars: 0.02

F/F (vertical) and 3 min (horizontal), and 100 μ m and 10 μ m (inset). Effect of MK-801 (1 μ g, i.t.) on NTX-precipitated (1 μ g, i.t.) (D) hyperalgesia and (E) touch-evoked dorsal horn pERK expression ($n = 5-10$). (F) Spinal cord cAMP levels after intrathecal vehicle ($n = 14-18$), CTOP (100 ng; $n = 6$) or NTX (1 μ g; $n = 6-10$). (G) Effect of MK-801 (1 μ g, i.t.) on NTX-precipitated spinal cAMP overshoot ($n = 5$). (H-K) Effect of intrathecal NMDA (3 pmol; $n = 5-7$) or forskolin (1.5 μ g; $n = 6$) on (H and J) spontaneous nocifensive behaviors and (I and K) spinal cAMP levels. (L) Progression of mechanical hyperalgesia and (M) effect of intrathecal NTX in $AC1^{-/-}$ and wild-type mice 21d after CFA ($n = 5-9$). Effect of NB001 (1.5 μ g, i.t.) on NTX-precipitated (N) hyperalgesia ($n = 4-7$), (O) spinal cAMP levels ($n = 6-9$), and (P) affective pain ($n = 6-12$). (Q) Schematic of cellular pathways involved in endogenous opioid withdrawal and pain reinstatement. $\star P < 0.05$.

Fig. 3. Spinal MORs acquire constitutive activity after injury

(A,B,C) Effects of NTX (1 μ M or 1 μ g), 6 β -naltrexol (1 μ M or 10 μ g) or co-administration of 6 β -naltrexol+NTX in sham and CFA-21d mice on (A) $[Ca^{2+}]_i$ in spinal cord slices ($n = 5-6$), (B) spinal cAMP levels ($n = 6-11$) and (C) hyperalgesia ($n = 6-7$). (D) Effect of intrathecal 6 β -naltrexol and/or NTX on hyperalgesia in Paw Incision-21d mice ($n = 6-7$). (E) Effect of intrathecal β -funaltrexamine (β -FNA; 2.5 μ g) on hyperalgesia ($n = 6-7$). (F) Representative radiograms and (G) dose-response effects of β -FNA on basal GTP γ S³⁵ binding in lumbar ipsilateral dorsal horn; inset: binding E_{max} ($n = 7-9$). ★ $P < 0.05$. All data shown as mean $\pm$ s.e.m. See fig. S9 for full time course data of panels C-E.

Fig. 4. Prolonged endogenous MOR signaling generates psychological and physical dependence (A) Behavioral signs of psychological withdrawal (aversion associated with spontaneous pain), reflected by place preference for intrathecal lidocaine upon naloxone administration. *Left:* Intragroup chamber analysis for intrathecal saline (5 μ l) or lidocaine (0.04%) in sham and CFA-21d mice treated with intraperitoneal saline or naloxone (3 mg/kg). *Right:* Intergroup difference score analysis illustrating time spent in intrathecal lidocaine-paired chambers. ($n = 6$ per group). (B,C) Behavioral signs of physical withdrawal, recorded for 60 min after injection of NTX (3 mg/kg), NMB (3 mg/kg) or vehicle. ($n = 6 - 7$). (D) Progression paw edema and (E) effects of repeated subcutaneous vehicle or NTX (3 mg/kg) on hyperalgesia over 105 days after CFA. ($n = 7$ per group) (F) Effect of repeated NTX (3 mg/kg) on the number of precipitated escape jumps over 77 days after CFA. ($n = 8$). ★ $P < 0.05$. All data shown as mean $\pm$ s.e.m.