ANESTHESIOLOGY

Combination of a δ-opioid Receptor Agonist and Loperamide Produces Peripherally-mediated Analgesic Synergy in Mice

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The adverse effects of opioids are largely mediated by central μ-opioid receptors
- Central μ- and δ-opioid receptors synergistically provide analgesia

What This Article Tells Us That Is New

- The administration of a selective δ-opioid agonist, oxymorphindole, and a peripherally-restricted μ-agonist, loperamide, provided synergistic analgesia in a mouse inflammatory pain model
- The use of combinations of peripherally-restricted opioid ligands may provide analgesia with reduced side effects when compared with centrally acting opioids

ABSTRACT

Background: The long-term use of opioids for analgesia carries significant risk for tolerance, addiction, and diversion. These adverse effects are largely mediated by μ-opioid receptors in the central nervous system. Based on the authors' previous observation that morphine and δ-opioid receptor agonists synergize in spinal cord in a protein kinase Cε–dependent manner, they predicted that this μ-opioid receptor–δ-opioid receptor synergy would take place in the central terminals of nociceptive afferent fibers and generalize to their peripheral terminals. Therefore, the authors hypothesized that loperamide, a highly efficacious μ-opioid receptor agonist that is excluded from the central nervous system, and oxymorphindole, a δ-opioid receptor agonist that was shown to synergize with morphine spinally, would synergistically reverse complete Freund's adjuvant–induced hyperalgesia.

Methods: Using the Hargreaves assay for thermal nociception, the von Frey assay for mechanical nociception and the complete Freund's adjuvant–induced model of inflammatory pain, we tested the antinociceptive and antihyperalgesic effect of loperamide, oxymorphindole, or the loperamide–oxymorphindole combination. Animals (Institute for Cancer Research [ICR] CD1 strain mice; n = 511) received drug by systemic injection, intraplantar injection to the injured paw, or a transdermal solution on the injured paw. Dose–response curves for each route of administration and each nociceptive test were generated, and analgesic synergy was assessed by isobolographic analysis.

Results: In naïve animals, the loperamide–oxymorphindole combination ED_{50} value was 10 times lower than the theoretical additive ED_{50} value whether given systemically or locally. In inflamed animals, the combination was 150 times more potent systemically, and 84 times more potent locally. All combinations showed statistically significant synergy when compared to the theoretical additive values, as verified by isobolographic analysis. The antihyperalgesia was ablated by a peripherally-restricted opioid antagonist.

Conclusions: From these data we conclude that the loperamide–oxymorphindole combination synergistically reverses complete Freund's adjuvant–induced inflammatory hyperalgesia. The authors also conclude that this interaction is mediated by opioid receptors located in the peripheral nervous system.

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Chronic pain is generally thought of as pain without apparent biologic value, persisting beyond normal tissue healing time, and not amenable to treatments based on specific remedies.[1] The gold standard for the treatment of chronic pain has been opioids (*e.g.*, morphine, oxycodone *etc.*) that exert their analgesic effect through their interaction with the μ–opioid receptor, primarily in the central nervous system (CNS). Long–term use of centrally active opioids carries substantial risk for undesirable effects, namely tolerance, addiction, hyperalgesia, and respiratory depression. In the peripheral nervous system, μ–opioid receptors are expressed by nociceptive dorsal root ganglion neurons,[2] as well as the peripheral terminals of primary afferent fibers,[3]

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and are known to modulate nociceptive signaling.[4–7] These observations motivated development of opioid therapeutics with chemical properties either restricting their access to the CNS[8,9] or optimizing their activity in inflamed peripheral tissues.[10] Loperamide, a μ–opioid receptor agonist that is a substrate for the transport molecule P-glycoprotein and therefore unable to cross the blood–brain barrier, has been shown to have weak analgesic and antihyperalgesic properties on its own,[9,11] thought to be mediated at the peripheral terminals of afferent fibers. Loperamide is available over the counter for control of diarrhea and manifests a therapeutic index as an analgesic relative to constipation of about unity, rendering it ineffective as a pain therapeutic in humans. Fortunately, μ–opioid agonists synergize with select other analgesics when coadministered.[12] In combination they exhibit a statistically significant shift in potency, allowing subtherapeutic doses of two drugs to be given to achieve full antinociception or antihyperalgesia. Importantly, interactions between analgesics have been shown not to cause a parallel synergy in side effects such as sedation and cardiovascular effects.[13] In theory, therefore, analgesic synergy may provide peripheral opioid-mediated antinociception without adverse effects in the CNS or the gut.

Our laboratory recently published that the μ–opioid receptor agonist morphine synergized with the δ–opioid receptor agonist oxymorphindole[14] when administered intrathecally.[15] It is known that opioids exert their analgesic effect partly by binding opioid receptors on the central terminals of primary afferent fibers in the spinal dorsal horn. Importantly, the synergism in this study was dependent on the epsilon isoform of protein kinase C, an intracellular signaling molecule present in 95% of primary afferent fibers.[15] We reasoned that, because the synergistic interaction depended on intracellular actions, μ–opioid receptors and δ–opioid receptors must colocalize in individual neuronal compartments, possibly the central terminals of primary afferent neurons. Because primary afferents are pseudo-unipolar neurons, with terminals in both the CNS and peripheral nervous system, it follows that μ–opioid receptors and δ–opioid receptors might also colocalize on the peripheral terminals of these neurons. Therefore, we investigated whether μ–opioid receptor–δ–opioid receptor synergy between loperamide and oxymorphindole could be (1) localized electrophysiologically to the central terminals of nociceptors, and (2) demonstrated at the peripheral terminals of nociceptors by measuring thermal withdrawal latencies using the complete Freund's adjuvant–induced mouse model of inflammatory pain. We hypothesized that the combination of loperamide and oxymorphindole would synergistically reverse complete Freund's adjuvant–induced hyperalgesia.

Materials and Methods

Animals

For behavioral experiments, adult male Institute for Cancer Research (ICR) CD1 mice (25 to 35 g; n = 511) were housed four to a cage and maintained on a 12-h light/dark cycle, with *ad libitum* access to food and water. Testing was performed during the light phase. The University of Minnesota Institutional Animal Care and Use Committee (Minneapolis, Minnesota) approved all protocols employing animals. Immediately following experiments, animals underwent carbon dioxide euthanasia.

For electrophysiologic experiments, $Na_v1.8\text{-}ChR2^+$ mice were created as described previously.[16] Conditional expression of channelrhodopsin-2 (ChR2) was targeted to $Na_v1.8+$ sensory neurons by crossing heterozygous $Na_v1.8–$ channelrhodopsin-2 mice with wild-type C57BL/6 mice purchased from Jackson Laboratories (USA). This cross yielded approximately 10% of offspring expressing channelrhodopsin-2, and $Na_v1.8\text{-}ChR2^+$ mice were identified by phenotyping for a nocifensive reaction to illumination of hind paws with 470 nm light (5 mW/mm²) from a light-emitting diode (Plexon, USA) with an attached fiber optic cable.

Spinal Cord Slicing Procedure

$Na_v1.8\text{-}ChR2^+$ mice (N = 8) were anesthetized using an overdose of isoflurane. Transcardial perfusion was performed using oxygenated (5% CO_2, 95% O_2), high sucrose/kynurenic acid artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 95, KCl 1.8, KH_2PO_4 1.2, $CaCl_2$ 0.5, $MgSO_4$ 7, $NaHCO_3$ 26, glucose 15, sucrose 50, and kynurenic acid 1. Spinal cords were removed *via* dorsal laminectomy, and the ventral and dorsal spinal roots were removed. The ventral side of the spinal cord was glued to an agar block and sliced into 400 μm sections using a vibrating microtome (VT1200S; Leica Biosystems, Germany). Slices were incubated at 37°C for 1 h in oxygenated aCSF containing (in mM): NaCl 127, KCl 1.8, KH_2PO_4 1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, and glucose 15. Slices were then moved to a chamber with oxygenated aCSF at room temperature until recordings were performed.[17]

Electrophysiologic Recordings

Slices were placed in a recording chamber and perfused with oxygenated aCSF at 30°C. Glass patch pipettes were filled with a solution containing (in mM): K-gluconate 135, KCl 5, $MgCl_2$ 2, $CaCl_2$ 0.5, HEPES 5, EGTA 5, ATP-Mg 5, and GTP-Na 0.5. Lamina I/II neurons were visualized using differential interference contrast optics on an Olympus BX50WI (USA) microscope, and whole cell patch clamp configuration was obtained. An Axopatch 200b amplifier (Molecular Devices, USA) was used to record membrane currents at a holding potential of –65 mV. Miniature excitatory postsynaptic currents were acquired using a Digidata 1322A and PClamp 8.0 software (Molecular Devices), and miniature excitatory postsynaptic current data were analyzed using MiniAnalysis 6.0.7 (Synaptosoft, USA). α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–mediated miniature excitatory postsynaptic currents were isolated by perfusing the slice with aCSF

containing tetrodotoxin 1 μM, picrotoxin 100 μM, DL-APV 100 μM, and strychnine 5 μM. To evoke glutamate release from $Na_v1.8$-expressing primary afferent terminals, 470 nm light (0.75 mW/mm^2) was continuously shone on the slice through the $40\times$ objective. Data obtained in our lab not shown here indicate that continuous illumination of tissue results in a consistent and static increase of miniature excitatory postsynaptic current frequency over a 20 min period. Next, increasing concentrations of drug were included in the bath at 3-min intervals to yield cumulative concentration–response curves.

Drug Preparation and Administration

The compounds used were: loperamide HCl (Sigma, USA), oxymorphindole HCl,[14] β–funaltrexamine,[18] naltrindole,[19] and naloxone methiodide (Sigma). Stock solutions were prepared with 5% Cremophor EL and 5% dimethyl sulfoxide in H_2O. All drugs were diluted to testing concentrations with 0.9% sterile saline (or aCSF for electrophysiology) such that final Cremophor and dimethyl sulfoxide concentrations were less than 1%; the topical vehicle was further diluted 1:1 with 95% ethanol. The routes and volumes of administration were: intrathecal, 5 μL; intraplantar, 30 μL; subcutaneous, 10 μL per gram; and topical, 20 μL. Intrathecal injections were performed as previously described.[20] For intraplantar injections and topical administration, animals were lightly anesthetized with 2.5% isoflurane and the drugs were administered to the left hind paw.

Behavioral Measures

Central thermal antinociception was assessed using intrathecal injections[20] and the warm water (52.5°C) tail immersion assay as described previously.[21] Briefly, each animal was gently held wrapped in a cloth and the tail dipped into a controlled temperature water bath. Withdrawal latency was recorded as the amount of time that passed before a rapid movement of the tail; cutoff was set to 12 s. Baseline latency was recorded before drug administration, and subsequent latencies were recorded 7 min after each dose, immediately before the next dose. Each agonist or combination was administered sequentially approximately every 7 min in increasing doses to generate a cumulative dose–response curve; each mouse received no less than three and no more than four doses.[22]

Peripheral thermal nociception was assessed using the Hargreaves assay as described previously.[23] Briefly, animals were placed on a heated glass floor (30°C) and a small plastic box restricted their movement. After allowing the animals to acclimate to the testing environment for a minimum of 15 min, a radiant heat lamp was shone on the left hind paw until the animal withdrew the paw. Paw withdrawal latencies were measured by a plantar stimulator antinociception meter (IITC Lifesciences, USA), and a cutoff time of 20 s was used to prevent tissue damage. An average of three paw withdrawal latencies was taken, with a minimum of 30 s

between tests. Baseline latencies were recorded before drug administration, and subsequent latencies were recorded 15 min after injection for intraplantar and topical experiments, and 45 to 60 min after injection for subcutaneous experiments. Initial dose-ranging studies were not blinded; subsequent replicate experiments with an experimenter blinded to treatment yielded similar results.

Mechanical hypersensitivity was assessed using the von Frey assay. Briefly, mice were placed on a wire mesh grid under a glass enclosure and allowed to acclimate for 30 min before testing. Hypersensitivity was tested by using an electronic von Frey device (IITC Life Sciences). The tip of the stimulator was pressed to the plantar surface of both the left and right hind paws until the animal withdrew its paw from the tip, typically with a flinching behavior. The amount of force required for the response was recorded in grams by the IITC Lifesciences stimulator.

Respiratory depression was measured using a MouseOx Plus (Starr Lifesciences, USA). Briefly, animals were shaved on the back and sides of the neck, and recording collars were placed on the exposed skin. Animals were placed in beakers, where they were allowed free movement while readings were collected. Each animal was recorded for 1 h of baseline measurements before subcutaneous drug or vehicle injection. Following injection, oxygen saturation was measured for 1 h.[24,25]

Gastrointestinal motility was measured by counting the number of fecal pellets produced. Animals were given an subcutaneous injection of drug or vehicle and placed in a beaker for 1 h. After 1 h, animals were returned to their home cages, and the number of fecal pellets was manually counted.[26]

Complete Freund's Adjuvant–induced Hyperalgesia

After determining naïve paw withdrawal latencies, animals were lightly anesthetized using 2.5% isoflurane and an emulsion of complete Freund's adjuvant in saline (1:1; final volume 30 μL) was administered by intraplantar injection into the left hind paw. Three to five days after injection, a robust, inflammatory hyperalgesia was present, and hyperalgesic paw withdrawal latencies were determined.[27]

Topical Tolerance Paradigm

Animals were baselined on the Hargreaves assay, and subsequently given a unilateral injection of complete Freund's adjuvant into the left hind paw. Three days after administration of complete Freund's adjuvant, animals were randomized to two groups, receiving twice daily topical administration of vehicle or drug. After 4 days, all animals received sequential topical administrations of drug to generate cumulative concentration-response curves.

Data Analysis

For electrophysiologic experiments, data were analyzed as a percentage inhibition of blue light–evoked responses, given

by the equation: % inhibition = ([blue light frequency − baseline frequency] − [experimental frequency − baseline frequency])/(blue light frequency − baseline frequency) × 100. For behavioral experiments with noninflamed subjects, data were analyzed as a percentage of maximum possible effect (% MPE), given by the equation: % MPE = (experimental value − baseline)/(cutoff − baseline) × 100. For injury models, data were analyzed as a percentage of antihyperalgesia (% AH), given by the equation: % AH = ([postinjury value − baseline] − [experimental value − baseline])/(postinjury value − baseline) × 100. The ED_{50} of all agonists and combinations was calculated using the graded dose–response curve method.[28] Dose ratios for drug combinations were estimated based on comparison of previously determined single drug ED_{50} values. Two-tailed isobolographic analyses were performed using the numerical method,[29,30] as implemented by the JFlashCalc Pharmacologic Calculations Program software package (http://u.arizona.edu/~michaelo/jflashcalc.html; accessed December 12, 2018). *P* values for isobolographic analysis were determined by the JFlashCalc software program. For all isobolograms, error bars for theoretical additive and observed combination ED_{50} values represent the vector sum of vertical and horizontal confidence limits. Error bars in isobolograms are presented as 95% CI, and error bars for all other data are presented as SD. For analysis between multiple groups, either an ordinary one-way ANOVA or a repeated measures two-way ANOVA was performed. ANOVA calculations were performed using GraphPad Prism 7.0, and used the Shapiro–Wilk normality test, and Bonferroni multiple comparisons test where applicable. For all data, *P* values less than 0.05 were considered statistically significant. No statistical power calculation was conducted prior to the study, and the sample sizes for all experiments were based on our extensive previous experience with this design. No randomization methods were used to assign subjects to treatment groups, and animals were tested in sequential order. No animals were excluded from the study, and the data were monitored for statistical outliers, but no data were excluded from the analyses presented here.

Results

Having previously shown that morphine and oxymorphindole synergize spinally,[15] we first sought to assess whether loperamide would also synergize with oxymorphindole in an antinociceptive assay when given spinally. Figure 1A shows cumulative dose-response curves in naïve mice after an intrathecal injection. Either loperamide or oxymorphindole (0.1 to 10 nmol) given alone produced antinociception in the hot water tail flick assay; the 1:1 combination (0.01 to 1 nmol) was similarly effective. The ED_{50}s of the individual drugs were 5.4 nmol (loperamide) and 3.5 nmol (oxymorphindole), and the ED_{50} of the combination was 0.6 nmol (n = 6 per group). This measured ED_{50} for the combination differed significantly from the expected additive ED_{50} (*P* < 0.0001),

indicating that loperamide and oxymorphindole synergize when delivered spinally. This interaction is represented graphically by the isobologram in figure 1B.

Having demonstrated that loperamide and oxymorphindole synergized spinally, we sought to determine whether this interaction was mediated in the central terminals of primary afferent nociceptive fibers in the dorsal horn. Therefore, we conducted whole cell patch clamp recordings in spinal cord neurons located in the superficial laminae of the lumbar dorsal horn. For these recordings we used a transgenic mouse line bred to express channelrhodopsin-2, a light-activated cation channel, under the control of the promoter for the Nav1.8 isoform of voltage-gated sodium channels. Nav1.8 is primarily expressed by nociceptive afferents, and the majority of light-responsive fibers in this mouse line had been shown to be C polymodal nociceptors.[31] Therefore, we measured the frequency and amplitude of miniature excitatory postsynaptic currents driven by 470 nm light illuminating the field around the neuron being recorded through the 40× objective as a measure of presynaptic nociceptive afferent activity. Figure 1C shows representative traces of the baseline miniature excitatory postsynaptic current frequency, the frequency in the presence of blue light stimulation and the frequency in the presence of blue light and the loperamide–oxymorphindole combination (0.3 nM). Figure 1D shows the cumulative concentration-response curves for loperamide, oxymorphindole or a 1:1 combination to inhibit the miniature excitatory postsynaptic current frequency driven by blue light (n = 3 to 6 cells per drug or combination; N = 8). Either loperamide or oxymorphindole given alone inhibited miniature excitatory postsynaptic current frequency in a concentration-dependent manner, and the combination was 100-fold more potent than either drug given alone. This shift in potency was confirmed to be synergistic by isobolographic analysis (*P* = 0.002; fig. 1E). We interpret these data to indicate that loperamide and oxymorphindole are binding their respective receptors on the presynaptic terminals of primary afferents and inhibiting the release of glutamate from these central terminals. By the same token, we take the combination's shift in potency to indicate that the synergy between loperamide and oxymorphindole is also mediated within these central terminals.

Next, we tested the hypothesis that loperamide and oxymorphindole would display antinociceptive synergy when administered in the periphery, *i.e.,* on the peripheral terminals of nociceptors in the skin. To test this hypothesis, either drug or the 1:1 combination was given as an intraplantar injection in the hind paw of mice, and thermal nociceptive responses were tested on the Hargreaves assay 15 min later. Figure 2A shows the dose-response curves for loperamide, oxymorphindole, and their 1:1 combination in naïve mice. Consistent with the interaction observed spinally, the combination ED_{50} is approximately 10-fold less than either drug alone: combination ED_{50} value 4.6 nmol *versus* 57 nmol
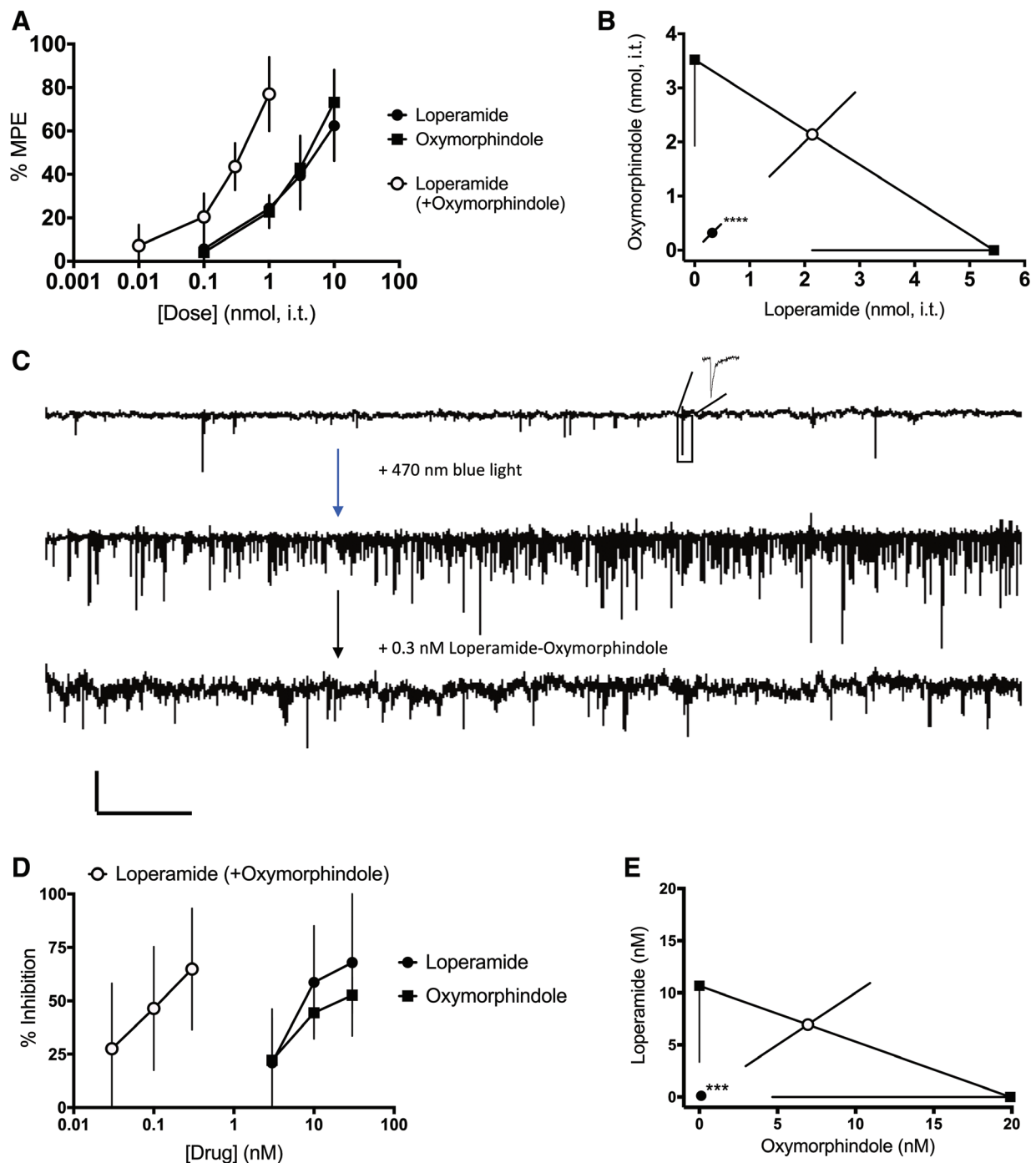
Fig. 1. Loperamide and oxymorphindole synergize on primary afferents in the spinal cord. (*A*) Dose–response curves showing the ability of loperamide, oxymorphindole, or their 1:1 combination to block nocifensive responses in the hot water tail flick assay (n = 6 animals per group). Data are shown as % MPE ± SD. (*B*) Isobolographic analysis of data from A, demonstrating a synergistic interaction. (*C*) Representative traces of mEPSC frequency at baseline and following application of 470 nm blue light. Scale bars 20 pA by 2.1 s. Inset shows a representative mEPSC. (*D*) Concentration-response curves showing the ability of loperamide, oxymorphindole, or their combination (1:1) to inhibit mEPSC frequency (n = 3 to 6 cells per group). Data are shown as a % inhibition. (*E*) Isobolographic analysis of the data from D, demonstrating a synergistic interaction. ***$P < 0.001$, ****$P < 0.0001$. mEPSC, miniature excitatory postsynaptic current; MPE, maximum possible effect.
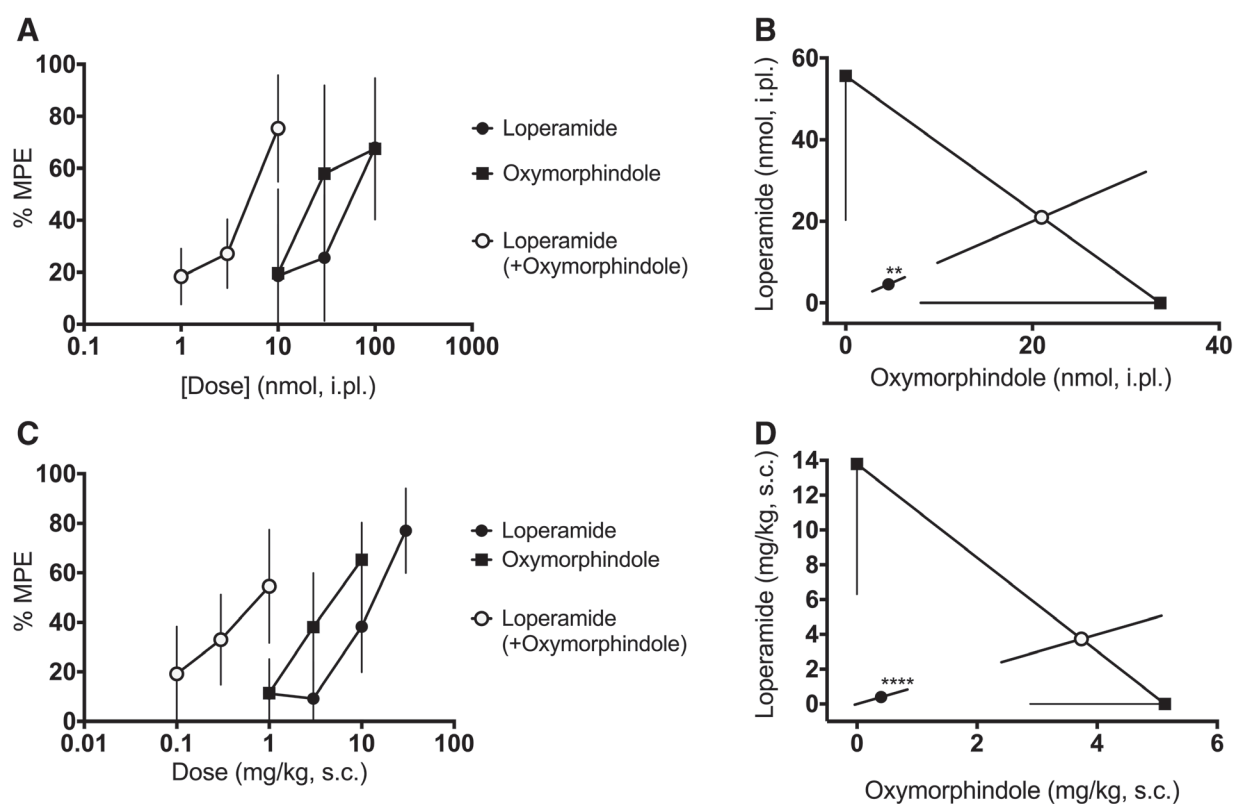
Fig. 2. Locally and systemically administered loperamide–oxymorphindole synergizes in naïve animals. (*A*) Dose–response curves showing the ability of intraplantar loperamide, oxymorphindole or their combination (1:1) to inhibit nociceptive responses in the Hargreaves assay (n = 6 per dose). Data are shown as % MPE ± SD. (*B*) Isobolographic analysis of the data from *A*, showing a synergistic interaction. (*C*) Dose–response curves for subcutaneous loperamide, oxymorphindole, or their combination (1:1; n = 5 per dose). Data are shown as % MPE. (*D*) Isobolographic analysis of the data from *C*, demonstrating a synergistic interaction. **$P < 0.01$, ****$P < 0.0001$. i.pl., intraplantar; MPE, maximum possible effect; s.c., subcutaneous.

for loperamide and 34 nmol for oxymorphindole (n = 6 per dose). This shift in potency was significantly synergistic ($P = 0.002$), as demonstrated in figure 2B. Next, we assessed the ability of systemically administered loperamide and oxymorphindole to synergize in naïve animals. Drugs were given as a subcutaneous injection, and nociceptive responses were measured on the Hargreaves assay 45 min later. The dose–response curves are shown in figure 2C. With the systemic route of administration, loperamide's and oxymorphindole's ED_{50} values were 14 mg/kg and 5.1 mg/kg, respectively, while the combination ED_{50} was 0.8 mg/kg (n = 5 per dose). This interaction was statistically validated as a synergistic interaction ($P < 0.0001$), as shown in figure 2D, with the shift in potency remaining at approximately 10-fold.

With the antinociceptive effects of the loperamide-oxymorphindole combination verified in central and peripheral terminals, and after systemic administration, we evaluated the ability of loperamide, oxymorphindole, or their combination to reverse an established inflammatory

pain state. Three to five days before testing, animals were given an intraplantar injection of complete Freund's adjuvant in the left hind paw, resulting in a robust inflammatory state and hyperalgesic thermal withdrawal latencies on the Hargreaves assay. After the confirmation of hyperalgesia, animals were treated with intraplantar drug or combination as previously described. Figure 3A shows the dose-response curves for intraplantar loperamide, oxymorphindole or their combination in complete Freund's adjuvant–inflamed hind paws. The ED_{50} values were 12 nmol for loperamide, 6.4 nmol for oxymorphindole and 0.1 nmol for their 1:1 combination (n = 5 to 6 per dose). Again, this approximately 100-fold shift in potency observed for the combination was significantly different from the theoretical additive combination ED_{50} (fig. 3B; $P < 0.0001$). Following the paradigm of the naïve study, we next repeated this experiment with a systemic route of administration. After subcutaneous injection, the observed ED_{50} values for loperamide, oxymorphindole, and their combination were 2.4, 1.1, and 0.01 mg/kg, respectively (n = 4 to 6 per dose).
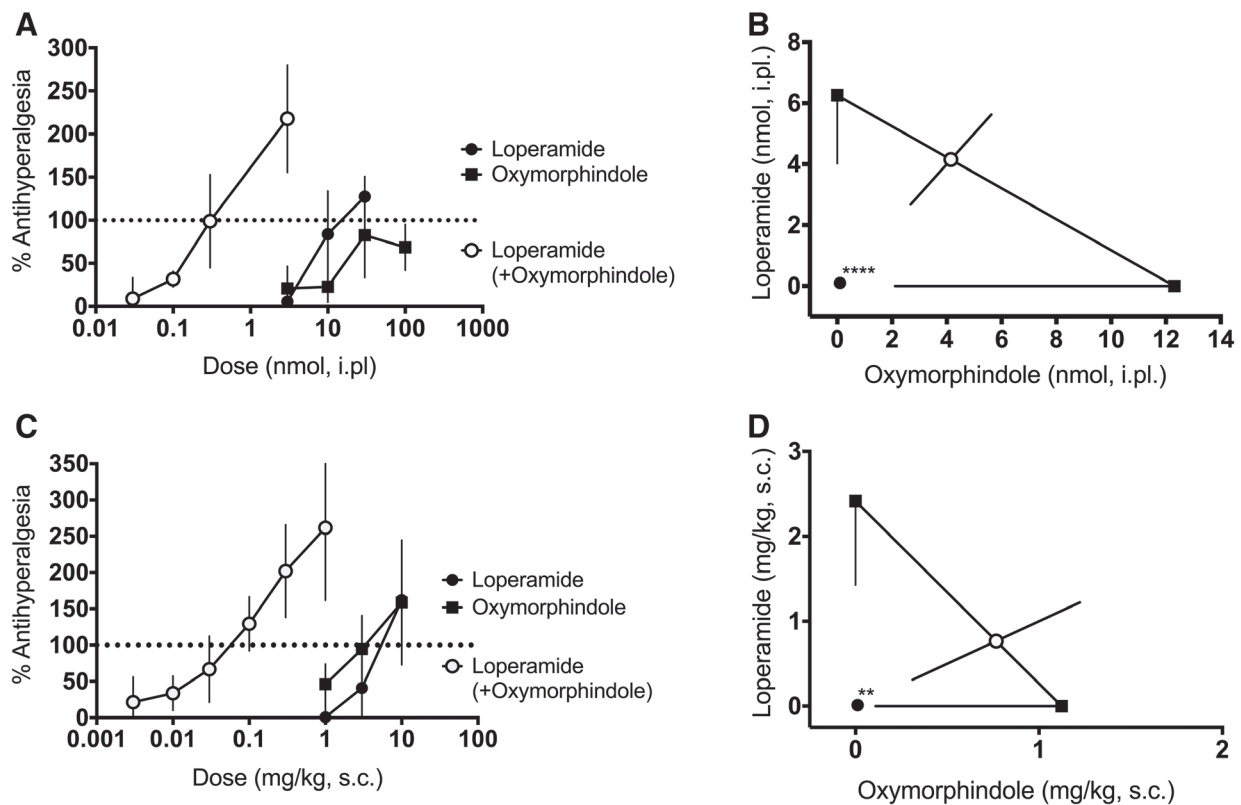

Fig. 3. Locally and systemically administered loperamide-oxymorphindole synergizes in inflamed animals. (*A*) Peripherally mediated thermal nociceptive responses in the Hargreaves assay were assessed. Following CFA-induced inflammation in the hind paw, subjects were given an intraplantar injection of loperamide, oxymorphindole or combination and postdrug nociceptive responses were shown as % antihyperalgesia, which was used to generate dose–response curves. (*B*) Isobolographic analysis of the data from *A*, showing a synergistic interaction compared to the theoretical additive ED_{50} value. (*C*) Dose–response curves for subcutaneous loperamide, oxymorphindole or combination following CFA-induced inflammation in the hind paw. Data are shown as % antihyperalgesia. (*D*) Isobolographic analysis of the data from *C*, demonstrating a synergistic interaction compared to the theoretical additive ED_{50} value. **$P < 0.01$, ***$P < 0.001$. CFA, complete Freund's adjuvant; i.pl., intraplantar; s.c., subcutaneous.

These dose–response curves are shown in figure 3C. The isobologram in figure 3D demonstrates that the interaction between systemically administered loperamide and oxymorphindole in inflamed mice is also synergistic ($P = 0.0013$), with an apparent leftward shift of 150-fold.

To confirm that the antinociceptive and antihyperalgesic effects observed in the previous experiments were being mediated by action at μ-opioid receptors and δ-opioid receptors, we tested the ability of a panel of opioid antagonists to block the synergism in complete Freund's adjuvant–inflamed mice. We chose naloxone methiodide, a pan-opioid antagonist that is peripherally-restricted; naltrindole, a δ-opioid receptor–selective antagonist; and β–funaltrexamine, a μ-opioid receptor–selective antagonist (n = 5 to 6 per dose). Naltrindole and naloxone methiodide were coadministered as an intraplantar injection with 0.3 nmol of the loperamide–oxymorphindole combination. β–Funaltrexamine was administered as an

intraplantar injection 24h before the combination. Animals were tested on the Hargreaves assay 15min after receiving the loperamide-oxymorphindole combination. When naloxone methiodide was coadministered with loperamide–oxymorphindole, there was a statistically significant reduction in antihyperalgesia, as calculated by ordinary one-way ANOVA ($P < 0.0001$; F[5, 44 = 30.91]). When we tested loperamide–oxymorphindole with naltrindole, we observed similar results. Coadministration of the combination with naltrindole resulted in a statistically significant reduction in antihyperalgesia (ordinary one-way ANOVA; $P < 0.0001$; F[5, 44] = 23.04). Finally, we pretreated animals with β–funaltrexamine 24h before loperamide–oxymorphindole administration, and in keeping with the other antagonists, we observed a statistically significant reduction in antihyperalgesia (ordinary one-way ANOVA; $P < 0.0001$; F[5, 44] = 20.59). The degree to which all the antagonists reversed loperamide-oxymorphindole's antihyperalgesia
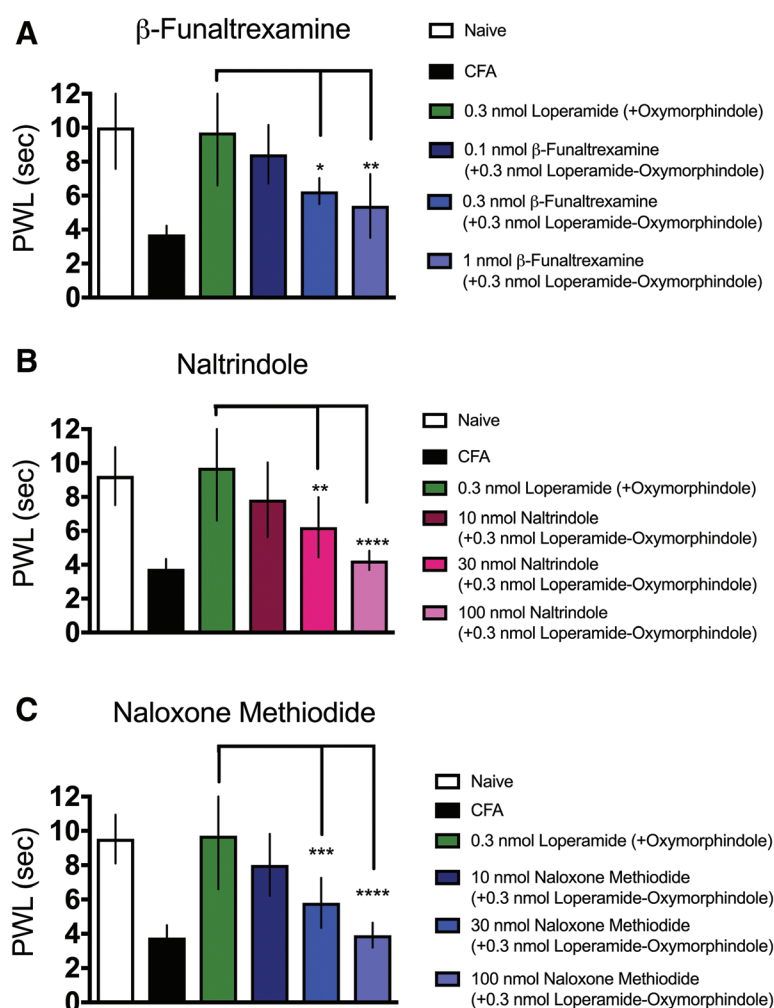
Fig. 4. Antagonism of locally-administered loperamide–oxymorphindole. Paw withdrawal thresholds using the Hargreaves assay were measured for naïve animals, inflamed animals, and animals treated with an intraplantar injection of 0.3 nmol loperamide and oxymorphindole. (*A*) Ability of β-funaltrexamine, an irreversible μ-opioid receptor antagonist, to inhibit loperamide–oxymorphindole antihyperalgesia. Three different doses of β-funaltrexamine were given 24 h before loperamide-oxymorphindole as an intraplantar injection. (*B*) Ability of naltrindole, a δ-opioid receptor antagonist, to inhibit loperamide–oxymorphindole antihyperalgesia. Increasing doses of naltrindole were given concurrently with 0.3 nmol of loperamide–oxymorphindole as an intraplantar injection. (*C*) Ability of naloxone methiodide, a peripherally-restricted opioid antagonist, to inhibit loperamide–oxymorphindole antihyperalgesia. Increasing doses of naloxone methiodide were given concurrently with 0.3 nmol loperamide–oxymorphindole as an intraplantar injection. (*A–C*) Paw withdrawal thresholds were measured using the Hargreaves assay and antagonist data were compared to 0.3 nmol loperamide–oxymorphindole using ordinary one-way ANOVA with Bonferroni test for multiple comparisons. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. CFA, complete Freund's adjuvant; PWL, paw withdrawal latency.

was dose-dependent. These data are presented in figure 4. We interpret these data to indicate that the antinociceptive and anti-hyperalgesic effects of the loperamide–oxymorphindole combination are mediated by peripheral μ–opioid receptors and δ–opioid receptors.

In order to refine the localization of the synergy between loperamide and oxymorphindole, we administered the drugs alone or in combination as topical solutions in complete Freund's adjuvant–inflamed animals. As shown in figure 5A, loperamide and oxymorphindole showed similar

potencies as topical solutions (in a 50% ethanol:50% water mixture), with EC_{50} values in this preparation of 230 and 170 μM, respectively. When combined, the shift in potency was comparable to that with intraplantar administration, with a combination EC50 of 3.4 μM, which corresponds to an approximately 50-fold shift in potency. This synergistic interaction was validated by isobolographic analysis (n = 6 per dose; $P < 0.0001$), as shown in figure 5B. Taken together, these data strongly suggest a cutaneous site of action for the combination, potentially in the epidermis.
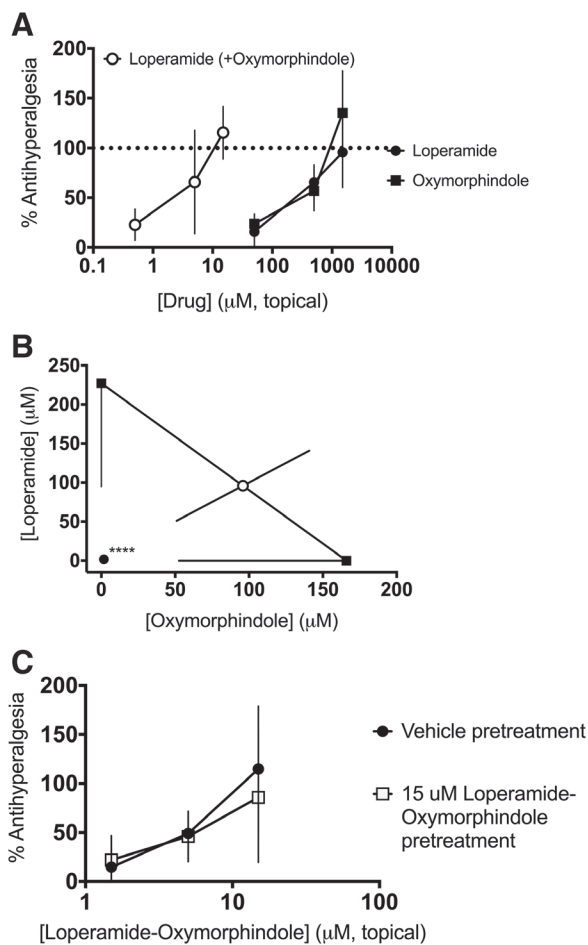
Fig. 5. Topically administered loperamide-oxymorphindole synergizes in inflamed animals. (*A*) Peripherally-mediated thermal nociceptive responses in the Hargreaves assay were assessed. Subjects were given a topical solution of loperamide, oxymorphindole or combination on the inflamed hind paw and post-drug responses are shown as % antihyperalgesia, which was used to generate concentration–response curves. (*B*) Isobolographic analysis of the data from *A*, showing a synergistic interaction compared to the theoretical additive value. ****$P < 0.0001$. (*C*) Antihyperalgesic tolerance to repeated administrations of topical loperamide-oxymorphindole was assessed. Subjects were given twice daily topical treatments of either vehicle or 15 µM loperamide–oxymorphindole for 3 days. On the fourth day, all animals received increasing concentrations of loperamide–oxymorphindole to generate cumulative dose-response curves.

Given that peripheral µ-opioid receptors have been recently implicated in the development of analgesic tolerance to classical opioids such as morphine,[32] we wanted to test whether the topically-delivered combination of loperamide–oxymorphindole would induce analgesic tolerance. Three days before testing, animals (n = 6 per group) were given an intraplantar injection of complete Freund's adjuvant in the left hind paw, resulting in a robust inflammatory

state and hyperalgesic thermal withdrawal latencies on the Hargreaves assay. Then animals received twice daily topical administrations of vehicle or 15 µM loperamide–oxymorphindole for four days. 24 h after the last administration, all animals received increasing concentrations of topically-administered loperamide–oxymorphindole and cumulative concentration-response curves were generated (fig. 5C). Data from this experiment were analyzed by repeated measures two-way ANOVA, and animals that received twice daily loperamide–oxymorphindole did not show a statistically significant difference from animals that received twice daily vehicle (F[1, 5] = 0.4582; $P = 0.53$ for treatment). Therefore, we conclude that repeated topical administration of loperamide–oxymorphindole does not induce analgesic tolerance in animals with inflammatory pain.

To rule out the possibility that the observed synergy in the above experiments was unique to radiant heat stimulation, we tested the ability of loperamide, oxymorphindole, and the loperamide–oxymorphindole combination to reverse complete Freund's adjuvant–induced hypersensitivity as measured by the von Frey mechanical sensitivity assay. Baseline measurements of mechanical withdrawal thresholds were taken before complete Freund's adjuvant administration, and three to five days before testing, animals were given an intraplantar injection of complete Freund's adjuvant in the left hind paw. Postinjury measurements confirmed that complete Freund's adjuvant administration reduced mechanical withdrawal thresholds only on the ipsilateral hind paw. Following the confirmation of hyperalgesia, animals were treated with subcutaneous drug or combination as previously described, and posttreatment von Frey measurements were recorded. Figure 6A shows the dose-response curves for loperamide, oxymorphindole, or their combination (1:1) in complete Freund's adjuvant–inflamed hind paws. In this assay, the observed ED$_{50}$ values were 20 mg/kg for loperamide, 12 mg/kg for oxymorphindole, and 0.1 mg/kg for the combination. This interaction was confirmed to be statistically significant synergy, and the resulting isobologram is shown in figure 6B ($P = 0.0002$). The magnitude of the potency shift mirrored what was observed using the Hargreaves assay, with the combination being approximately 100 times more potent than either drug alone. Therefore, the synergistic effect following loperamide–oxymorphindole administration is not specific to heat hyperalgesia, but generalizes to mechanical hypersensitivity as well.

Finally, we measured whether the combination of loperamide and oxymorphindole demonstrated typical acute opioid-induced side effects, namely respiratory depression and constipation. To test for respiratory depression, mice were given a subcutaneous injection of vehicle, fentanyl, or the loperamide–oxymorphindole combination, and vital signs were monitored using a pulse oximeter. As a positive control, fentanyl reduced arterial oxygen saturation in a dose-dependent and statistically significant manner
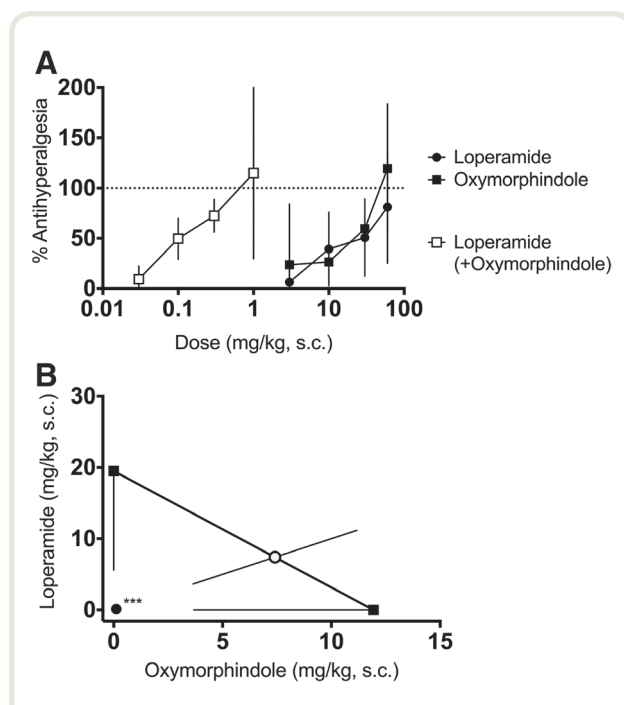
Fig. 6. Systemically administered loperamide–oxymorphindole synergistically reverses mechanical hypersensitivity in inflamed animals. (*A*) Peripherally-mediated mechanical nociceptive responses in the von Frey assay were assessed. Following complete Freund's adjuvant–induced inflammation in the left hind paw, subjects were given a subcutaneous injection of loperamide, oxymorphindole or combination and postdrug mechanical sensitivity responses were analyzed as % antihyperalgesia, which was used to generate dose–response curves. (*B*) Isobolographic analysis of the data from *A*, showing a synergistic interaction compared to the theoretical additive ED$_{50}$ value. ***$P < 0.001$.

(ordinary one-way ANOVA with Bonferroni test for multiple comparisons; $P = 0.004$; F[8, 63] = 3.268). Neither loperamide, oxymorphindole, nor their combination showed any statistically significant reduction of arterial oxygen saturation from baseline measurements (fig. 7A, n = 8 per group). Therefore, we conclude that the loperamide–oxymorphindole combination has a significantly lower risk for centrally-mediated adverse effects. We also assessed the loperamide-oxymorphindole combination for reduction of gastrointestinal transit, which is an expected effect for loperamide as well as other μ-opioid receptor agonists. Following subcutaneous injection of vehicle, fentanyl, loperamide, oxymorphindole, or the loperamide–oxymorphindole combination, fecal boli were counted for 1 h (n = 6 per group). The data are summarized in figure 7B and were analyzed by ordinary one-way ANOVA with Bonferroni test for multiple comparisons ($P < 0.0001$; F[7, 44] = 7.869). Whereas a high dose of loperamide or the combination did show a constipating effect, an antihyperalgesic dose of the combination did not; this result suggests a therapeutic window of the combination with respect to

constipation of 3 to 10. Loperamide and fentanyl predictably caused constipation, but oxymorphindole alone had no effect, consistent with previous work showing that μ-opioid receptors, but not δ-opioid receptors, contribute to inhibition of gastrointestinal transit.[33] All ED$_{50}$ values for drugs and combinations are presented in table 1.

Discussion

The results presented here clearly demonstrate that analgesic synergy between opioid agonists can be mediated by the peripheral nervous system without the involvement of spinal cord circuitry. By using a μ-opioid receptor agonist that is unable to penetrate the CNS together with local routes of administration, we have shown that the involvement of central opioid receptors is not necessary to achieve either robust antinociception in naïve animals, or antihyperalgesia in a mouse model of inflammatory pain. It is well known that the analgesic effect of opioids is enhanced following tissue injury,[6,7,34,35] μ-opioid receptors are upregulated,[36] and the binding efficiency of peripheral μ-opioid receptors increases.[37] Similar results have also been reported concerning the up-regulation and trafficking of the δ-opioid receptor after inflammation.[38] In humans, a meta-analysis of peripherally-delivered opioids for postoperative pain found that preoperative inflammation was a key factor in determining postoperative analgesic outcomes.[39] Accordingly, we report here that the potencies of both loperamide and oxymorphindole were increased following inflammation, such that ED$_{50}$ values following complete Freund's adjuvant are approximately three- to six-fold lower in inflamed than in naïve mice. This holds true for both drugs, as well as for both intraplantar and subcutaneous routes of administration. Therefore, the present results confirm that inflammation potentiates the peripheral analgesic action of both μ-opioid receptor and δ-opioid receptor agonists.

Interestingly, inflammation also magnified the synergism observed following coadministration of loperamide and oxymorphindole. For example, when the combination was administered as an intraplantar injection in naïve mice, the shift in potency from the single drug ED$_{50}$ value to the combination ED$_{50}$ value was approximately 10-fold. By contrast, in animals that had been previously inflamed with complete Freund's adjuvant, the intraplantar synergy was approximately 100-fold. When the drugs were given subcutaneously, the shifts in naïve and inflamed animals were approximately 10-fold and 150-fold, respectively. Because the synergistic shifts are relative to the single drugs in either injury state, the increased potency of the loperamide and/or oxymorphindole alone after inflammation is not sufficient to explain this additional potentiation.

One potential mechanism for this increased synergism is that the loperamide-oxymorphindole combination exerts its pharmacodynamic action through μ-opioid receptor–δ-opioid receptor heteromers, and the increased expression of μ-opioid receptors and δ-opioid receptors at
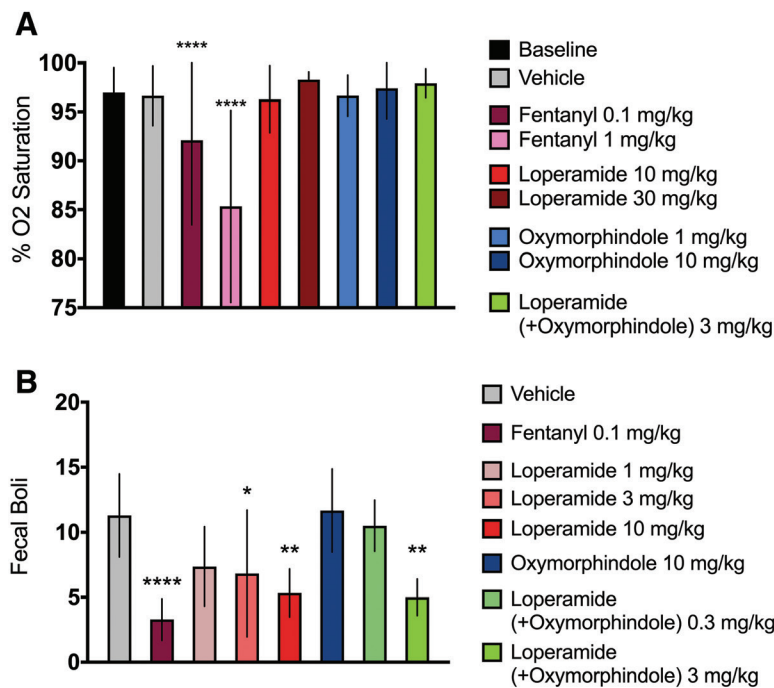
Fig. 7. Acute safety profile of loperamide-oxymorphindole. (*A*) Arterial blood oxygenation was continuously monitored using a STARR MouseOx Plus (Starr Life Sciences Corp., USA) to assess respiratory effects. After acclimation and baseline readings, animals were administered either saline, or increasing doses of fentanyl, loperamide, oxymorphindole, or loperamide–oxymorphindole, and oxygen saturation measured by pulse oximetry readings were recorded. Data were analyzed by ordinary one-way ANOVA with Bonferroni test for multiple comparisons. ****$P < 0.0001$ *versus* baseline. (*B*) Constipation was assessed by counting fecal boli produced for 1 h after drug or saline administration. Data were analyzed by ordinary one-way ANOVA with Bonferroni test for multiple comparisons. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ *versus* vehicle.

Table 1. ED_{50} Values of Loperamide, Oxymorphindole, and Their Combination Compared with the Theoretical Additive ED_{50}s by Three Routes of Administration in Naive and Inflamed States

	Systemic ED_{50}, mg/kg (Mean, 95% CI)		Intraplantar ED_{50}, nmol (Mean, 95% CI)		Topical EC_{50}, µM (Mean, 95% CI)	
	Naïve	**Inflamed**	**Naïve**	**Inflamed**	**Naïve**	**Inflamed**
Loperamide	14 (6.3 to 22)	2.4 (1.4 to 3.4)	57 (15 to 99)	6.4 (4 to 8.7)	n.d.	227 (94.7 to 359)
Oxymorphindole	5.1 (2.9 to 7.4)	1.1 (0.1 to 2.1)	34 (8 to 59)	12 (2.1 to 23)	n.d.	166 (52.6 to 279)
Loperamide + oxymorphindole (1:1 ratio)	0.8* (−0.08 to 1.7)	0.01* (0.004 to 0.02)	4.6* (2.9 to 6.3)	0.1* (0.06 to 0.1)	n.d.	3.4* (0.5 to 6.4)
Theoretical additive	7.5 (4.8 to 10)	1.5 (0.6 to 2.4)	42 (19 to 65)	8.4 (5.4 to 11)	n.d.	192 (102 to 282)

*Statistically significant difference between experimentally observed combination ED_{50} and the theoretical additive value; $P < 0.05$ by JFlashCalc (http://u.arizona.edu/~michaelo/jflashcalc.html) isobolographic analysis.
n.d., not done.

the cell membrane after inflammation allows for the formation of more heterodimers. In naïve animals, anywhere from 29 to 38% of unmyelinated sensory axons at the dermal–epidermal junction express either µ-opioid receptors or δ-opioid receptors,[40] and it follows that the increases in expression of µ-opioid receptors and δ-opioid receptors after inflammation would provide more opportunities for heterodimerization. There is *in vivo* evidence indicating that

coexpressing µ-opioid receptors and δ-opioid receptors allows for interactions between coadministered agonists, and that µ-opioid receptor–δ-opioid receptor heteromers activate different downstream signaling pathways.[41–43] These findings are supported by pharmacologic studies examining the ability of bivalent ligands—molecules consisting of two pharmacophores connected by a chemical linker—to selectively activate putative heteromers. For example, Daniels *et*

al. showed that a series of bivalent ligands with a μ–opioid receptor agonist and a δ–opioid receptor antagonist pharmacophore separated by different linker lengths caused different pharmacologic effects depending on the spacer length.[44] There was a specific spacer length that resulted in a 10-fold increase in potency and mitigated the development of tolerance, which is suggestive of a receptor pair that is able to stabilize in multiple conformations and utilize different signaling pathways. This idea is borne out by the finding that coadministration of the two monovalent pharmacophores did not recapitulate the analgesic effect of the bivalent ligand, as well as a recent finding from our laboratories that showed that different δ–opioid receptor agonists codelivered with morphine activate different signaling cascades.[15,44] In rhesus monkeys, the coadministration of naltrindole with classical μ–opioid receptor–selective agonists causes a rightward shift in potency, suggesting that the μ–opioid receptor agonists are at least partially exerting their analgesic effect through μ–opioid receptor–δ–opioid receptor heteromers.[45] In contrast to the aforementioned studies, Scherrer *et al.* have shown that μ–opioid receptors and δ–opioid receptors are rarely expressed in the same sensory neurons, and differentially modulated mechanical and heat pain,[46] which is in direct conflict with studies asserting the coexpression of μ–opioid receptor and δ–opioid receptor in the same cell.[47] Therefore, additional work is necessary to fully understand the mechanism underlying inflammation's role in enhancing loperamide–oxymorphindole synergy.

With regard to the antinociceptive mechanism of action of the loperamide–oxymorphindole combination, the data presented above reinforce the idea that binding to opioid receptors located on primary afferent neurons is required for antinociception. Either β-FNA, a μ–opioid receptor antagonist, or naltrindole, a δ–opioid receptor antagonist, was able to reverse the antihyperalgesic effect of the loperamide–oxymorphindole combination in a dose–dependent manner. Importantly, because the peripherally-restricted antagonist, naloxone methiodide, completely ablated the antihyperalgesia, we interpret these data to confirm that the synergistic interaction between loperamide and oxymorphindole is being mediated by μ–opioid receptors and δ–opioid receptors in the peripheral nervous system, and not in the spinal cord or other supraspinal opioid–targeting regions. The action at primary afferents is supported by our electrophysiologic data, which suggest that loperamide and oxymorphindole both act and synergize at presynaptic terminals of Nav1.8-expressing neurons innervating the dorsal horn. A report published in 2017 on the mouse line used for those experiments examined the *in vivo* response properties of cutaneous nociceptors that were responsive to blue light. The authors found that 77% of C fibers responsive to blue light were also activated by mechanical and thermal stimuli, suggesting that most Nav1.8-expressing primary afferents are C polymodal nociceptors.[31] That the profound synergy observed after intraplantar delivery generalized to the topical route indicates that the antihyperalgesic action of the combination is manifest superficially in the skin and suggests that local topical application to painful areas could prove to be clinically useful. The experimental characterization of topical opioids has precedent,[48] and we have extended that logic to incorporate synergism between topically applied opioids. Taking these data into account, we conclude that μ–opioid receptor and δ–opioid receptor action at the level of primary afferent nociceptors mediates the analgesic effect of the loperamide–oxymorphindole combination.

Finally, recent efforts to develop new opioid therapeutics have focused on reducing or eliminating classic opioid adverse effects, for example, tolerance, respiratory depression, and addiction. One popular method for mitigating opioid toxicity is to attempt to synthesize agonists at the μ–opioid receptor that preferentially activate G protein–mediated signaling pathways. The hypothesis underlying that research is that β-arrestin signaling is responsible for the nonanalgesic effects of μ–opioid receptor agonists. For example, a 2016 paper in *Nature* reported the structure-based optimization of PZM21, a μ–opioid receptor agonist that robustly activated G_i, but not β-arrestin.[49] In that report, the authors support the conclusion that biased agonism will reduce opioid toxicity with data showing that PZM21 is an effective analgesic that is devoid of both respiratory depression and morphine-like reinforcing activity in mice at analgesic doses. Alternatively, we have recently shown that analgesic synergy also represents an effective method of limiting side effects. Stone *et al.* published data establishing that morphine and clonidine, an $α_{2A}$-adrenergic receptor agonist, synergized in their analgesic effect, but not in sedative or cardiovascular effects.[13] The data presented here corroborate that finding, demonstrating that the loperamide-oxymorphindole combination, in contrast to the prescription opioid fentanyl, does not cause respiratory depression or constipation at therapeutic doses (*e.g.,* 0.3 mg/kg). Additionally, the loperamide–oxymorphindole combination did not cause respiratory depression at a supratherapeutic dose (*e.g.,* 3 mg/kg). The same supratherapeutic dose did cause some constipation due to the dose of loperamide in the combination being of the same order of magnitude as its constipatory dose alone.[50] Overall, because loperamide is restricted from the CNS, and we are able to drastically lower the doses given to achieve antinociception with synergy, these data uphold the idea that peripherally-mediated opioid synergy is a safe and effective strategy for further development. There are currently no peripherally-restricted opioid analgesics on the market for treatment of acute or chronic pain. The current report may represent an initial step toward the development of peripherally-restricted, synergistic analgesic combination pharmacotherapies for moderate to severe inflammatory pain.

Competing Interests

Mr. Bruce, Dr. Akgün, Dr. Portoghese, Dr. Fairbanks, and Dr. Wilcox have a related international patent pending (with title "Combination for treating pain"). An International Patent Application was published under the Patent Cooperation Treaty on September 28, 2017: International Publication Number WO 2017/165558 A1. The invention provides compositions and methods that can be used to treat pain with reduced central nervous system side effects like addiction liability and respiratory depression. The other authors declare no conflicts of interest.

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