Overexpression of μ-Opioid Receptors in Peripheral Afferents, but Not in Combination with Enkephalin, Decreases Neuropathic Pain Behavior and Enhances Opioid Analgesia in Mouse

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ABSTRACT

Background: The current study used recombinant herpes simplex virus type I to increase expression of μ-opiate receptors and the opioid ligand preproenkephalin in peripheral nerve fibers in a mouse model of neuropathic pain. It was predicted that viral vector delivery of a combination of genes encoding the μ-opioid receptor and preproenkephalin would attenuate neuropathic pain and enhance opioid analgesia. The behavioral effects would be paralleled by changes in response properties of primary afferent neurons.
Methods: Recombinant herpes simplex virus type 1 containing cDNA sequences of the μ-opioid receptor, human preproenkephalin, a combination, or *Escherichia coli lacZ* gene marker (as a control) was used to investigate the role of peripheral opioids in neuropathic pain behaviors.
Results: Inoculation with the μ-opioid receptor viral vector (n = 13) reversed mechanical allodynia and thermal hyperalgesia and produced leftward shifts in loperamide (ED_{50} = 0.6 ± 0.2 mg/kg *vs.* ED_{50} = 0.9 ± 0.2 mg/kg for control group, n = 8, means ± SD) and morphine dose-response curves (ED_{50} = 0.3 ± 0.5 mg/kg *vs.* ED_{50} = 1.1 ± 0.1 mg/kg for control group). In μ-opioid receptor viral vector inoculated C-fibers, heat-evoked responses (n = 12) and ongoing spontaneous activity (n = 18) were decreased after morphine application. Inoculation with both μ-opioid receptor and preproenkephalin viral vectors did not alter mechanical and thermal responses.
Conclusions: Increasing primary afferent expression of opioid receptors can decrease neuropathic pain-associated behaviors and increase systemic opioid analgesia through inhibition of peripheral afferent fiber activity. **(Anesthesiology 2018; 128:967-83)**

NEUROPATHIC pain is a debilitating condition that affects more than 4 million individuals in the United States alone.[1] Chronic neuropathic pain is often refractory to current analgesic therapies or requires escalating doses of opioids that result in treatment-limiting side effects. Driven by the characterization of peripheral opioid ligands and receptors,[2,3] peripheral opioid receptor–mediated analgesia has been demonstrated in experimental models of inflammatory pain,[4] neuropathic pain,[5–7] and possibly clinical settings.[7–9] Recognition is growing of an important role for peripheral opioid analgesia in neuropathic pain, suggesting that the peripheral opioid system may yield novel analgesic approaches with a more limited side-effect profile.[4] Currently available peripherally active opioid agonists have limited utility as analgesics because they decrease intestinal motility. An alternative strategy is to directly modify the peripheral opioid system in the tissues of interest for the modulation of pain, namely the peripheral nervous system, using virally mediated gene transfer.

What We Already Know about This Topic

- Opioid signaling through μ-opioid receptors is diminished after nerve injury
- Augmenting μ-opioid receptor and/or enkephalin levels in afferent neurons using viral vectors may reduce neuropathic sensitization

What This Article Tells Us That Is New

- Using a mouse model of neuropathic pain, it was shown that increasing μ-opioid receptor expression in peripheral neurons reduced nociceptor activity and left-shifted loperamide and morphine dose-response curves
- Unexpectedly, inoculation with both μ-opioid and preproenkephalin-containing herpes viruses was ineffective in reducing nerve injury–induced pain behaviors

Virally mediated gene transfer with herpes simplex virus type 1 (HSV-1) vectors has been shown to effectively increase the expression of the μ-opioid receptor (mOR)[10] or the endogenous opioid ligand enkephalin in afferent neurons[11] with

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concomitant behavioral changes in nociception and analgesia.[12,13] Virally mediated expression of mOR increases analgesia produced by systemic morphine[6,8] or the peripherally active opioid agonist loperamide.[10] In animal models, virally mediated expression of enkephalin is antinociceptive in capsaicin-induced thermal hyperalgesia,[14–16] trigeminal neuralgia,[17] and spinal nerve ligation (SNL) models of neuropathic pain.[18] Several of these studies have demonstrated reversal of antinociception with the nonspecific opioid antagonist naloxone, suggesting that enkephalin-induced antinociception is dependent upon signaling through opioid receptors.[14,17,19] This raises the possibility for an additive antinociceptive and analgesic effect when HSV-1 is used to simultaneously overexpress both enkephalin and mOR to alleviate chronic neuropathic pain.

This study is a side-by-side comparison of recombinant HSV-1 driven over expression of mOR, the endogenous opioid ligand preproenkephalin, or a combination of the two viral vectors in a mouse L5 SNL model of chronic neuropathic pain. We defined the time course post-nerve injury over which herpes simplex virus (hsv) vectors that encode the mOR (hsvMOR) and/or preproenkephalin (hsvPPE) can (1) increase mOR and/or preproenkephalin in primary afferent neurons, (2) modulate mechanical allodynia and thermal hyperalgesia, and (3) enhance both systemic (morphine) and peripheral (loperamide) analgesia. In addition, (4) we determined whether any of these behavioral changes could be established at the primary afferent level by myelinated or unmyelinated fibers as evidence of peripherally mediated opioid analgesia in neuropathic pain.

Materials and Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of South Carolina School of Medicine (Columbia, South Carolina) and by the Johns Hopkins Animal Care and Use Committee (Baltimore, Maryland). Efforts were made throughout the experiment to minimize animal discomfort and to reduce the number of animals used. Female and male Swiss-Webster mice (5 to 6 weeks old, 20 to 30 g; Charles River, USA) were used. To date, no sex differences have been reported regarding the effects of inoculation, and this hypothesis was not directly tested in this study. The animals were housed in a standard 12 h light/12 h dark environment with food and water *ad libitum*. The animals were randomly assigned to treatment groups using simple randomization.

Timeline

The experimental timeline is shown in figure 1. For immunohistochemistry, the mice underwent an L5 SNL followed by intraplantar inoculation with hsv control (hsvCON), hsvMOR, hsvPPE, or hsvMOR + preproenkephalin (PPE) on day 7 postsurgery (n = 4 per group per time point). A separate group of mice underwent sham surgery followed by intraplantar inoculation with hsvCON on day 7 postsurgery (n = 4 per time point). On days 1, 9, and 16 postinoculation, hind paw skin, L3–L6 dorsal root ganglia (DRG), and lumbar spinal cord were collected for immunohistochemical analysis of mOR and enkephalin immunoreactivity. Track tracing of peripheral nerve fibers using wheat germ agglutinin-biotin and cholera B toxin–biotin indicate that at least 70% of dorsal root ganglion neurons become infected with the HSV-1 virus (data not shown).

For behavioral assessment, baseline mechanical paw withdrawal thresholds (PWTs) and thermal paw withdrawal latencies (PWLs) were obtained before nerve ligation. Development of thermal hyperalgesia and mechanical allodynia was confirmed on day 7 after L5 SNL, before intraplantar injection with hsvCON (n = 8), hsvMOR (n = 13), hsvPPE (n = 9), hsvMOR + PPE (n = 8), buffer that was used to harvest the viral vectors from the infected cells (n = 8), or buffer that was used to harvest the viral vectors, which included cell debris from the culture (n = 8). Paw volume was measured on days 1, 3, 5, and 7 postinfection using a plethysmometer (IITC Life Science Inc., USA) according to the manufacturer's instructions. An experimenter blinded to group assessed thermal hyperalgesia and mechanical allodynia for 15 days postinjection. Cumulative loperamide and morphine dose-response curves were generated on days 16 and 19 postinjection, respectively.

For *ex vivo* recordings, mice underwent an L5 SNL followed by either no injection or intraplantar inoculation with hsvCON or hsvMOR on day 7 postsurgery. Recordings were done to correspond with behavioral experiments (fig. 1), 7 to 14 days after inoculation. Experimenters were blinded to virus infection throughout all behavioral and histochemical experiments and analysis.

Recombinant HSV-1 Vector Infection

As previously described,[10,15] recombinant KOS strain HSV-1 viruses encoding the human hsvPPE and rat hsvMOR gene in the sense orientation under control of the human cytomegalovirus immediate-early promoter/enhancer were used (fig. 1). Two similarly constructed vectors encoding the *Escherichia coli* lac *Z* gene served as controls (hsvCON). In hsvMOR and one of the control virus constructs (hsvCON with green fluorescent protein [GFP]), the cDNA for GFP was included as a reporter gene. Herpes simplex viruses are ideal for targeting nerve fibers, because they are neurotrophic and establish latent infection mainly in the trigeminal and dorsal root ganglia.[20] On the day of infection, the mice were anesthetized with isoflurane for intraplantar administration of virus into the left hind paw. Vehicle that was used to isolate virus from cell culture (10% sucrose/PBS-D), vehicle applied to a mock-infected culture (MOCK), or vehicle containing hsvPPE, hsvMOR, or hsvCON (2×10^7 plaque-forming unit [pfu]/μl) was administered subcutaneously in the left plantar hind paw (10 μl). In all experiments, the virus suspensions were prepared to contain 1×10^7 pfu/μl virus encoding GFP + 1×10^7 pfu/μl virus not encoding GFP. For instance, hsvPPE alone was balanced with hsvCON (with GFP) and hsvMOR alone was balanced with hsvCON (without GFP). The control group included both hsvCON (with GFP) and hsvCON (without GFP).

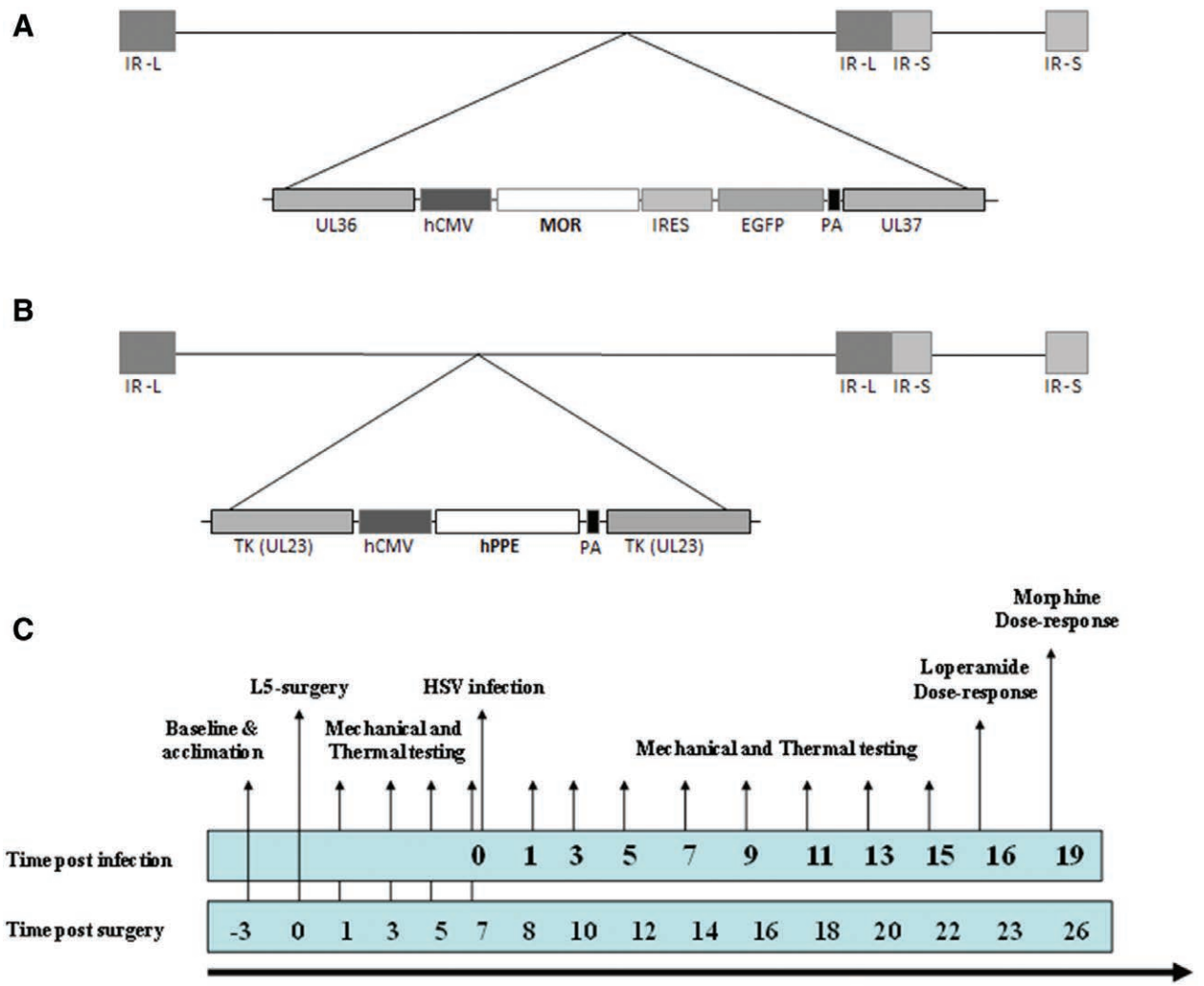

Fig. 1. Viral constructs and study timeline. (*A*, *B*) Schematic diagram of herpes simplex virus that encodes μ-opioid receptor (hsvMOR) recombinant herpes vector (*A*), expression of β-galactosidase (*Escherichia coli lacZ*), and preproenkephalin (PPE) (*B*) are driven by the human cytomegalovirus (hCMV) immediate-early enhancer-promoter as previously described by Zhang *et al.*[10] (*C*) Before spinal nerve ligation surgery, the mice were acclimated to the blinded experimenter and test environment. The animals were tested for basal paw mechanical withdrawal thresholds and heat threshold latencies. All groups were assessed for mechanical allodynia and thermal hyperalgesia on days 1, 5, 7, 8, 10, 12, 14, 16, 18, and 22 after L5 ligation surgery. Viruses were administered at day 7 postsurgery. Cumulative loperamide dose-response curves were generated at 16 days after viral administration. Cumulative morphine dose-response curves were generated at 19 days after viral administration. Electrophysiology studies were carried out in separate groups of animals with the same injury and inoculation timeline. Tissue was harvested at 7 to 14 days after inoculation in correlation with behavioral studies. HSV = herpes simplex virus; IR = internal repeat; IRES = internal ribosomal entry site; EGFP = enhanced green flourescent protein; hPPE = human preproenkephalin; L = long; MOR = μ-opioid receptor; PA = polyadenylation signal; S = short; TK = tymadine kinase; UL36 and UL37 = herpes simplex virus genes for tegument proteins.

L5 SNL

The mice were anesthetized with isoflurane in O_2 carrier (3% induction, 1.5% maintenance). The fur overlying L3–S3 was shaved, and the skin was cleaned with povidone iodine. A small incision was made to the skin and fascia overlaying L5–S1. The paravertebral musculature was retracted from the vertebral transverse processes. The L6 transverse process was partially removed, exposing the L4 and L5 spinal nerves. The L5 spinal nerve was lifted slightly and transected. The wound was irrigated with saline and closed in two layers with a 3-0 polyester suture (fascial plane) and surgical skin staples or vicryl sutures. The sham surgical procedure was identical, but the spinal nerve was not ligated.

Immunohistochemistry: mOR and Enkephalin Immunoreactivity

On days 1, 9, and 16 postinfection, the mice were deeply anesthetized for transcardiac perfusion with 0.1 M phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Lumbar spinal cords and

ipsilateral L3–L6 DRG were removed and postfixed in 4% paraformaldehyde for 24 h followed by cryoprotection in 30% sucrose in 0.1 M phosphate buffer. Left plantar hind paw skin was removed and postfixed in 4% paraformaldehyde for 4 h followed by cryoprotection in 30% sucrose in 0.1 M phosphate buffer. For assessment of paw edema, skin samples were collected on day 1 postinfection for hematoxylin and eosin histochemistry.

Lumbar spinal cords (30 μm), DRG (12 μm), and skin (12 μm) were serially sectioned. DRG and skin were thaw-mounted onto slides for slide-mounted immunohistochemistry. Spinal cords were placed into Tris-buffered saline (TBS) with 1% Tween 80 (TBST) in 24-well plates for free-floating immunohistochemistry as previously described. Spinal cord sections were rinsed in TBS and quenched for endogenous peroxidase activity and antigen recovery in mentholic peroxide for 15 min. The samples were rinsed three times (10 min each) and then blocked for 20 min at room temperature with normal donkey serum (1.5%) in TBST. This was followed by a 24-h incubation at 4°C with polyclonal rabbit anti-mOR (1:1,000; Neuromics, USA) or polyclonal rabbit anti-met-enkephalin (1:750; Immunostar, USA) primary antibody in 1% normal donkey serum in TBST. The next day, the samples were rinsed three times (10 min each) with TBST. Samples were incubated for 1.5 h at room temperature with biotinylated affinity-purified donkey anti-rabbit immunoglobulin G secondary antibody (1:1,000; Jackson ImmunoResearch, USA) in 1.5% normal donkey serum in TBST. The samples were washed three times (10 min each) and incubated for 1 h at room temperature with horseradish peroxidase–conjugated streptavidin (1:1,600; Jackson ImmunoResearch) in TBST. The samples were washed four times (10 min each) and developed with diaminobenzidine. After substrate–chromogen reaction, spinal cord tissues were mounted on slides. All slides were then dehydrated and coverslipped.

Dorsal root ganglia and skin were processed as described above, but the biotinylated secondary antibody was replaced with a Texas Red–conjugated affinity-purified donkey anti-rabbit immunoglobulin G (1:200; Jackson ImmunoResearch). After a 2-h incubation at room temperature in the dark, the slides were washed three times (10 min each) in TBS, coverslipped with VectaShield (Vector Laboratories, USA), and stored at 4°C until analysis.

Immunohistochemistry of DRG, skin, or spinal cords was performed in a single run. Tissues across serial sections were analyzed and averaged by an investigator blinded to the experimental conditions and virus infections. Spinal cord section imaging and analysis were carried out as described previously.[10] Briefly, digital images were obtained for each spinal cord slice. ImageJ version 1.26 (National Institutes of Health, USA) was used to determine the optical density of mOR immunoreactivity (mOR-ir) and met-enkephalin immunoreactivity (Enk-ir) within laminae I–III of ipsilateral L3–L6 spinal cords. Density was averaged across animals in a treatment group (n = 4 tissues per spinal level per mouse). The mOR-ir was variable between neurons *versus* the epidermal nerve fibers and spinal cord, which is not surprising given that mOR staining differences with respect to localization have been reported in previous studies.[21]

In DRG, mOR-ir and Enk-ir cell bodies were counted and categorized into small (4 to 20 μm), medium (22 to 40 μm), and large (42 to 77 μm) diameter. Only cells in which the nucleolus was present were sized. Similarly, cell bodies expressing GFP were counted by size. Because the hsvMOR virus included the cDNA for GFP, we normalized the number of the colabeled mOR-ir- and GFP-expressing cell bodies as a percentage of total cell bodies within each DRG. The data are presented as a percentage of the total number of cell bodies positive for mOR + GFP or enkephalin across L3–L6 DRG.

In skin sections, we counted the number of mOR-ir, Enk-ir, and GFP-positive dermal nerve innervations. For hsvMOR–infected mice, we quantified the percentage of mOR + GFP–colabeled nerve innervations. Because the hsvPPE construct did not encode for GFP, we did not count the number of GFP–positive epidermal nerve terminals.

Capsaicin-induced cFos Expression

Two hours postcapsaicin (intraplantar, 20 μl of 0.15% capsaicin dissolved in vehicle containing 10% ethanol, 10% Tween 80, and 80% saline; Sigma, USA) administration, the mice were deeply anesthetized and perfused transcardially with 0.1 M phosphate buffered saline (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the spinal cords were removed and postfixed in 4% paraformaldehyde for 24 h and then placed in 30% sucrose in 0.1 M phosphate buffer for cryoprotection (n = 8 mice per virus treatment).

The spinal cords were sectioned (20 μm), and free-floating cFos immunohistochemistry was performed as detailed above for the mOR and enkephalin immunohistochemistry. The primary antibody (polyclonal rabbit anti-cFos at a 1:5,000 dilution in TBST; Sigma) and the secondary antibody (biotin-SP–conjugated AffiniPure donkey anti-rabbit IgG at a 1:1,000 dilution in TBST; Jackson ImmunoResearch) were used. The number of cFos-positive neurons in laminas I and II of L3–L6 spinal cord sections were counted by an investigator blinded to infection (5 sections per level per animal).

Behavioral Studies

Mechanical Allodynia. The animals were acclimated to the experimenter and testing environment before behavioral testing. The mice were placed in plastic boxes over a mesh floor, and their basal responses to mechanical stimuli were quantified as the total number of paw withdrawals to an ascending series of calibrated von Frey filaments (0.16, 0.4, 1.0, 2.0, and 4.0 g; Stoelting Company, USA) applied to the plantar surface of the ipsilateral hind paw. Each von Frey filament

was applied five consecutive times, and the maximum possible number of withdrawal responses was 25. The filament force that caused more than one withdrawal incidence of five stimulations was considered the PWT. %PWT was calculated by dividing the measured PWT by the presurgery basal PWT. Allodynia was characterized as withdrawal of the paw to these normally nonnoxious stimuli (*i.e.*, a decrease in the %PWT).

Thermal Hyperalgesia. Thermal PWL to radiant heat stimuli was measured using a plantar stimulator analgesia meter (IITC model 390). Animals were placed under separate plastic boxes on a heated glass floor. Radiant heat was applied from below to the plantar surface of the ipsilateral hind paw, and an electronic timer measured the time until the animal removed its paw from the thermal stimulus. PWL was defined as the average latency in three sets of a single stimulation. We waited at least 10 min between each set to prevent sensitization. We used a maximum exposure of 20 s to prevent any potential tissue damage. %PWL was calculated by dividing the measured PWL by the presurgery basal PWL. Hyperalgesia was characterized as an enhanced withdrawal of the paw (*i.e.*, shorter PWL) to this the radiant heat.

Pharmacologic Studies: Opioid Dose-response Curve

Cumulative dose-response curves were generated for subcutaneous loperamide (20% Cremophor EL in sterile saline) and subcutaneous morphine (in sterile saline). First, PWLs were measured at 10 min after vehicle administration. Cumulative doses of loperamide (0.17, 0.5, 1, 1.5, 2, 3, and 4 mg/kg) or morphine (0.25, 0.5, 1, 1.5, 2, 3, and 4 mg/kg) were then administered in 15-min intervals, and PWLs were measured 10 min after each administration. Loperamide and morphine were administered subcutaneously in the ipsilateral flank through a 29-gauge insulin syringe.

Spontaneous Behavioral Assessment: Capsaicin Injection

Transient receptor potential vanilloid 1 expression and activity has been implicated in multiple chronic pain states, including neuropathic pain.[22] Because intradermal capsaicin-injection protocols have been used to as neuropathic pain models in humans,[23] we chose to monitor spontaneous nocifensive behaviors and thermal withdrawal thresholds in mice after capsaicin injection. At 4 weeks after virus infection, baseline PWLs were measured. Capsaicin (20 µl of 0.15% capsaicin dissolved in vehicle containing 10% ethanol, 10% Tween 80, and 80% saline; Sigma) was subcutaneously administered into the left plantar hind paw of mice (n = 10 mice per treatment). The mice were returned to the plastic boxes, and the first 5 min after capsaicin were videotaped using a digital camcorder. Thermal PWLs were measured in 10-min intervals after capsaicin for 60 min. The videos were scored by a blinded observer who analyzed the time spent licking, flicking, and holding the hind paw. Pain scores were calculated using the formula: [2(seconds spent in licking/flicking of the paw) + seconds spent holding the paw]/(60 · n), where n is the recorded time in minutes.

Ex Vivo *Electrophysiologic Studies*

One to two weeks after inoculation, the mice were euthanized with pentobarbital (50 mg/kg, intraperitoneally), and the hairy skin of the hind paw, together with the saphenous or sural nerve, was carefully dissected and transferred to an *in vitro* system described in detail previously.[24] The skin was mounted corium side up in an organ bath and superfused (750 ml/h) with synthetic interstitial fluid (SIF) heated to ~32°C and continuously bubbled with carbogen to obtain a pH of 7.4. The nerve was threaded through a hole from the organ bath into a mineral oil–filled recording chamber containing a splitting platform. Under a microscope and with fine watchmaker forceps, epi- and perineurium were carefully removed, and the nerve was teased into smaller bundles that were placed onto a recording electrode positioned above the splitting platform. Nerve bundles were teased into smaller filaments until single-fiber activity could be recorded. Cutaneous receptive fields (RFs) were first localized by applying mechanical stimuli to the skin with a blunt glass rod. After localizing the RF, the investigator applied electrical stimuli at the RF with a concentric electrode (up to 1 mA, 100 µs) to determine conduction latency, which, together with the conduction distance, was used to calculate conduction velocity (CV). Fibers with a CV of less than 1 m/s were classified as C-fibers, those with a CV between 1 and 10 m/s were classified as Aδ-fibers, and those with a CV more than 10 m/s were classified as Aβ-fibers.

Mechanical thresholds were determined with an ascending series of von Frey filaments. The smallest filament to produce a response in two of four trials was regarded as threshold. RFs were isolated with a 10-mm steel ring from the surrounding preparation to restrict the effect of the thermal stimuli and drug effects to the area of interest. SIF was removed from within the ring, and heat sensitivity was assessed by using superfused heated SIF at the receptive field in a resistive heating perfusion system.[24] Immediately after heat testing, the ring was refilled with SIF, and the unit was allowed to rest for 10 min before being tested for cold sensitivity. Cold SIF was superfused at the RF through a perfusion system as described in detail previously.[24] Mechanical sensitivity was tested by applying an ascending series of suprathreshold mechanical stimuli (0.1 to 8 g or 0.98 to 78.5 mN for 2 s, separated by 1 min) to the RF with a blunt probe (1-mm diameter) connected to a computer-controlled mechanical stimulator (model 305C; Aurora Scientific, Canada).

After baseline mechanical and thermal testing was completed, SIF within the ring was replaced by a 2 µM morphine SIF solution, which was maintained for 5 min. This concentration of morphine (morphine sulfate; Sigma–Aldrich, USA) was equivalent to the systemic 1 mg/kg dose that had antinociceptive effects in previous behavioral experiments and is accepted in the range of opiate concentrations for use

in a skin nerve preparation.[25] Five minutes after morphine application, we retested the responsiveness to mechanical and thermal stimuli using the same sequence and timing as described for baseline testing with the exception that morphine SIF solution was applied in the rest periods between stimuli.

For data analysis, all action potentials were filtered, amplified, digitized, and stored on a computer using DAPSYS (Brian Turnquist, Bethel University, St. Paul, Minnesota; http://www.dapsys.net, accessed November 1, 2017). DAPSYS was also used to control the mechanical stimulator and to record temperatures from heat and cold stimulation. Fibers were recorded from skin in an hsvMOR–inoculated mouse under SIF conditions and under morphine conditions. These afferents responded to mechanical and heat stimuli or only to mechanical stimuli and according to previously established criteria would be classified as mechano–heat-sensitive and mechanosensitive C-fibers, respectively. The data were analyzed only from the units where the thermal and mechanical stimulation protocol was completed.

The total number of evoked action potentials to a given mechanical or thermal stimulus, the instantaneous peak frequency, and thermal thresholds (defined as the temperature at which the first action potential was recorded, or a 30% increase in activity if the unit had spontaneous activity) were used for data analysis. Evoked responses for thermal and mechanical stimuli included a poststimulus period of 5 and 3 s, respectively. The peak instantaneous frequency data were median-smoothed to account for data variance introduced by a single action potential. Spontaneous activity was also measured and compared between SNL groups after morphine application by using the mean evoked action potentials per minute under SIF and morphine conditions.

Statistical Analysis

We performed the appropriate one-way, two-way, or repeated measure ANOVA followed by *post hoc* analysis (Bonferroni) to determine the significance of virus-mediated changes in behavior, immunohistochemistry, and histochemistry. Differences in dose-response curves were determined by using repeated-measures ANOVA. ED_{50} was calculated by using nonlinear regression to generate a sigmoidal dose-response curve. The goodness of fit of the curve was determined by R^2 values between 0.91 and 0.96. $P < 0.05$ was considered significant. All statistical analyses were carried out with Prism 4.0 (GraphPad Software Inc., USA). The data are presented as means ± SD, and $P < 0.05$ was considered statistically significant. Sample sizes were based on previous behavioral and immunohistochemistry studies.[10,26]

To compare pre- and postmorphine electrophysiology data, and to compare results between different virus-treatment groups, we carried out statistical tests where appropriate with Statistica software. Fibers from mice that were noninoculated or hsvCON–inoculated demonstrated similar mechanical and thermal thresholds (data not shown) and were collapsed *post hoc* into a control group (no treatment and hsvCON). We used factorial ANOVA to compare data in uninjured and injured animals (two levels) and vector treatment (two levels). The data are presented as mean ± SD, and $P < 0.05$ was considered statistically significant. Because the evoked responses to thermal and mechanical stimuli vary considerably across fibers, we normalized the data for analysis. For example, the number of evoked action potentials and peak instantaneous frequency to a given stimulus at baseline (premorphine) or postmorphine was normalized to: [Post/(Pre + Post)] = fraction of the response stimulus (*i.e.*, if morphine had no effect, responses at baseline and after morphine would each contribute approximately 50% of the total response). Thresholds are presented as postmorphine – premorphine in °C (temperature data) or grams (mechanical data). The percentage of change in spontaneous activity is represented as: [(Post – Pre)/Pre] × 100.

Results

Enk-ir and mOR-ir in Plantar Hind Paw Skin

Hind paw infection with viral vectors produced mild to moderate short-term inflammation that resolved within 3 days postinfection (see figure, Supplemental Digital Content 1, http://links.lww.com/ALN/B602, paw volume and inflammation data). Nerve injury did not alter the number of Enk-ir innervations in plantar hind paw skin compared with that of sham surgery controls (fig. 2). Infection with hsvMOR, hsvPPE, and hsvMOR + PPE increased the number of Enk-ir innervations on day 16 postinfection (ANOVA; fig. 2A). Nerve injury decreased the number of colocalized mOR-ir and GFP-positive innervations in the plantar hind paw skin of hsvCON–infected mice compared with that of sham mice (fig. 2B). Infection with hsvMOR, hsvPPE, and hsvMOR + PPE increased the number of mOR-ir and GFP colocalized innervations compared with that of sham and hsvCON (ANOVA; fig. 2B), 16 days postinoculation.

Enk-ir and mOR-ir in L3-L6 DRG

Nerve injury did not change the number of medium, large, or total Enk-ir or mOR-ir DRG cells compared with that of sham surgery controls (fig. 3). The number of large, medium, small, and total Enk-ir DRG cells was higher in hsvPPE and hsvMOR + PPE mice than in sham or hsvCON mice. hsvMOR mice also had increased the number of small Enk-ir DRG cells on day 16 postinfection compared with sham and hsvCON (two-way ANOVA, factors: group × neuron size; fig. 3A). Infection with hsvMOR produced a large increase in the number of large and medium DRG cells that colocalized mOR-ir and GFP compared with the percentage in sham and hsvCON–inoculated animals. hsvPPE and hsvMOR + PPE also produced a large increase in the number of

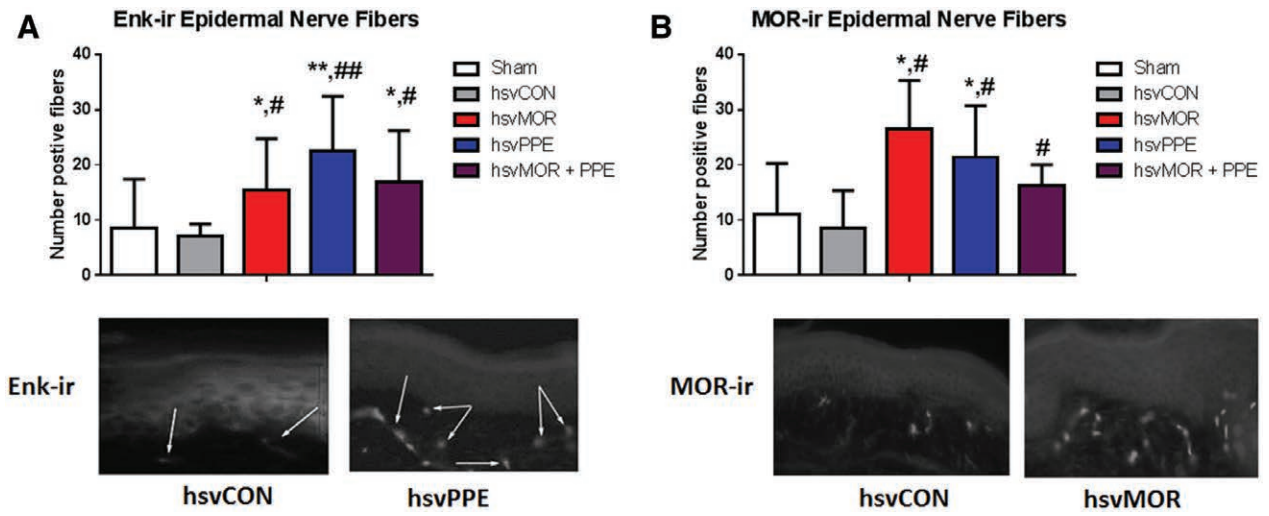

Fig. 2. The number of enkephalin immunoreactivity (ENK-ir) and colabeled μ-opioid receptor immunoreactivity (MOR-ir) and green fluorescent protein–positive epidermal nerve fibers in plantar hind paw skin increases after herpes simplex virus that encodes μ-opioid receptor (hsvMOR), herpes simplex virus that encodes preproenkephalin (hsvPPE), and hsvMOR + preproenkephalin (PPE) inoculation. (*A*, *top*) Quantification of Enk-ir–positive nerve fibers of epidermis on day 16 after viral inoculation. There is a significant increase in Enk-ir in hsvMOR, hsvPPE, and hsvMOR + PPE treatment groups compared to sham and control virus (ANOVA, F[4, 19] = 2.3, $P = 0.01$; hsvCON). (*Bottom*) Histology images of Enk-positive epidermal nerve fibers from forepaw footpad skin after treatment with control virus (*left*) and hsvPPE (*right*). (*B*, *top*) Quantification of MOR-ir–positive nerve fibers of epidermis on day 16 after viral inoculation. (*Bottom*) Histology images of MOR–positive epidermal nerve fibers from the forepaw footpad skin after treatment with control virus (*left*) and hsvMOR (*right*). There is a significant increase in MOR-ir in hsvMOR, hsvPPE, and hsvMOR + PPE treatment groups compared to sham (ANOVA, F[4, 19] = 3.5, $P = 0.032$). Bonferroni *post hoc* test was used for multiple comparisons. #significant at $P < 0.05$ *versus* sham; ##significant at $P < 0.01$ *versus* sham; *significant at $P < 0.05$ *versus* control virus-treated (hsvCON); **significant at $P < 0.01$ *versus* control virus-treated (hsvCON). CON = control; Enk = enkephalin; hsv = herpes simplex virus; ir = immunoreactivity; MOR = μ-opioid receptor.

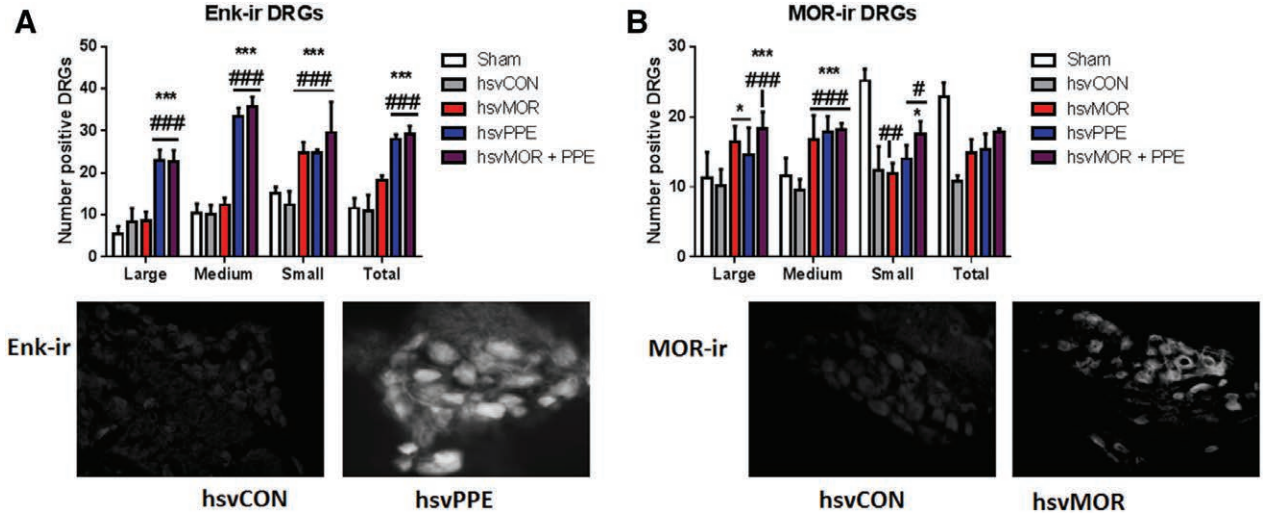

Fig. 3. Inoculation with herpes simplex virus that encodes μ-opioid receptor (hsvMOR), herpes simplex virus that encodes preproenkephalin (hsvPPE), or hsvMOR + preproenkephalin (PPE) increases the number of enkephalin immunoreactivity (ENK-ir) and colabeled μ-opioid receptor immunoreactivity (MOR-ir) and green fluorescent protein–positive dorsal root ganglion cells. (*A*) Quantification of Enk-ir–positive dorsal root ganglia (DRGs) on day 16 after viral inoculation. There is a significant increase in Enk-ir in hsvPPE and hsvMOR + PPE treatment groups compared to sham and control virus (hsvCON, two-way ANOVA, factors: group × neuron size, F[12, 60] = 7.5, $P < 0.001$). (*Bottom*) Histology images of enkephalin-positive dorsal root ganglion with control virus (*left*) and hsvPPE (*right*). (*B*) Quantification of MOR-ir–positive DRGs on day 16 after viral inoculation. (*Bottom*) Histology images of MOR–positive dorsal root ganglion with control virus (*left*) and hsvMOR (*right*). There is a significant increase in MOR-ir in large and medium sized DRGs of hsvMOR, hsvPPE, and hsvMOR + PPE treatment groups compared to sham and control virus (hsvCON, two-way ANOVA, neuron size, F[12, 60] = 10.5, $P < 0.001$). Bonferroni *post hoc* test was used for multiple comparisons. #significant at $P < 0.05$ *versus* sham; ##significant at $P < 0.01$ *versus* sham; ###significant at $P < 0.001$ *versus* sham; *significant at $P < 0.05$ *versus* control virus-treated (hsvCON); **significant at $P < 0.01$ *versus* control virus-treated (hsvCON); ***significant at $P < 0.001$ *versus* control virus-treated (hsvCON). CON = control; Enk = enkephalin; hsv = herpes simplex virus; ir = immunoreactivity; MOR = μ-opioid receptor.

medium and large DRG cells that colocalized mOR-ir and GFP 16 days postinfection compared with that of sham and hsvCON–inoculated animals (two-way ANOVA, factors: group × neuron size; fig. 3B). Because of the large decrease in mOR-ir in small diameter DRG after nerve injury, the total number of mOR-ir DRGs did not significantly differ across treatment groups (two-way ANOVA, factors: group × neuron size, $P = 0.064$).

Enk-ir and mOR-ir in Laminae I–III of the Lumbar Spinal Cord

Nerve injury produced dynamic changes in Enk-ir in laminae I-III of the lumbar spinal cord across the time course (fig. 4A). Overall, hsvPPE and hsvMOR + PPE increased Enk-ir in laminae I-III on day 16 postinfection, which was significantly higher than in sham or hsvCON–infected mice (two-way ANOVA, factors: group × lamina, fig. 4A). Nerve-injured mice exhibited an increase in mOR-ir in laminae I–III on day 16 postinfection compared with that of sham mice (two-way ANOVA, factors: group × lamina; fig. 4B). However, hsvMOR markedly increased mOR-ir in laminae I–III when compared to that of sham and hsvCON mice. hsvPPE and hsvMOR + PPE caused an increase in mOR-ir compared with that of sham and hsvCON–infected mice (fig. 4B).

Mechanical Allodynia and Thermal Hyperalgesia

Our previous studies and preliminary data indicate that mechanical and thermal sensitivity 4 weeks after hsvMOR, hsvPPE, and hsvMOR + PPE inoculation result in similar evoked thresholds (data not shown). The focus of this study was to investigate the efficacy of elevated opiate receptors and/or opiate peptides after nerve injury, and therefore SNL was used as a model for neuropathic pain in humans. All mice developed equivalent mechanical allodynia and thermal hyperalgesia by day 7 after L5 SNL, as evidenced by decreases in percent paw withdrawal thresholds and latencies from baseline (day 0). The mice inoculated with vehicle, MOCK, or hsvCON demonstrated similar allodynia across the entire time course of the study (data not shown) and were collapsed into a control group. hsvMOR reversed mechanical allodynia to preligation PWTs by day 5 postinfection (experimental day 12; *vs.* control at the same time point; fig. 5A). For the remainder of the study, PWTs remained significantly higher in the hsvMOR group than in the control group (repeated measures ANOVA; fig. 5A). By day 9 postinfection (experimental day 16) with hsvMOR, PWTs exceeded preligation thresholds, showing the development of analgesia (fig. 5A). By day 9 postinfection with hsvPPE, mechanical allodynia was reversed to levels equivalent to preligation PWTs (*vs.* control at the same time point and *vs.* hsvPPE).

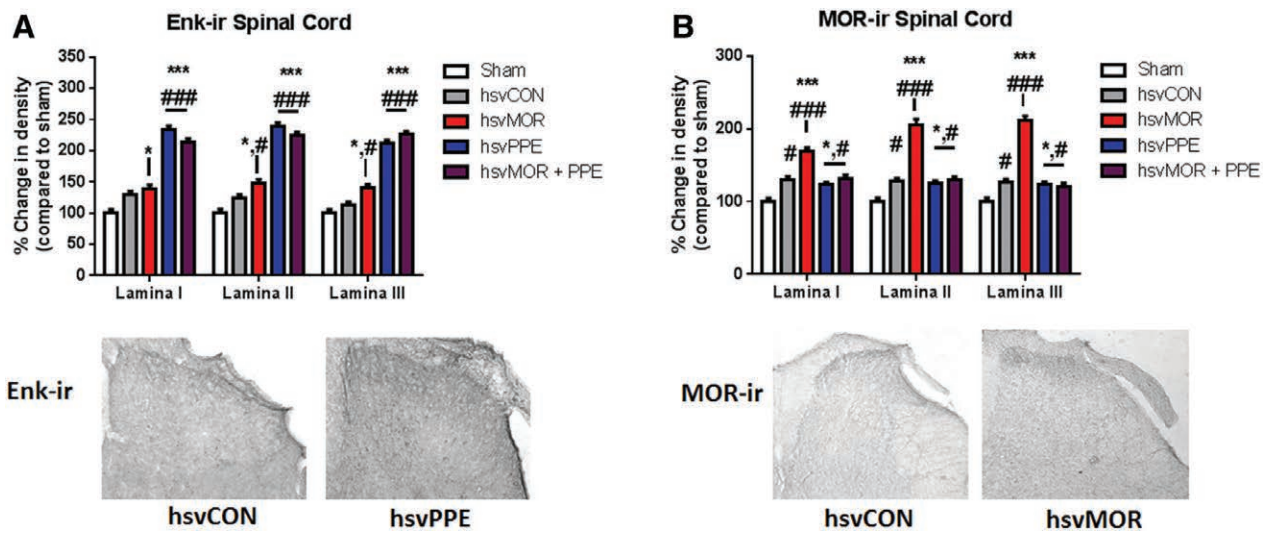

Fig. 4. The optical density of enkephalin immunoreactivity (Enk-ir) and colabeled μ-opioid receptor immunoreactivity (MOR-ir) and green fluorescent protein–positive areas of laminae I–III of the dorsal lumbar spinal cord increases after herpes simplex virus that encodes μ-opioid receptor (hsvMOR), herpes simplex virus that encodes preproenkephalin (hsvPPE), and hsvMOR + preproenkephalin (PPE) inoculation. (*A*) Quantification of Enk-ir–positive areas of the dorsal horn on day 16 after viral inoculation. There is a significant increase in Enk-ir in hsvPPE and hsvMOR + PPE treatment groups compared to sham and control virus (two-way ANOVA, factors: group × lamina, $F[8, 45] = 7.1$, $P < 0.001$, hsvCON). (*Bottom*) Histology images of enkephalin-positive areas of the dorsal horn of the spinal cord with control virus (*left*) and hsvPPE (*right*). (*B*) Quantification of MOR-ir–positive areas of the dorsal horn on day 16 after viral inoculation. (*Bottom*) Histology images of MOR–positive areas of the dorsal horn of the spinal cord with control virus (*left*) and hsvMOR (*right*). There is a significant increase in MOR-ir in all lamina of hsvMOR, hsvPPE, and hsvMOR + PPE treatment groups compared to sham and control virus (two-way ANOVA, factors: group × lamina, $F[8, 45] = 18.7$, $P < 0.001$; hsvCON). Bonferroni *post hoc* test was used for multiple comparisons. #significant at $P < 0.05$ *versus* sham; ##significant at $P < 0.01$ *versus* sham; ###significant at $P < 0.001$ *versus* sham; *significant at $P < 0.05$ *versus* control virus-treated (hsvCON); **significant at $P < 0.01$ *versus* control virus-treated (hsvCON); ***significant at $P < 0.001$ *versus* control virus-treated (hsvCON). CON = control; Enk = enkephalin; hsv = herpes simplex virus; ir = immunoreactivity; MOR = μ-opioid receptor.

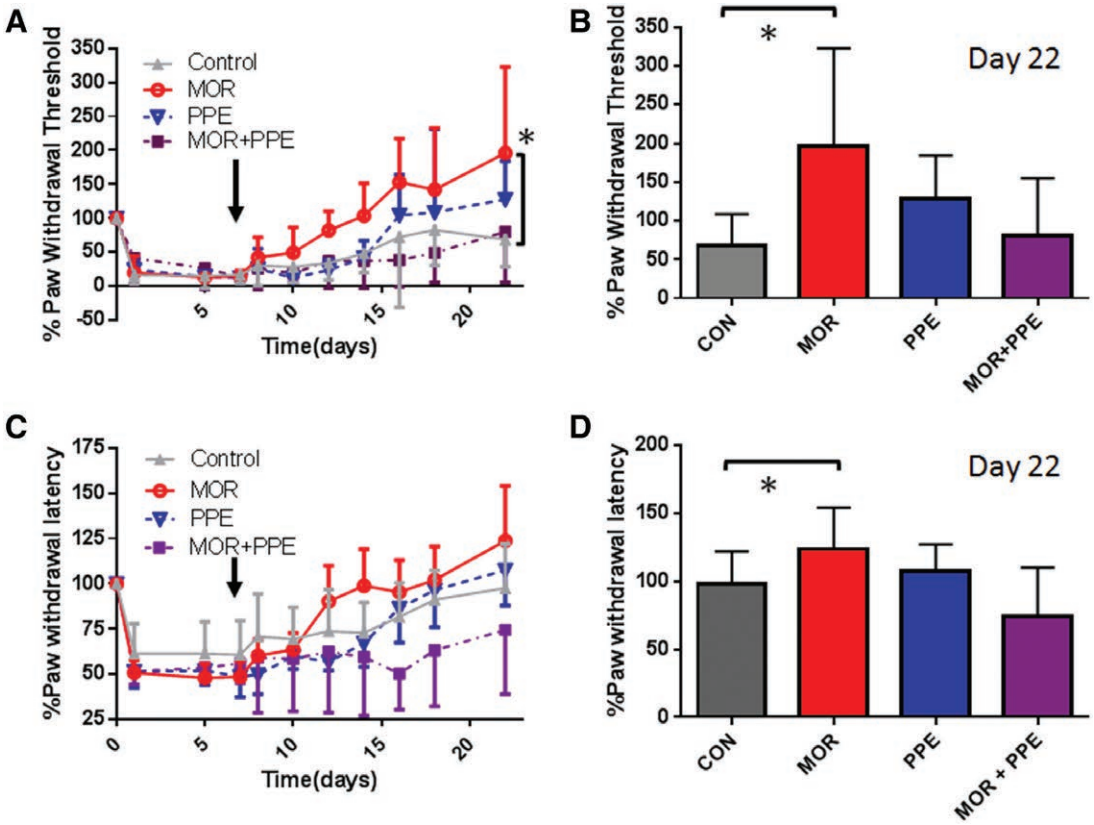

Fig. 5. Mechanical allodynia and thermal hyperalgesia after L5 spinal nerve ligation surgery are attenuated in herpes simplex virus that encodes μ-opioid receptor (hsvMOR) inoculated mice. At 7 days after nerve injury (*arrow*), μ-opioid receptor (MOR), preproenkephalin (PPE), MOR + PPE combination, or control (CON) virus was administered into the intraplantar aspect of the ipsilateral hind paw; mechanical allodynia and thermal hyperalgesia were then assessed more than 15 days. (*A*) MOR and PPE virus infections reversed mechanical allodynia after 12 and 16 days, respectively. The MOR + PPE virus–infected mice had an allodynia reversal time course comparable to that of control virus–infected mice. Paw withdrawal threshold increased significantly over time for MOR virus-inoculated animals (repeated measures ANOVA, F[30, 500] = 3.9). *$P < 0.001$ *versus* control group. (*B*) The paw withdrawal threshold after MOR infection was significantly increased compared to control virus infection at the conclusion of the behavioral study (day 22, ANOVA, F[3, 50] = 8.3). *$P < 0.001$ *vs*. control group. (*C*) MOR and PPE virus infections reversed thermal allodynia after 12 and 16 days, respectively, compared to 21 days for control virus infection. Mice administered the MOR + PPE virus combination did not have allodynia reversal during the time course of the experiment. (*D*) The thermal threshold after MOR infection was significantly increased compared with that after control virus infection at the conclusion of the behavioral study (day 22, ANOVA, F[3, 50] = 5.9). *$P = 0.022$ *vs*. control group. Bonferroni *post hoc* test was used for multiple comparisons.

In contrast, hsvMOR + PPE did not change mechanical allodynia compared with that of control (repeated measures ANOVA; fig. 5A). By experimental day 22, hsvMOR paw withdrawal thresholds exceeded preligation thresholds (fig. 5B). In contrast, hsvPPE did not reverse mechanical allodynia (fig. 5A) or thermal hyperalgesia (fig. 5C) as compared to control (repeated measures ANOVA, $p = 0.16$ and $p = 0.99$, respectively). Furthermore, hsvMOR + PPE had no effect on the duration of thermal hyperalgesia compared with that of the control group (repeated measures ANOVA, $p = 0.12$; fig. 5D).

Peripheral and Systemic Opioid Analgesia

To examine virus-mediated changes in opioid analgesia, we tested the peripherally active opioid agonist, loperamide, and the peripherally and centrally active opioid agonist, morphine, on SNL-induced thermal hyperalgesia at 16 days postinfection. The mice inoculated with vehicle, MOCK, or hsvCON demonstrated similar loperamide and morphine dose-response curves and were collapsed into a single control group for statistical analysis (data not shown). hsvMOR produced a leftward shift in the loperamide dose-response curve compared with that of the control group (fig. 6A). The ED_{50} for loperamide was 0.9 ± 0.2 mg/kg in control mice and 0.6 ± 0.2 mg/kg in control virus-treated (hsvCON) mice (table 1). In contrast, loperamide was not analgesic at any of the doses tested in mice infected with hsvPPE or hsvMOR + PPE. An ED_{50} for loperamide could not be calculated for hsvPPE or hsvMOR + PPE.

Similarly, hsvMOR produced a leftward shift in the morphine dose-response curve compared with that of the control group (fig. 6B). The ED_{50} for morphine was 1.1 ± 0.1 mg/kg in control mice and 0.3 ± 0.5 mg/kg in hsvMOR–infected

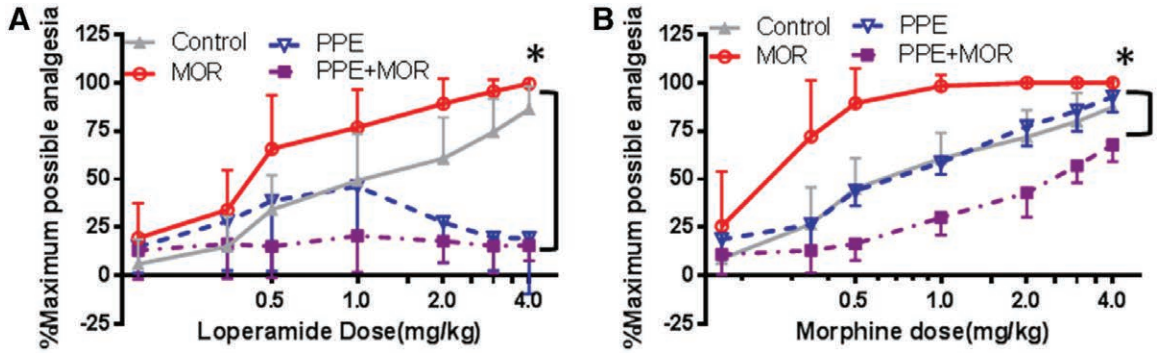

Fig. 6. Loperamide and morphine dose response effect on paw withdrawal latency to heat is significantly shifted after intraplantar administration of herpes simplex virus that encodes μ-opioid receptor (hsvMOR) *versus* herpes simplex virus that encodes preproenkephalin (hsvPPE) and hsvMOR + preproenkephalin (PPE) in nerve-injured mice. (*A*) The loperamide dose-response curve was significantly shifted to the left in animals infected with μ-opioid receptor (MOR) virus (day 16 postinfection). *$P < 0.001$ compared with control. The loperamide dose-response curve in PPE–infected mice was comparable to that of control virus–infected mice at low doses (0.17 to 1.0 mg/kg); however, at higher cumulative doses, the PPE–infected mice were nonresponsive to loperamide. MOR + PPE virus–infected mice were largely nonresponsive to loperamide. (*B*) The morphine dose-response curve was shifted to the left in animals infected with MOR (day 19 postinfection). *$P < 0.001$ compared with control. The morphine dose-response curve was shifted to the right in animals infected with MOR + PPE as compared to that in controls. In contrast, the morphine dose-response curve of PPE–infected mice was comparable that of control virus–infected mice. The percentage of maximum analgesia was calculated based on the formula: (measured PWL [s] – predrug PWL [s])/(20 – predrug PWL [s]) × 100. Bonferroni *post hoc* test was used for multiple comparisons. PWL = paw withdrawal latency.

Table 1. Effective Doses (ED_{50} Values) of Morphine and Loperamide Heat Latency Paw Withdrawal 16 Days after Intraplantar Administration of Virus (Means ± SD)

	ED_{50} ± SD (95% CI)	
	Morphine	Loperamide
hsvCON (n = 8)	1.1 ± 0.1 (0.9–1.3)	0.9 ± 0.2 (0.6–1.2)
hsvMOR (n = 13)	0.3 ± 0.5 (0.3–1.2)*	0.6 ± 0.2 (0.1–1.1)*
hsvPPE (n = 9)	0.9 ± 0.1 (0.7–1.2)	NA
hsvMOR + PPE (n = 8)	1.7 ± 0.2 (1.3–2.0)*	NA

The EC_{50} values were compared to those of the control group (hsvCON).

*Significant at $P < 0.001$.

hsvMOR = herpes simplex virus that encodes μ-opioid receptor; hsvPPE = herpes simplex virus that encodes preproenkephalin; NA = not applicable; PPE = preproenkephalin.

mice (table 1). hsvPPE had no effect on morphine analgesia, as shown by an overlapping dose-response curve ($P > 0.05$) and equivalent ED_{50} with control. hsvMOR + PPE produced a rightward shift in the morphine dose-response curve compared to that of control and increased the ED_{50} for morphine to 1.7 ± 0.2 mg/kg (table 1).

Capsaicin-induced Spontaneous Behaviors, Thermal Hyperalgesia, and Neuronal Activity

hsvCON–infected mice had a pain score of 0.58 ± 0.15 (average ± SD) calculated from spontaneous pain-associated behaviors exhibited during the first 5 min after intraplantar capsaicin administration (fig. 7A). A statistically significant decrease in pain score was observed in mice infected with hsvMOR (0.38 ± 0.11), hsvPPE (0.27 ± 0.09), or hsvMOR + PPE (0.35 ± 0.11) as compared to hsvCON (fig. 7A). Ten minutes postcapsaicin, all groups developed heat hyperalgesia evidenced by the decrease in paw withdrawal latency (fig. 7B). Heat hyperalgesia resolved more quickly, and maximal heat hyperalgesia was significantly attenuated in animals infected with hsvMOR, hsvPPE, and hsvMOR + PPE as compared to hsvCON.

cFos was used as a marker of neuronal activation and was assayed in separate animals 2 h after capsaicin, a time point when cFos should be maximally induced (fig. 7C). There was a statistically significant reduction in the number of cFos-positive neurons in hsvMOR–, hsvPPE–, and hsvMOR + PPE–inoculated animals, as compared to hsvCON–infected mice. hsvMOR–infected mice had significantly fewer cFos positive neurons compared to hsvPPE– and hsvMOR + PPE–inoculated mice (fig. 7D).

Changes in Physical Properties/Responsiveness of Primary Afferent Fibers after SNL

Neuropathic hyperalgesia was reversed (fig. 5), and behavioral responses after opioid administration (fig. 6) were shifted significantly to the left in hsvMOR–inoculated animals compared with those in hsvCON–inoculated animals. Animal groups inoculated with hsvPPE and hsvMOR + PPE did not show such effects. Therefore, we did not include them in the subsequent electrophysiologic experiments that focused on the effects of hsvMOR inoculation on receptive properties of afferent fibers.

Sensitization in primary afferent fibers has been postulated to contribute to the pain phenotype seen in many peripheral neuropathies, especially after nerve injury. We evaluated baseline properties and evoked activity of C-, Aδ-, and Aβ-fibers recorded from animals with or without nerve injury that either were not inoculated or were inoculated with control or hsvMOR virus (table 2). Compared to

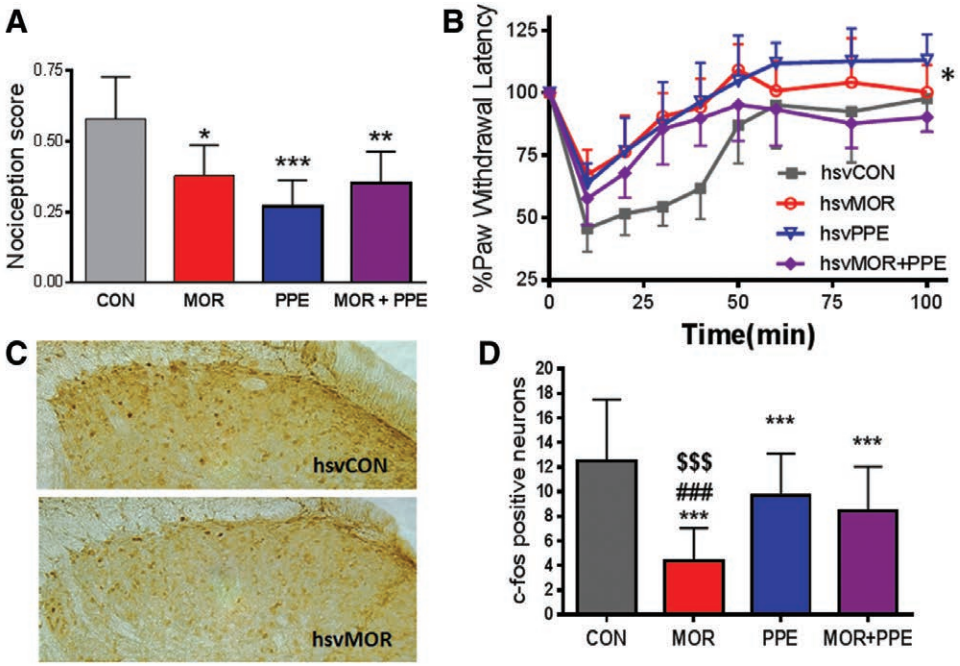

Fig. 7. Herpes simplex virus (hsv) vectors that encode μ-opioid receptor (hsvMOR) or preproenkephalin (hsvPPE), and hsvMOR + preproenkephalin (PPE) decreased capsaicin-induced nociception and neuronal activation at 4 weeks after topical hind paw infection. (*A*) hsvMOR, hsvPPE, and hsvMOR + PPE produced lower capsaicin-induced nociception scores 5 min after subcutaneous capsaicin administration in the left hind paw, in comparison to hsvCON. **P* < 0.05 *versus* hsvCON; ***P* < 0.01 *versus* hsvCON; ****P* < 0.001 *versus* hsvCON. (*B*) hsvMOR, hsvPPE, and hsvMOR + PPE attenuated capsaicin-induced thermal hyperalgesia compared to hsvCON. ***P* < 0.01 hsvMOR *versus* hsvCON; ****P* < 0.001 hsvMOR *versus* hsvCON; #*P* < 0.05 hsvPPE *versus* hsvCON; ###*P* < 0.001 hsvPPE *versus* hsvCON; $$$*P* < 0.001 hsvMOR + PPE *versus* hsvCON. (*C*) cFos expression in the dorsal horn of the lumbar spinal cord at 2 h after capsaicin administration was greater in hsvCON as compared to hsvMOR infection. (*D*) hsvMOR, hsvPPE, and hsvMOR + PPE infection decreased the number of cFos-positive neurons compared to hsvCON. ****P* < 0.001 compared to hsvCON; ###*P* < 0.001 compared to hsvPPE; $$$*P* < 0.001 compared to hsvMOR + PPE. CON = control; MOR = μ-opioid receptor.

fibers recorded from uninjured animals, C-fibers recorded from nerve-injured animals had significantly higher heat thresholds, lower cold thresholds, and slower CVs (table 3) with no significant effects caused by vector. Additionally, we observed no significant baseline changes in Aδ- or Aβ-fibers after nerve injury or with respect to viral vector inoculation (data not shown).

Upon mechanical stimulation, Aβ- and Aδ-fibers from animals in the nerve-injured control groups (SNL) exhibited significantly increased responses to suprathreshold stimulation compared with those of the uninjured controls (no treatment and hsvCON); the difference became more apparent across larger forces (repeated measures ANOVA, factors: injury status × force; fig. 8A and Supplemental Digital Content 2, http://links.lww.com/ALN/B603; Aβ-receptive field responses to mechanical stimulation data, respectively). Suprathreshold mechanical responses did not differ between Aδ-fibers recorded from lesioned or uninjured animals that were inoculated with hsvMOR after injury (fig. 8B), and the responses in these groups were not statistically different from those observed in uninjured control animals (compare to fig. 8A). Interestingly, C-fibers from lesioned animals did not show more robust responses to suprathreshold mechanical stimuli than did fibers from uninjured controls (fig. 8, C and D).

Decrease in Evoked Mechanical and Thermal Responses in hsvMOR Fibers after Morphine Application

The sensitivity to evoked stimulation was measured in primary afferent nerve fibers before and after morphine application in uninjured and nerve-injured animals. Regardless of injury status, Aβ-fibers recorded from animals in the hsvMOR inoculation group displayed a significant decrease in the mechanically evoked responses across forces after morphine application (see figure, Supplemental Digital Content 3, http://links.lww.com/ALN/B604, hsvMOR inoculated Aβ-fiber responses data), and the total responses postmorphine were significantly smaller than their corresponding premorphine responses (paired *t* test). Mechanical responses recorded from Aβ-fibers in the control groups (no treatment and hsvCON) were similar to those after morphine application. We observed no significant decrease in mechanically evoked responses for Aδ-fibers or C-fibers postmorphine, regardless of vector treatment or injury status (data not shown).

Table 2. Populations of Units Based on Response Properties

Unit Type	No Treatment	hsvCON	hsvMOR	SNL	SNL hsvCON	SNL hsvMOR
C-fibers	n = 11	n = 23	n = 18	n = 20	n = 23	n = 16
CMH	4	2	3	6	4	2
CMHC	2	12	6	6	3	2
CMC	0	1	6	2	6	3
CM	2	3	0	4	2	2
CH/CC/CHC	3	5	3	2	8	7
Aδ-fibers	n = 11	n = 8	n = 7	n = 14	n = 11	n = 17
ADM	9	7	7	10	8	11
ADMC	0	1	0	0	1	1
ADMHC	0	0	0	0	0	1
ADMH	2	0	0	4	2	4
ADH	0	0	0	0	0	0
Aβ-fibers	n = 13	n = 7	n = 14	n = 16	n = 17	n = 17

ADH = heat-sensitive Aδ-fiber; ADM = mechanosensitive Aδ-fiber; ADMC = mechano–cold-sensitive Aδ-fiber; ADMH = mechano–heat-sensitive Aδ-fiber; ADMHC = mechano-–heat and –cold-sensitive Aδ-fiber; CC = cold-sensitive C-fiber; CH = heat- and cold-sensitive C-fiber; CHC = heat- and cold-sensitive C-fiber; CM = mechanosensitive C-fiber; CMC = mechano–cold-sensitive C-fiber; CMH = mechano–heat-sensitive C-fiber; CMHC = mechano–heat- and –cold-sensitive C-fiber; CON = control; hsv = herpes simplex virus; MOR = μ-opioid receptor; SNL = spinal nerve ligation.

Table 3. Baseline Properties of C-fibers (Means ± SD)

Group	Von Frey Threshold (mbars)	Heat Threshold (°C)*	Cold Threshold (°C)†	Mechanical Threshold (g)	Conduction Velocity (m/s)‡
No vector and hsvCON (n =34)	1.8 ± 0.6	40.1 ± 4.5	14.0 ± 6.4	1.8 ± 0.8	0.65 ± 0.2
hsvMOR (n = 18)	1.4 ± 0.8	38.2 ± 5.9	12.9 ± 6.4	1.9 ± 1.7	0.74 ± 0.25
SNL no vector and hsvCON (n = 43)	1.5 ± 0.7	41.2 ± 3.9§	17.3 ± 9.8§	3.0 ± 2.3	0.65 ± 0.26#
SNL hsvMOR (n = 16)	1.6 ± 0.4	41.8 ± 5.6§	17.2 ± 6.0‖	1.6 ± 0.5	0.48 ± 0.08#

1 Millibar (mbar) = 0.0001 mN force.

*Significant increase in SNL (SNL no vector and hsvCON; SNL hsvMOR) *versus* uninjured fibers (no vector and hsvCON; hsvMOR). †Significant decrease in SNL (SNL no vector and hsvCON; SNL hsvMOR) *versus* uninjured fibers (no vector and hsvCON; hsvMOR). ‡Significant decrease in SNL (SNL no vector and hsvCON; SNL hsvMOR) *versus* uninjured fibers (no vector + hsvCON and hsvMOR). §ANOVA: $F(1, 74) = 5.7$, $P < 0.01$. ‖ANOVA: $F(1, 64) = 6.2$, $P < 0.01$. #ANOVA: $F(1, 107) = 6.4$, $P < 0.05$.

CON = control; hsv = herpes simplex virus; MOR = μ-opioid receptor; SNL = spinal nerve ligation.

Compared to C-fibers from uninoculated animals or animals inoculated with control virus, heat-sensitive C-fibers from animals inoculated with hsvMOR showed a significant decrease in the fraction of evoked action potentials postmorphine (ANOVA, factor: vector; fig. 9A). The heat threshold postmorphine was significantly greater in C-fibers from hsvMOR–inoculated animals than in control fibers (ANOVA, factor: vector; fig. 9B). Although the fraction of evoked potentials and heat thresholds before and after morphine application did change with statistical significance in hsvMOR–inoculated fibers compared with that in controls, the numerical difference was very small, suggesting that some fibers may be affected more than others or that the peripheral terminals of heat-sensitive C-fibers are not readily affected by hsvMOR inoculation. Overall, spontaneous activity was found in more than 50% of C-fibers after nerve injury, and this activity was significantly decreased after morphine application in hsvMOR fibers but not in control fibers (fig. 9, C and D). Only one fiber in the SNL hsvMOR group had spontaneous activity above ten action potentials per min, whereas seven of the fibers in the no treatment and hsvCON groups had spontaneous activities above that rate. After morphine incubation, the remaining C-fiber spontaneous activity was significantly decreased in the hsvMOR–inoculated fibers compared to that in fibers recorded from nerve-lesioned, uninoculated, or control virus-inoculated animals (% change = [(Post – Pre)/Pre] × 100, unpaired *t* test, $P < 0.05$; fig. 9D; example traces shown in fig. 9, E and F).

Discussion

Expression of mOR and Enkephalin after Nerve Injury

Immunoreactivity for mOR in epidermal nerve fibers in the plantar hind paw skin, and small-diameter DRG cells decreased in hsvCON nerve injured mice compared to sham mice, in agreement with previous reports of a nerve injury–induced loss of mOR.[27–29] mOR expression is regulated by several factors during chronic pain, including DNA methylation at the mOR promotor and histone deacetylation, which are both involved in mOR gene silencing.[30,31] Whether HSV-1 inoculation has any effect on these epigenetic factors in the peripheral nervous system is unknown, but regulatory mechanisms for the control of mOR expression could be a future target for gene therapy.

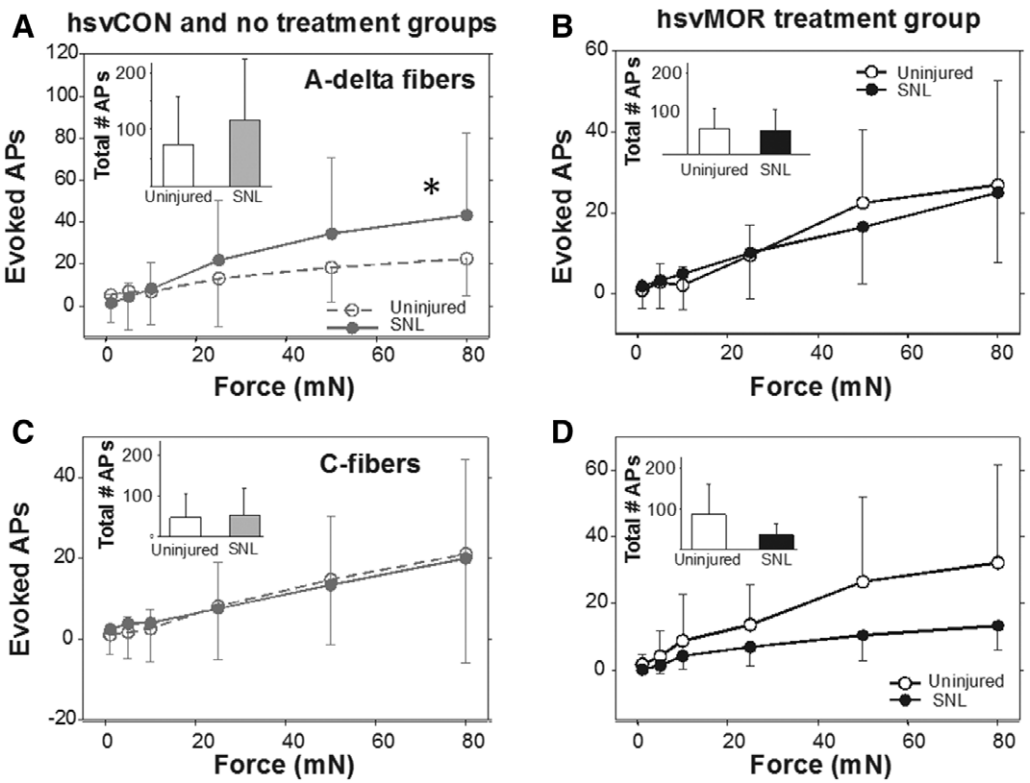

Fig. 8. Nerve injury enhances mechanically evoked responses of myelinated primary afferent fibers. Evoked mechanical stimuli were delivered to the receptive fields of nerve fibers by a feedback-controlled mechanical stimulator in naïve mice and mice after SNL. (*A*) Aδ-fiber responses in control fibers (no treatment and hsvCON) to mechanical stimulation were increased after SNL compared with those of uninjured fibers across increasing forces of mechanical stimulation (repeated measures ANOVA: F[5, 215] = 3.3, *P* = 0.0014). (*Inset*) The total number of mechanically evoked action potentials was slightly higher in nerve-injured Aδ-fibers than in uninjured fibers. (*B*) Aδ-fiber responses in hsvMOR–inoculated mice did not differ between injured and uninjured fibers across increasing forces of mechanical stimulation. (*C*) C-fiber mechanical responses in control fibers (no treatment and hsvCON) were not increased after nerve injury across forces. (*D*) C-fiber mechanical responses in from hsvMOR inoculated fibers were not increased after nerve injury. AP = action potential; CON = control; hsv = herpes simplex virus; MOR = μ-opioid receptor; SNP = spinal nerve ligation.

In contrast, enkephalin expression was not changed in the plantar hind paw skin, and DRG cells in hsvCON nerve-injured mice compared to sham mice. Although exogenous administration of enkephalins[32] and virally mediated expression of preproenkephalin[15,16,19,33,34] reduces pain in experimental models, the role for endogenous preproenkephalin in the initiation and maintenance of neuropathic pain has not been well studied.[17]

Effect of Nerve Lesion on Receptive Properties of Cutaneous Afferents

Spinal nerve ligation increased the response to suprathreshold mechanical stimulation in Aδ-fibers, whereas SNL increased the response only at the highest force for Aβ-fibers (Supplemental Digital Content 2, http://links.lww.com/ALN/B603), and responses in C-fibers did not differ between unlesioned and SNL animals treated with control virus. Changes in peripheral nerve sensitivity have also been found in Aδ- and C-fibers in a spared nerve injury mouse model,[35] indicating that mechanical allodynia may be mediated by both myelinated and unmyelinated fibers. Because we only found modest changes in heat sensitivity after SNL, peripheral sensitization of C-fibers to heat stimuli is unlikely to explain behavioral signs of heat hyperalgesia that has been observed after SNL in mice. In contrast, cold thresholds in C-fibers were increased by about 3°C (table 3), and this change may contribute to signs of cold hyperalgesia that develops after SNL[36] and in neuropathic pain patients.[37] After SNL, we observed a considerable number of C-fibers with spontaneous activity. Spontaneous activity in intact C-fibers after SNL has previously been observed in non-human primate[38] and rat,[39,40] and such activity may induce spontaneous pain behavior and contribute to the initiation and maintenance of central sensitization.

Overexpression of mOR

The attenuation of evoked responses indicate that tactile and heat sensitivity are decreased after hsvMOR inoculation. Future studies utilizing conditioned place preference, gait analysis, and innate behavioral quantification (*e.g.*, digging, locomotion) could help provide further insight on the effects

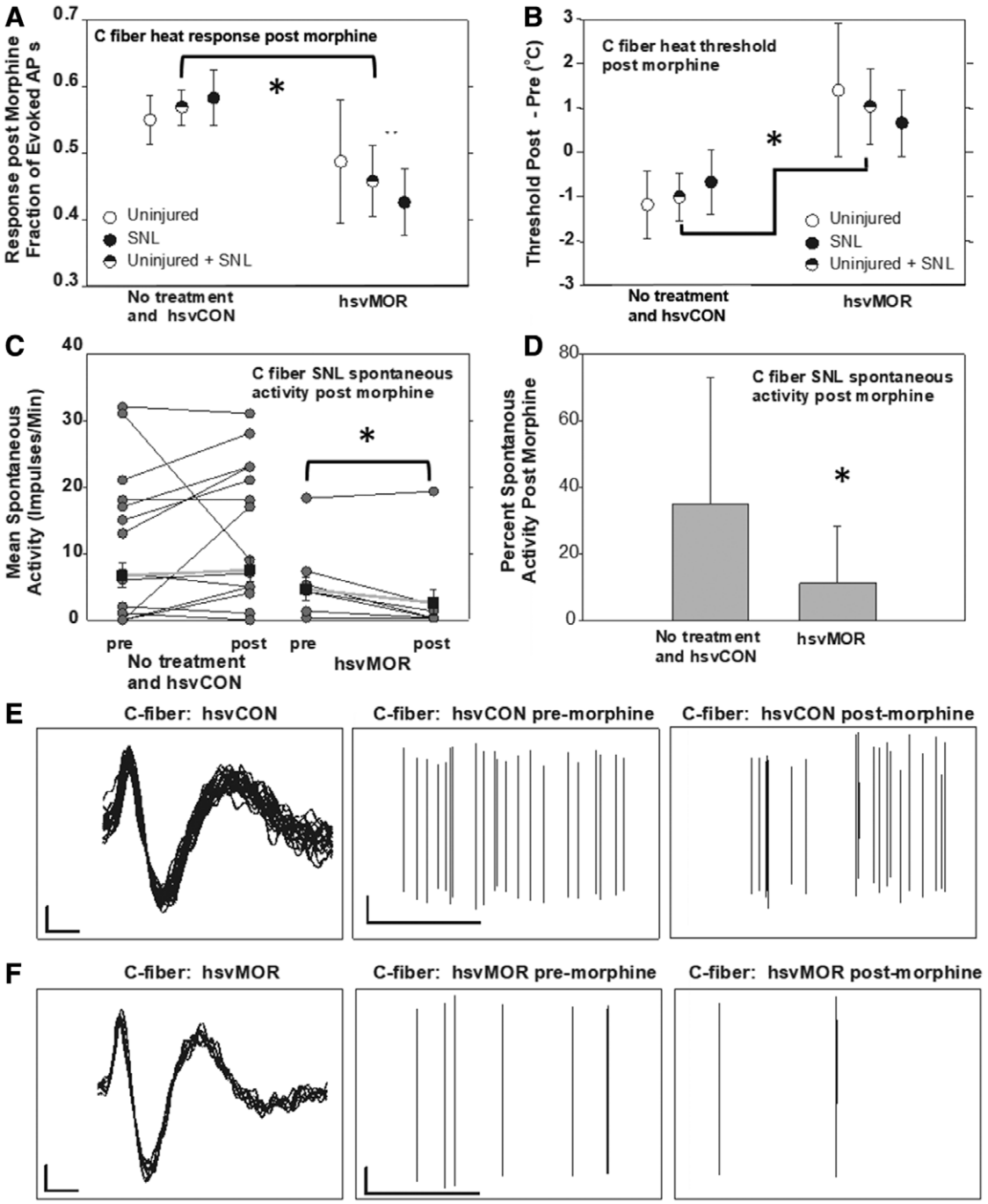

Fig. 9. Morphine application causes a significant decrease in evoked and spontaneous C-fiber activity in mOR (hsvMOR) viral vector-inoculated fibers. (*A*) The fraction of heat-evoked action potentials after morphine application was lower in hsvMOR–inoculated fibers than in uninoculated and hsvCON–inoculated fibers ($F[1, 69] = 4.2$). *$P = 0.044$. The data are presented as the fraction change after morphine application compared to premorphine values. Overall the heat responses in C-fibers were quite variable, and the data were pooled to reduce variance, especially in uninjured animals. Uninjured + SNL = units combined from uninjured animals and SNL. (*B*) Heat threshold in hsvMOR–inoculated fibers increased significantly after morphine application compared with that in uninoculated and hsvCON–inoculated fibers (ANOVA, factor: vector, $F[1, 71] = 4.5$). *$P = 0.038$. (*C*) The mean spontaneous activity (impulses/min) was significantly less in C-fibers from hsvMOR–inoculated mice with SNL after morphine application (*post*) than before morphine application (*pre*) (paired *t* test). *$P = 0.028$. The averaged data are shown in *black squares*. (*D*) Spontaneous activity decreased significantly after morphine application in hsvMOR–inoculated fibers (unpaired *t* test). *$P = 0.048$. (*E*) Example traces of C-fiber spontaneous activity from hsvCON–infected animals. (*Left*) Waveform traces. *Vertical bar* = 2 mV; *horizontal bar* = 0.5 ms. (*Middle*) Before morphine application. *Vertical bar* = 2 mV; *horizontal bar* = 20 ms. (*Right*) Five minutes after morphine application. (*F*) As in (*E*) for example traces of C-fiber spontaneous activity from hsvMOR–infected animals. (*Left*) Waveform traces. (*Middle*) before morphine application. (*Right*) Five minutes after morphine application. CON = control; hsv = herpes simplex virus; MOR = μ-opioid receptor; SNP = spinal nerve ligation.

of enhanced mORs with the viral vector on ongoing, spontaneous pain, although negative results have been reported for multiple types of these behavioral assays, making data interpretation somewhat challenging.[41]

Similar to previous studies,[10] virus-driven mOR expression was observed in large and medium afferents and led to significant increases in mOR expression in laminae I–III. This presence of mOR in large diameter afferents coupled with the more diffuse mOR immunoreactivity in the spinal cord dorsal horn suggested that hsvMOR may allow for expression of mOR in myelinated fibers. Interestingly, hsvMOR animals also had increased levels of Enk-ir in epidermal nerve fibers, DRG cells, and dorsal horn of the spinal cord, which could indicate changes in PPE production in keratinocytes and immune cells after infection.

Effects of hsvMOR Inoculation on Properties of Cutaneous Afferents

Inoculation with hsvMOR in Aδ-fibers appears to prevent the SNL-induced increase to suprathreshold stimulation in this fiber class (compare fig. 8, A and B). This effect may contribute to the "spontaneous" reversal in paw withdrawal von Frey thresholds that was observed in animals inoculated with hsvMOR (fig. 5A). Furthermore, the incidence of C-fibers with spontaneous activity was reduced in lesioned animals inoculated with hsvMOR (fig. 9C). This decrease may in turn reduce central sensitization and thereby contribute to the reversal in mechanical paw withdrawal thresholds that was observed in nerve-lesioned, hsvMOR–inoculated animals (fig. 5A). The mechanically evoked activity in C fibers was increased in uninjured hsvMOR animals *versus* animals with SNL (fig. 8D). This increase is surprising, because hsvCON animals do not show an increased sensitivity to mechanical stimulation (fig. 8C). It is possible that overall neuronal sensitivity and changes in evoked responses are more pronounced in recordings from hsvMOR animals than hsvCON animals, because of increased neuroinflammation and changes in intracellular signaling after nerve injury.[42,43]

Opioid Analgesia

hsvMOR inoculation produced a leftward shift in loperamide and morphine analgesia, and these data are similar to previous hsvMOR studies in uninjured[10] and injured mice.[26] Previous studies have demonstrated that peripheral opioid receptors, endogenous or virally inserted, are a key site of action for opioid analgesia, because their efficacy can be reduced *via* naloxone[17,19,44] and methyl-naltrexone.[7,45] Taken together, these results suggest that increased expression of mOR on peripheral and spinal afferent nerve terminals may be important sites of action for opioid analgesia after nerve injury.

Spontaneously Evoked Behaviors and cFos Expression after Capsaicin Exposure

In the present study, infection with hsvMOR, hsvPPE, or hsvMOR + PPE decreased capsaicin-induced behaviors. This outcome is not unexpected, because peripheral administration of opiates attenuates capsaicin pain in humans.[46] hsvMOR produced maximal inhibition of capsaicin-induced cFos expression as compared to hsvPPE and hsvMOR + PPE (fig. 7D). The decrease in cFos activation of hsvMOR could be directly due to attenuation of nociceptor activation, a decrease in paw flinching/licking, and/or animal movement after capsaicin injection. Regardless, the cFos data suggest that excitatory inputs and presynaptic modulation/postsynaptic hyperpolarization of secondary sensory neurons are likely mediated by μ-opiate receptors after noxious stimulation (*e.g.*, capsaicin injection).

Effects of Morphine on Properties of Cutaneous Afferents

The effects of morphine on heat-evoked responses in C-fibers (fig. 9) were minimal, whereas in contrast, the increased behavioral effects of morphine after hsvMOR inoculation (fig. 6) may instead be due to an increased effect in the dorsal horn of the spinal cord because hsvMOR inoculation led to an increased, time-dependent expression of mOR in the dorsal horn (fig. 5). Morphine also reduced the responses to suprathreshold mechanical stimulation in Aβ-fibers recorded from animals inoculated with hsvMOR (Supplemental Digital Content 3, http://links.lww.com/ALN/B604). The behavioral effects of morphine inhibition of Aβ-fibers mechanosensitivity in the SNL model are not clear; however, such inhibition may be beneficial in patients with postherpetic neuralgia or traumatic nerve injury that present with dynamic mechanical allodynia.[37]

Overexpression of PPE

Opioid peptides, enkephalin in particular, stimulate cytokine release and immunocyte chemotaxis and can induce changes that signal for immunocyte activation.[47] The effects of opioid peptides that are locally released from immune cells[48] could explain the increase in Enk-ir in hsvMOR–infected animals as soon as day 1 postinfection (see tables, Supplemental Digital Content 4–6, http://links.lww.com/ALN/B605; tables for Enk-ir and mOR-ir in epidermal nerve fibers, dorsal root ganglia, and lumbar spinal cord, respectively). These data suggest that trafficking of enkephalin vesicles in primary afferent neurons may be initially enhanced[3] and that expression observed at day 1 postinfection is likely from nonneuronal cells.

Previous studies using an HSV proenkephalin sequence showed reductions in mechanical allodynia in a L5 spinal nerve ligation model in rats.[18] Surprisingly, in this study, infection with hsvPPE did not reverse thermal hyperalgesia and had no effect on morphine analgesia in nerve-injured mice. This is different from previous studies showing a reduction of neuropathic pain in rodent models of nerve injury[18,49] and capsaicin-induced pain.[15] It is noteworthy that our behavioral studies were conducted at least 2 weeks after intradermal injection of the virus and nerve injury, and experiments included female mice.

Overexpression of PPE and mOR

Surprisingly, coinfection with hsvPPE and hsvMOR decreased enkephalin and mOR expression in nerve fibers and did not reverse mechanical allodynia or thermal hyperalgesia. The analgesic effects hsvMOR + PPE was predicted to be additive to those of hsvMOR and hsvPPE. It is possible that an up-regulation of an inhibitory G-protein–coupled receptor and an inhibitory peptide in nerve fibers could alter receptor activity and downstream signaling pathways[50] and/or increase expression of pronociceptive signaling molecules not investigated in this study. Furthermore, coinfection shifted loperamide and morphine dose-response curves to the right, suggestive of opioid receptor desensitization, which could produce a physiologic state similar to opioid tolerance.

Potential Clinical Implications

Our preclinical findings suggest that enhancing peripheral mOR expression after nerve injury, using viral vectors, attenuates mechanical allodynia and thermal hyperalgesia through their effects on primary afferent signaling. These data suggest that important factors such as the transgene sequence, species, timing postinoculation, biologic sex, route of administration, and etiology of chronic pain are all important factors to consider in preclinical studies as this therapy moves closer to the clinic. Previous preclinical studies using hsvPPE have been recently tested in clinical trials for intractable cancer pain,[51,52] indicating that there is some translational potential for this viral vector system using hsvMOR. The peripheral application of hsvMOR presents a novel, translatable therapeutic strategy for the treatment of neuropathic pain with minimal CNS adverse effects that may result from enhanced endogenous opioid analgesia and a decrease in the dose of exogenous opioids.

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Competing Interests

The authors declare no competing interests.

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