

Enhanced Peripheral Analgesia Using Virally Mediated Gene Transfer of the μ-Opioid Receptor in Mice

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Background: **The use of opioids to treat pain is often limited by side effects mediated through the central nervous system. The current study used a recombinant herpes simplex virus type 1 to increase expression of the μ-opioid receptor (μOR) in primary afferent neurons. The goal of this strategy was to enhance peripheral opioid analgesia.**

Methods: **Cutaneous inoculation with herpes simplex virus containing μOR complementary DNA (cDNA) in antisense (SGAMOR) or sense (SGMOR) orientation relative to a constitutive promoter, or complementary DNA for *Escherichia coli* lac Z gene as a control virus (SGZ) was used to modify the levels of μOR in primary afferents. The effects of altered μOR levels on peripheral analgesia were then examined.**

Results: **At 4 weeks after SGAMOR and SGMOR infection, decreased and increased μOR immunoreactivity was observed in ipsilateral dorsal hind paw skin, lumbar dorsal root ganglion cells, and superficial dorsal horns, respectively, compared with SGZ. This change in μOR expression in mice by SGAMOR and SGMOR was accompanied at the behavioral level with a rightward and leftward shift in the loperamide dose–response curve, respectively, compared with SGZ.**

Conclusions: **This gene therapy approach may provide an innovative strategy to enhance peripheral opioid analgesia for the treatment of pain in humans, thereby minimizing centrally mediated opioid side effects such as sedation and addiction.**

THE efficacy of opioids in treating chronic noncancer pain remains controversial. Initial reports suggested that patients with cancer pain, including those with a neuropathic component, might benefit from opioid treatment,[1,2] but subsequent studies implied that neuropathic pain may be resistant to opioids.[3,4] Recent studies with oral[5–8] and intrathecal[9,10] opioids have demonstrated significant opioid-induced analgesia in patients with neuropathic pain. However, the required doses of opioids may be higher than for the treatment of acute nociceptive pain, and side effects associated with high doses of opioids such as sedation and cognitive dysfunction may limit their usefulness.[8,11–14] Therefore, strategies which increase the potency of opioid analgesia may be a viable therapeutic strategy to bypass the side effects of high-dose opioid therapy.

Traditionally, the analgesic effects of opioids were thought to be a result of their actions on the central nervous system. However, a growing body of evidence indicates that part of the analgesic effects of opioids, after tissue injury and inflammation, is mediated by peripheral opioid receptors.[15–19] In inflammatory states, topical or local administration of a peripheral opioid agonist, loperamide, produces antihyperalgesia without the side effects associated with systemic opioid administration.[20–22] Therefore, the development of analgesic strategies that increase peripheral opioid analgesia and limit opioid-induced side effects are possible.[7]

To this end, genetic strategies to increase the production of endogenous opioid receptor ligands[23] have been found to reduce pain induced by inflammation[24–27] and nerve injury[28] in experimental animals. More recently, direct injection of an adenoassociated virus encoding opioid receptors into the dorsal root ganglia or sciatic nerve has been shown to increase morphine analgesia.[29–31] As compared with adenoassociated virus, the use of recombinant herpes simplex virus (HSV) is advantageous in that it naturally targets primary afferent neurons after inoculation of the skin, thus bypassing the need for surgical delivery of virus. In this study, recombinant HSV constructs with the μ-opioid receptor (μOR) complementary DNA (cDNA) inserted in the sense (SGMOR) or antisense (SGAMOR) orientation relative to promoter were used to selectively increase or decrease expression of the μOR in primary afferent neurons. We have previously shown that insertion of μOR cDNA in the antisense orientation decreased expression of the μOR in the spinal terminals of primary afferents, corresponding to a rightward shift in the dose–response curve for the μOR-specific agonist [d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin (DAMGO) delivered intrathecally.[32] The current study builds on these previous findings to determine whether the SGMOR construct can (1) increase μOR expression in primary afferent neurons and (2) enhance peripheral opioid analgesia. In the current study, we show a leftward shift in loperamide analgesia and an increased expression of μOR in the skin, dorsal root ganglion, and spinal terminals of primary afferent neurons after topical inoculation with the SGMOR viral construct, suggesting the utility of HSV-1 constructs in selectively enhancing peripheral opioid analgesia.

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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. Efforts were made throughout the experiment to minimize animal discomfort and to reduce the number of animals used. Female Swiss-Webster mice (5–6 weeks old, 20–25 g; Charles River, Wilmington, MA) were used. This strain and sex have been used in the historic studies that characterized the restriction of the KOS strain of HSV-1 to the peripheral nervous system.[33,34] Animals were housed in a standard 12–12 h light–dark environment with food and water *ad libitum*.

Recombinant HSV-1 Vector Construction and Application

A replication defective KOS strain of HSV-1 virus was used in this study. The KOS strain is an avirulent strain that is restricted to the peripheral nervous system specifically at the level of the dorsal root ganglia.[33] The virus was made replication defective by deletion of the immediate early gene ICP4 preventing the viral lytic cycle.[35] Viruses were prepared as previously described,[27] except that transgene cassettes were inserted between the HSV-1 UL36 and UL37 genes. Use of this insertion site in the viral genome takes advantage of a unique *Spe*I restriction site to increase the efficiency of recombinant virus generation.[36] A schematic of the recombinant viruses encoding the rat μ-opioid gene (provided by Huda Akil, Ph.D., Professor, Department of Psychiatry, University of Michigan, Ann Arbor, Michigan) in antisense orientation (SGAMOR) or sense orientation (SGMOR) relative to the cytomegalovirus promoter is shown in figure 1. A second, similarly constructed vector encoding the *Escherichia coli* lac Z gene served as a control (SGZ). In all constructs, the cDNA for green fluorescent protein (GFP) was included as a reporter gene.

Animals were deeply anesthetized with 400 mg/kg tribromoethanol (intraperitoneal). Dorsal hind paws were treated with Nair (Church and Dwight Co., Inc., Lakewood, NJ) to remove the hair. Both dorsal and plantar hind paws were scarified (the outermost layer of dead keratin cells was removed) with medium-coarse sandpaper, whereupon saline vehicle (10 μl) or vehicle containing SGMOR, SGAMOR, or SGZ (1×10^7 pfu, 10 μl) was topically applied to both the dorsal and plantar hind paw. The scarification allows the virus to have access to the epidermal nerve fibers that it infects.

Immunohistochemistry

Previous work has shown significant viral vector–mediated effects on spinal opioid analgesia at 4 weeks after cutaneous viral inoculation.[32] As previously described,[32] 4 weeks after virus application, mice (n = 3–4/treatment) 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–L5 dorsal root ganglia were removed and postfixed in 4% paraformaldehyde for 24 h followed by cryoprotection in 30% sucrose in 0.1 M phosphate buffer. Left dorsal hind paw skin was also removed and postfixed in 4% paraformaldehyde for 4 h followed by cryoprotection in 30% sucrose in 0.1 M phosphate buffer.

Lumbar spinal cords (30 μm), dorsal root ganglia (12 μm), and skin (12 μm) were serially sectioned. Dorsal root ganglia and skin were thaw-mounted onto slides for slide-mounted immunohistochemistry. Spinal cords were placed into Tris-buffered saline (TBS) with 1% Tween 80 (TBS+) in 24-well plates for free-floating immunohistochemistry. Immunohistochemistry was done using the avidin–biotin method as previously described.[24] Briefly, sections were rinsed in TBS and quenched for endogenous peroxidase activity and antigen recovery in mentholic peroxide for 15 min. Samples were rinsed three times (10 min each) and then blocked for 20 min at room temperature with normal donkey serum (1.5%) in TBS+. Blocking was followed by a 24-h incubation at 4°C in polyclonal rabbit anti-μOR (1:1,000; Neuromics, Minneapolis, MN), 1% normal donkey serum in TBS+. The following day, samples were rinsed three times (10 min each) with TBS+. Samples were then incubated for 1.5 h at room temperature in biotinylated affinity-purified donkey anti-rabbit immunoglobulin G (1:1,000; Jackson Immunoresearch, West Grove, PA), 1.5% normal donkey serum in TBS+. Samples were washed three times (10 min each) and then incubated for 1 h at room temperature in horseradish peroxidase–conjugated streptavidin (1:1,600; Jackson ImmunoResearch) in TBS+. Samples

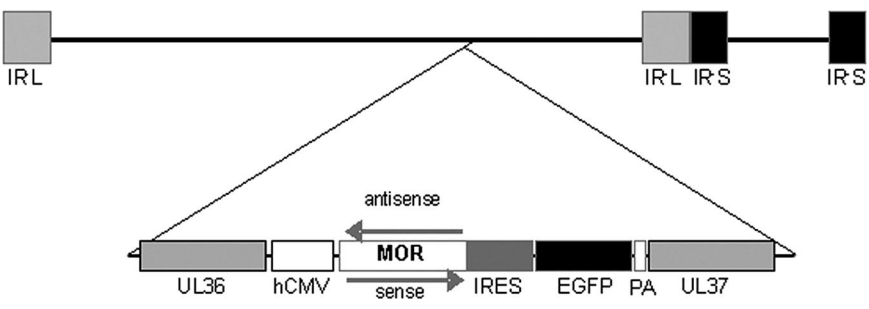

Fig. 1. Schematic diagram of recombinant herpes viruses. The transgene expression cassette is inserted between herpes genes UL36 and UL37. The μ-opioid receptor (μOR) complementary DNA is inserted in either the sense or the antisense orientation relative to the cytomegalovirus (hCMV) immediate early enhancer–promoter. EGFP = enhanced green fluorescent protein; IR = internal repeat; IRES = internal ribosome entry site; L = long; PA = polyadenylation signal; S = short.

were washed four times (10 min each) and developed with diaminobenzidine (Sigma, St. Louis, MO). After substrate–chromogen reaction, spinal cord tissues were mounted on slides. All slides were then dehydrated and coverslipped for image analysis.

Alternatively, a second set of skin samples was processed as above but the biotinylated secondary antibody was replaced with a Texas Red–conjugated affinity-purified donkey anti-rabbit immunoglobulin G (1:200; Jackson ImmunoResearch) and incubated for 2 h at room temperature in the dark. This was followed by three washes (10 min each) in TBS and a brief rinse in distilled water before application of VectaShield (Vector Laboratories, Burlingame, CA) and a coverslip. Coverslips were sealed with clear nail polish, and slides were stored at 4°C until pictures were taken.

Image Analysis

All tissues were stained in a single immunohistochemical run and analyzed by someone who was blinded to experimental conditions. In skin sections, the number of μOR-immunoreactive (μOR-ir) nerve terminals in the epidermis was counted. Similarly, the number of green fluorescent nerve terminals in the epidermis was counted. Overlap of the μOR with GFP was quantified.

In dorsal root ganglia, the number of μOR-ir cell bodies was counted. Cell bodies were categorized as large diameter (42–77 μm), medium diameter (22–40 μm), or small diameter (4–20 μm). Similarly, the number of green fluorescent cell bodies was counted and categorized by size. Overlap of the μOR with GFP was quantified. Using phase contrast microscopy, the total number of large-, medium-, and small-diameter neurons in each dorsal root ganglion slice was counted. The percentages of cells that were positive for μOR, GFP, or both μOR and GFP were calculated.

Image analysis of spinal cords was similar to that described elsewhere.[37] In brief, for spinal cord sections, a digital image of each slice was used to determine optical density with Image J version 1.26 (National Institutes of Health, Bethesda, MD). Density of μOR-ir within laminae I and II of lumbar regions L1, L2, L3, L4, L5, and L6 was averaged across animals in a treatment group (n = 3–5 tissues per spinal level/animal). Density of μOR-ir in laminae I and II on the side ipsilateral to hind paw application of HSV-1 vectors was expressed as a percent difference relative to that of the contralateral spinal cord.

Dose–Response Curves

In the current study, subcutaneous loperamide (20% Cremophor EL [Fluka Biochemika, Ludwigshafen, Germany] in sterile saline vehicle) was used to generate cumulative dose–response curves at 4 weeks after virus application. The experimenter was blinded to the viral treatment.

Mice were lightly anesthetized (800 mg/kg urethane intraperitoneal). Radiant heat (0.9°C/s) was applied to the dorsolateral and dorsomedial surfaces of the left hind paw until a paw withdrawal response was evoked. This method of testing thermal hyperalgesia in lightly anesthetized rodents has been previously described.[32,38,39] A maximal response latency of 20 s was used to prevent tissue sensitization and damage. The response latency for the lateral and medial aspects of the hind paw was collected with a 2-min interval between surfaces. Thermal latency for the hind paw was the average of the responses from the lateral and medial surfaces. Three baseline (before drug administration) thermal latencies were measured with 10 min between each measure. Subcutaneous vehicle was injected, and thermal latencies were measured at 10 min after vehicle. Cumulative doses of subcutaneous loperamide (0.17, 0.5, 1, 1.5, 2, 3, and 4 mg/kg) were administered in 15-min intervals, and thermal paw withdrawal latencies were measured 10 min after each injection (n = 5–6/treatment). Loperamide was administered using a 29-gauge insulin syringe placed in the same location in the hind paw.

Statistics

Analyses of variance followed by *post hoc* analyses (Bonferroni) were performed to determine the significance of HSV-mediated changes in GFP and μOR-ir. Differences in dose–response curves were determined using repeated-measures analyses of variance. EC_{50} for loperamide was calculated and compared between groups only after establishing that the Hill slopes of the dose–response curves were parallel. $P < 0.05$ was considered significant. All statistical analyses were performed using Prism 3.0 (GraphPad Software Inc., San Diego, CA).

Results

Viral-mediated Changes in μOR Expression

Topical infection of the hind paw with HSV-1 constructs containing the μOR cDNA inserted in the sense (SGMOR) or antisense (SGAMOR) orientation relative to the cytomegalovirus promoter produce significant changes in μOR expression in skin from the medial dorsal hind paw, L3–L5 dorsal root ganglia, and the dorsal horn of the lumbar spinal cord at 4 weeks after infection (figs. 2–8). As detailed in the methods and shown schematically in figure 1, the viral constructs in this study contain cDNA for μOR in sense or antisense direction (or β-galactosidase), an internal ribosome entry site, and GFP. Internal ribosome entry site is a nucleotide sequence that allows for translation initiation in the middle of a messenger RNA sequence. Therefore, GFP protein expression can be used as a control for viral infection to determine (1) whether the three viral vectors produce similar patterns of expression and (2) whether the changes in μOR-ir terminals in the skin are a result of infection with our HSV-1 viral constructs.

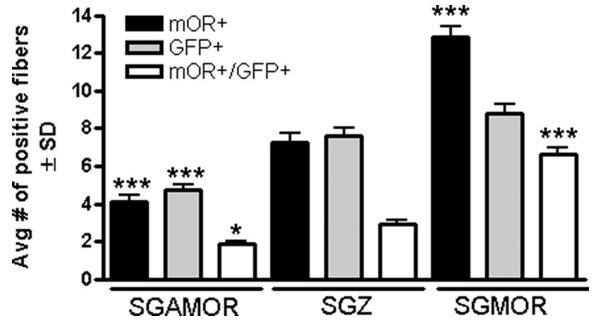

Fig. 2. Quantitative changes in expression of μ-opioid receptor (μOR), green fluorescent protein (GFP), and overlapping μOR immunoreactivity (-ir) with GFP in dorsal hind paw skin at 4 weeks after topical hind paw infection with herpes simplex virus encoding the gene for the μOR in antisense (SGAMOR) or sense (SGMOR) direction relative to the cytomegalovirus promoter, or the viral control encoding β-galactosidase (SGZ). Compared with control SGZ infection, infection with SGAMOR decreases the number of μOR-ir afferent terminals in the epidermis of the medial dorsal hind paw skin. In contrast, infection with SGMOR increases the number of μOR-ir afferent terminals in the epidermis of the medial dorsal hind paw skin. The number of μOR-ir terminals, GFP-positive terminals, and overlap of μOR-ir and GFP were counted in 4-mm sections of skin. Data are presented as average ± SD. *,* $P < 0.05$, 0.001 compared with SGZ (one-way analysis of variance followed by *post hoc* Bonferroni).**

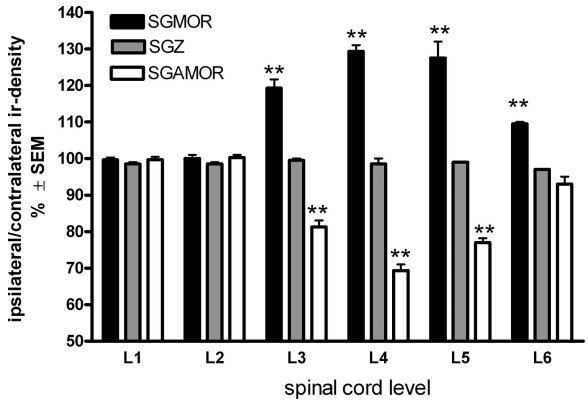

Fig. 4. Changes in expression of μ-opioid receptor (μOR) in dorsal horn of the lumbar spinal cord at 4 weeks after topical hind paw infection with herpes simplex virus encoding the gene for the μOR in antisense (SGAMOR) or sense (SGMOR) direction relative to the cytomegalovirus promoter, or the viral control encoding β-galactosidase (SGZ). Virally mediated changes in μOR are dependent on spinal cord level with maximal changes in μOR expression seen in L3–L6 spinal cord as a percentage immunoreactivity in the uninfected contralateral side. ** $P < 0.01$ compared with the density of μOR immunoreactivity in SGZ-infected same-level lumbar spinal cord (one-way analysis of variance followed by *post hoc* Bonferroni).

Expression of μOR in Skin

The numbers of μOR-ir and GFP-positive nerve terminals in the epidermis of the medial dorsal skin were counted in equivalent lengths of skin from the medial dorsal left hind paw (4.2 ± 0.67-mm sections in SGAMOR, 4.1 ± 0.54-mm sections in SGZ, and 4.2 ± 0.63-mm sections in SGMOR).

SGMOR increased μOR-ir in medial dorsal hind paw skin as compared with SGZ (figs. 2 and 3). An average (± SD) of 7.3 ± 2.27 μOR-ir terminals were observed in 4-mm sections of medial dorsal hind paw skin from SGZ-treated animals. The SGMOR virus increased μOR-ir terminals to 12.9 ± 2.52 ($P < 0.001$ compared with SGZ) in equivalent lengths of medial dorsal hind paw skin. In contrast, the SGAMOR virus decreased μOR-ir terminals to 4.1 ± 1.83 ($P < 0.001$ compared with SGZ) in equivalent lengths of medial dorsal hind paw skin.

Similar numbers of GFP-positive terminals in the medial dorsal hind paw skin were observed with SGZ and SGMOR (figs. 2 and 3). Approximately 75% of the GFP-positive terminals in SGMOR-infected skin colocalizes with μOR-ir. In SGMOR skin, these μOR/GFP-positive terminals account for the 75% increase in μOR-ir in skin from SGMOR *versus* SGZ infected animals. In contrast, in SGZ-infected skin, only approximately 39% of GFP-positive terminals colocalize with μOR-ir.

Approximately 30% fewer GFP-positive terminals were observed with SGAMOR as compared with either SGZ or SGMOR (figs. 2 and 3). Of those GFP-positive terminals, 60% do not colocalize with μOR-ir and would account for the 55% loss of μOR-ir terminals in SGAMOR- *versus* SGZ-infected skin.

Expression of μOR in Lumbar Spinal Cord

The SGMOR virus increased μOR-ir by 10–30% in L3–L6 dorsal horn (figs. 4 and 5). An increase in μOR-ir

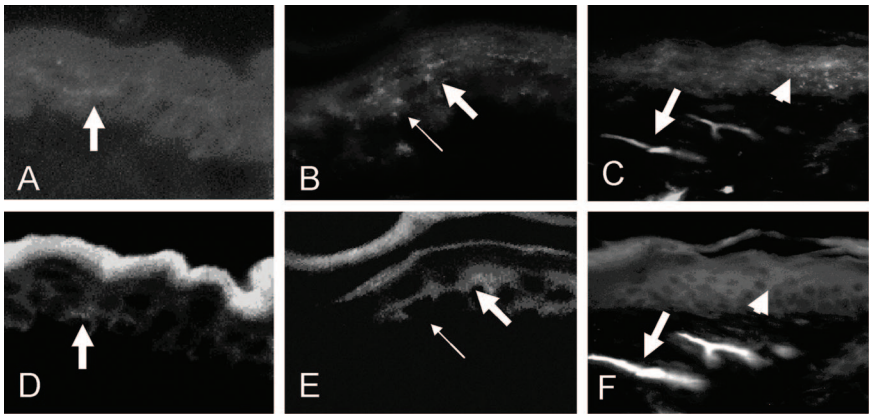

Fig. 3. Expression of μ-opioid receptor (μOR), green fluorescent protein (GFP), and overlapping μOR immunoreactivity (-ir) with GFP in dorsal medial hind paw skin at 4 weeks after topical hind paw infection with SGAMOR, SGZ, or SGMOR. *A–C* show μOR-ir nerve terminals in the epidermis of the dorsal medial hind paw. *D–F* show GFP in the same section of tissue for which μOR-ir is shown in *A–C*. *A* and *D* show decreased μOR-ir in SGAMOR-infected skin. *B* and *E* show μOR-ir overlapped with GFP approximately half of the time in skin from SGZ-treated animals. *C* and *F* show GFP and μOR-ir colocalized in SGMOR-infected skin. *Small arrows* point out GFP and μOR-ir that do not overlap. *Large arrows* point out areas GFP and μOR-ir that are overlapping.

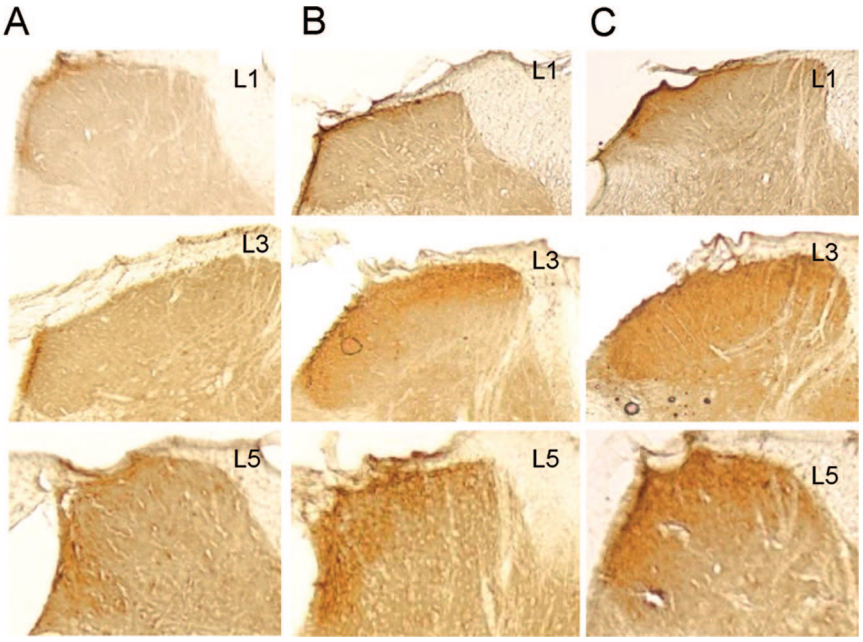

Fig. 5. Immunoreactive μ-opioid receptor (μOR) in the ipsilateral dorsal horn of the lumbar spinal cord at 4 weeks after topical hind paw infection with herpes simplex virus encoding the gene for the μOR in antisense (SGAMOR) or sense (SGMOR) direction relative to the cytomegalovirus promoter, or the viral control encoding β-galactosidase (SGZ). *A* shows decreased μOR immunoreactivity in the ipsilateral L3 and L5 dorsal horns after SGAMOR infection when compared with infection with the control SGZ virus in *B*. *C* shows increased μOR immunoreactivity in ipsilateral L3 and L5 dorsal horns as compared with infection with the control SGZ virus in *B*.

was found in the superficial layers of the dorsal horn of the spinal cord on the side ipsilateral to SGMOR infection as compared with the uninfected side. In contrast, the SGAMOR virus decreased μOR-ir by 20–30% in L3–L5 dorsal horn at 4 weeks. The SGZ virus had no effect on μOR-ir in the L1–L6 spinal cord. The greatest changes in μOR expression were observed in L3–L5 spinal cord corresponding to dorsal hind paw innervation. Of interest, the laminar distribution of the increase in μOR-ir was dependent on the spinal cord level. In L3–L5 spinal cord, a diffuse increase in μOR-ir in lamina I–III was observed as compared with lamina I–II expression in SGZ animals (figs. 5B and 5C).

Expression of μOR in Dorsal Root Ganglion Cells

Based on the findings in the lumbar spinal cord showing the largest changes in gene expression in L3–L5, the analysis of μOR expression in the cell bodies of the primary afferent neurons focused on the L3–L5 dorsal root ganglia. Cell counts were categorized into total, large-diameter, medium-diameter, or small-diameter cells positive for μOR and/or GFP. Small cells were defined as

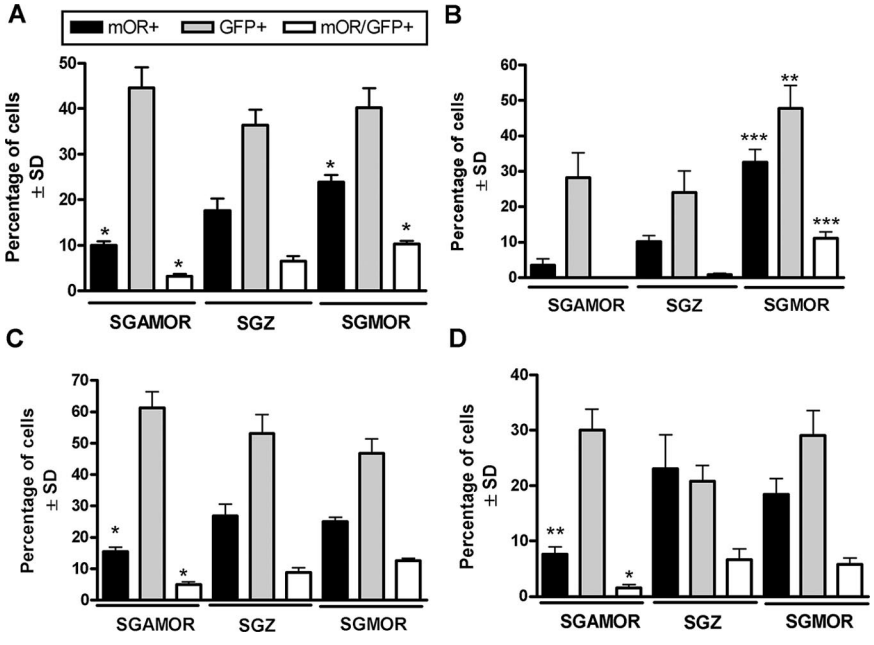

Fig. 6. Changes in expression of μ-opioid receptor (μOR), green fluorescent protein (GFP), and overlapping μOR immunoreactivity (-ir) with GFP in L3–L5 dorsal root ganglia at 4 weeks after topical hind paw infection with herpes simplex virus encoding the gene for the μOR in antisense (SGAMOR) or sense (SGMOR) direction relative to the cytomegalovirus promoter, or the viral control encoding β-galactosidase (SGZ). Compared with control SGZ infection, infection with SGAMOR decreases the percentage of μOR-ir neurons in the L3–L5 dorsal root ganglia. In contrast, infection with SGMOR increases the percentage of μOR-ir neurons in the L3–L5 dorsal root ganglia. *A* shows that the percentage of total cells that express μOR-ir or both μ-OR and GFP in the same cell increases with SGMOR infection and decreases with SGAMOR infection as compared with control SGZ infection. *B* shows an increased percentage of μOR-ir–positive, large-diameter neurons that also colocalize with GFP-positive cells at 4 weeks after SGMOR infection. *C* shows that the percentage of μOR-ir–positive, medium-diameter cells is decreased after SGAMOR infection. *D* shows that the percentage of μOR-ir–positive, small-diameter cells is decreased after SGAMOR infection. *, **, *** $P < 0.05$, 0.01, 0.001 compared with the number of cells in SGZ-infected control L3–L5 dorsal root ganglia (one-way analysis of variance followed by *post hoc* Bonferroni).

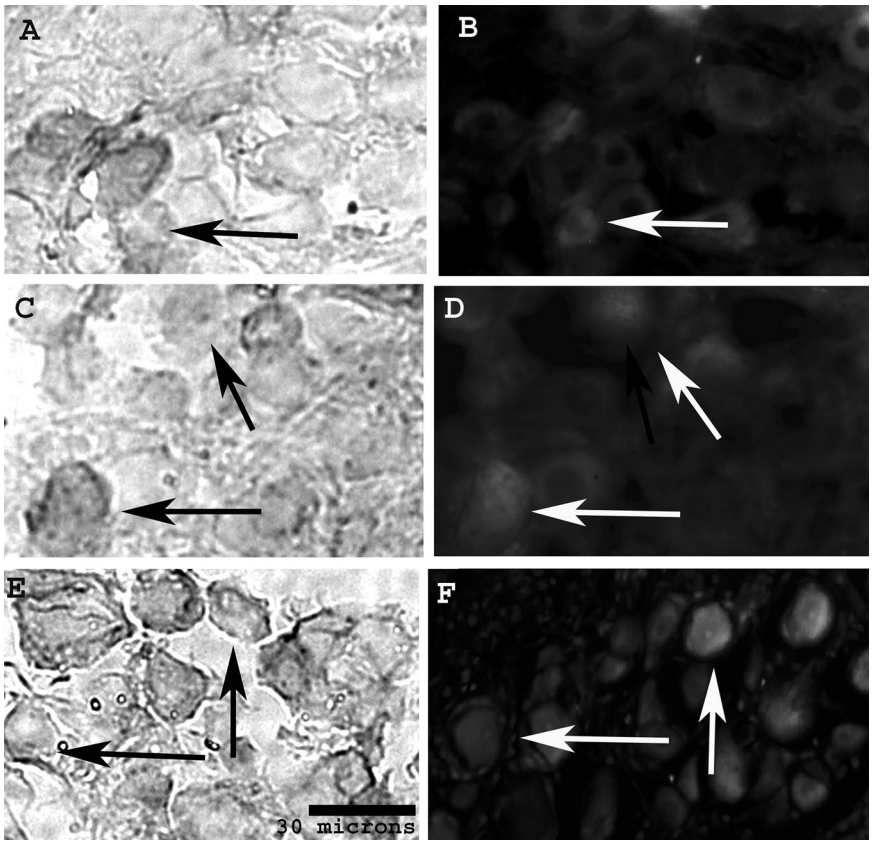

Fig. 7. Immunoreactive μ-opioid receptor (μOR) in dorsal root ganglion at 4 weeks after topical hind paw infection with herpes simplex virus encoding the gene for the μOR in antisense (SGAMOR) or sense (SGMOR) direction relative to the cytomegalovirus promoter, or the viral control encoding β-galactosidase (SGZ). *A*, *C*, and *E* show μOR immunoreactivity (-ir) in L5 dorsal root ganglion. *B*, *D*, and *F* show green fluorescent protein (GFP)–positive cells in identical L5 dorsal root ganglion sections as imaged for μOR-ir in *A*, *C*, and *D*. *A* and *B* show representative sections from SGAMOR-infected animals. *A* shows an absence of μOR-ir in a cell that is positive for GFP in *B* (*arrows*). *C* and *D* show representative sections from SGZ-infected animals. *C* shows a μOR-ir–positive cell and a μOR-ir–negative cell, both of which are GFP positive in *D* (*arrows*). *E* and *F* show representative sections from SGMOR-infected animals. *E* shows two μOR-ir–positive cells, both of which are GFP positive in *F* (*arrows*). *Bar* = 30 μm.

having diameters of 4–20 μm. Medium cells had diameters of 22–40 μm. Large cells had diameters of 42–77 μm. The size profiles of the cell populations did not change across L3, L4, and L5 dorsal root ganglia or across viral treatments (data not shown). The percentages of cells that were positive for μOR-ir and GFP were counted in the L3, L4, and L5 dorsal root ganglia. The data collected within each level were analyzed separately and did not show any differences according to lumbar level, so the data shown include cell counts for L3–L5.

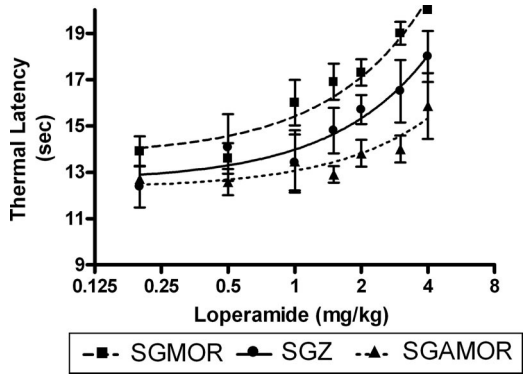

Fig. 8. Increased potency of loperamide analgesia after topical infection with SGMOR (sense μOR) compared with infection with the SGZ control virus. The loperamide dose–response curve was shifted to the left in animals infected with SGMOR as compared with SGZ. The loperamide dose–response curve was shifted to the right in animals infected with SGAMOR (antisense μOR) as compared with SGZ.

All three viral infections resulted in a similar percentage of total cells that were GFP positive regardless of cell size (fig. 6A). In contrast, approximately 50% more large-diameter cells expressed GFP after SGMOR viral infection as compared with SGZ and SGAMOR (fig. 6B). GFP expression was similar across treatments in medium-diameter (fig. 6C) and small-diameter (fig. 6D) cells. Three quarters of the cells infected with HSV-1 and expressing GFP were medium-diameter cells (figs. 6A and C).

SGAMOR reduced the percentage of μOR-ir cells by 43% compared with SGZ (fig. 6A). SGAMOR reduced μOR-ir mainly in medium- and small-diameter neurons (figs. 6C and D and fig. 7). The reduction in μOR-ir was paralleled by a decrease in the percentage of medium- and small-diameter cells expressing both GFP and μOR.

In contrast, SGMOR increased the percentage of μOR-ir cells by 35% compared with SGZ. SGMOR increased the percentage of μOR-ir large-diameter neurons (figs. 6B and 7). The increase in μOR-ir was paralleled by an increase in the percentage of large-diameter cells expressing both GFP and μOR. This corresponds with the more diffuse termination pattern of μOR in the L3–L5 spinal cord (fig. 5C).

Opioid Analgesia

Thermal paw withdrawal latencies at baseline were not significantly different at 13.0 ± 2.2, 12.8 ± 2.2, and 12.9 ± 3.7 s for SGAMOR, SGZ, and SGMOR, respec-

tively. Similarly, thermal paw withdrawal latencies were not significantly different after vehicle administration at 11.8 ± 1.5, 10.6 ± 1.3, and 11.9 ± 1.5 s for SGAMOR, SGZ, and SGMOR. Subcutaneous administration of loperamide was used to determine whether virus-mediated changes in μOR expression in primary afferent fibers can alter peripheral opioid analgesia. The antisense virus, SGAMOR, shifted the loperamide dose–response curve to the right relative to SGZ control infection (fig. 8). The dose–response curves obtained did not significantly depart from parallel, so the EC_{50}s could be compared directly. Therefore, application of SGAMOR significantly increased the loperamide EC_{50} for producing analgesia (3.9 ± 0.19; 95% confidence interval, 3.49–4.25 for SGAMOR *vs.* 2.1 ± 0.19; 95% confidence interval, 1.67–2.44 for SGZ; $P < 0.01$). An approximate twofold increase in the loperamide EC_{50} was observed in the ipsilateral SGAMOR-treated hind paw compared with SGZ animals.

In contrast, the SGMOR virus shifted the loperamide dose–response curve to the left relative to SGZ control infection (fig. 8). The SGMOR virus significantly decreased the loperamide EC_{50} for producing analgesia (1.2 ± 0.11; 95% confidence interval, 0.96–1.42 for SGAMOR *vs.* 2.1 ± 0.19; 95% confidence interval, 1.67–2.44 for SGZ; $P < 0.01$). An approximate twofold decrease in the loperamide EC_{50} was observed in the ipsilateral SGMOR-treated hind paw compared with SGZ animals.

Discussion

As far as we can tell, this is the first study using HSV-1 to increase the endogenous expression of a receptor and to show that increase in peripheral skin afferent terminals and the cell bodies in the dorsal root ganglia, as well as in the central spinal terminals. This is a significant finding because it shows not only that the receptor is being made but also that it is being transported both anterograde and retrograde and hence can influence pain and analgesia at peripheral as well as at spinal sites. Using the antisense construct, this study shows a decrease in μOR-ir in the skin, dorsal root ganglia, and lumbar spinal cord. Most important is the finding that the change in μOR-ir is accompanied by a change in function as measured by a shift in the loperamide dose–response curve.

These results show that similar viral constructs produce nearly equal overexpression or knockdown of μOR-ir across the same spinal cord segments. At the behavioral level, SGMOR and SGAMOR result in equivalent leftward and rightward shifts in the loperamide dose–response curve (approximately ½ or 2× compared with control SGZ), respectively. The 30% knockdown of the μOR with the SGAMOR virus was approximately double that found previously with a different HSV-based antisense μOR virus.[32] One likely reason is that in the current study, both the dorsal and plantar hind paw were inoculated, whereas previously, only the dorsal hind paw was inoculated.[32] This suggests that larger changes in μOR expression can be achieved by infecting a larger area of skin. The current study also suggests that infection of a larger area of skin decreases protein expression in more spinal segments (*e.g.*, L3–L5 compared with L4–L5), which is likely related to inclusion of skin that is innervated by both the sciatic and saphenous nerves, thus resulting in expression across a broader range of lumbar segments.

A 30% reduction or increase in μOR-ir in the dorsal horn of the spinal cord is a substantial change in presynaptic μOR expression from the infected/tested section of hind paw skin. It is estimated that approximately 21% of dorsal root ganglia neurons contain μOR,[40] and these give rise to half of the μOR expression in the spinal cord (from rhizotomy studies[37,41]). The substantial topographical overlap in sensory input in the dorsal horn means that only a fraction of the opioid receptor–positive terminals in a given spinal cord section innervate the corresponding infected hind paw skin (*e.g.*, for any given nerve root, 22–25% of primary afferents terminate in the corresponding spinal segment[37,42]). Therefore, opioid receptors in any given lumbar spinal cord section are likely to be on cells other than primary afferents innervating the infected hind paw skin, so the 30% change in expression in the current study is physiologically relevant as evidenced by the shift in the dose–response curve to intraplantar loperamide.

The increase in the percentage of cells showing μOR-ir after infection with SGMOR, especially the large-diameter cells, suggests that this viral approach can produce μOR expression in cells that previously did not express μOR. HSV-1 has been shown to deliver genes to both small- and large-diameter dorsal root ganglia neurons after inoculation of the skin.[26,43] The more diffuse staining pattern seen in L3–L5 spinal cord after SGMOR infection would suggest the possibility of expressing μOR in large-diameter primary afferent fibers, possibly Aβ fibers, with termination patterns in deeper lamina and that do not normally express the μOR. In the current study, using the SGMOR virus, it is more difficult to determine whether we are increasing the amount of μOR in primary afferents that already express the protein. In addition, there is a limitation to the sensitivity of immunohistochemical procedures to detect low levels of protein expression. Therefore, in some of the cells that are GFP positive, there may be an increase in μOR, but it is below the level of detection using immunohistochemical techniques. Similarly, when using the SGAMOR virus, small decreases in μOR expression are possible but cannot be quantified with the immunohistochemical techniques used in the current study.

The postulate that HSV-based strategies alter opioid analgesia at the level of primary afferent neurons is further supported by our previous study showing that an HSV-based antisense μOR virus produces a rightward shift in the dose–response curve for intrathecal administration of DAMGO.[32] The demonstration of peripheral opioid analgesia in experimental models of inflammatory pain,[19,44,45] in clinical studies,[46,47] and in experimental models of neuropathic pain[17,48–50] has been followed by the development of peripheral opioid agonists. Two peripherally selective μOR agonists have been well studied, the quaternary derivative of morphine, *N*-methyl morphine, and the antidiarrheal agent loperamide.[18,21,51]

Loperamide is selective for the μOR and does not penetrate the brain in appreciable amounts.[21] Pharmacokinetic studies show minimal accumulation of drug in the brain after intravenous injections of doses that approach the lethal dose.[52] The peripheral selectivity of loperamide is thought to be secondary to its lipophilicity and ability to serve as a substrate for the multidrug-resistant transporter.[52,53] Clinical studies have shown that loperamide does not possess abuse potential or dependence liability.[54] Local injection of loperamide in the inflamed, but not contralateral, paw attenuates Freund adjuvant–induced hyperalgesia[20] with a potency comparable to that of morphine (ED_{50} = 21 μg). Similar effects have been reported with a topical administration of 5% loperamide cream in a model of burn-induced hyperalgesia.[22] The behavioral effects of loperamide were observed in the absence of measurable concentration of the drug in blood.[22]

A potential disadvantage with peripherally acting opioids is that they are likely to share the gastrointestinal side effects common to all opioids, *e.g.*, reduced gut motility. This side effect can be minimized (1) by local administration of the peripheral opioid agonist in the region of hyperalgesia, *e.g.*, by topical application, and (2) by use of appropriate doses of an orally administered, low-bioavailability peripheral opioid antagonist to block the gastrointestinal side effects, or (3) by decreasing the dose of peripheral opioid agonist. HSV-mediated gene transfer offers one technique to selectively increase opioid receptor expression specifically in primary afferent neurons so that lower doses of peripheral opioid agonists can produce adequate analgesia. One of the limitations of the current study is that it uses only a single acute nociceptive test of thermal responses. Whereas this is inherently suited for examining functional consequences of changes in opioid receptor expression on small- and medium-diameter nociceptive afferents, it will miss testing functional changes in large-diameter afferents, which we have shown can express μOR after topical infection with the SGMOR virus. Future studies should be undertaken that will assess the effects of virus-induced changes in μOR receptor expression on multiple acute nociceptive modalities as well as inflammatory and neuropathic pain models.

Conclusions

Opioids have been demonstrated to be effective in the treatment of a number of chronic pain conditions, but a significant proportion of patients withdraw from opioid use because of central nervous system side effects. Understanding the role of peripheral opioid receptors and the efficacy of strategies that enhance μORs on primary afferent fibers will help to develop novel therapeutic strategies for controlling chronic pain. As with adenoassociated virus, studies of marker genes suggest HSV-mediated gene expression in rodents for several months.[26,27,55] The current study shows that HSV vectors can be used to decrease expression of μOR in small- and medium-diameter primary afferent fibers or, conversely, to increase μOR expression in large-diameter primary afferent neurons. Furthermore, these changes in receptor expression had a functional consequence on peripheral opioid analgesia effectively reducing or enhancing analgesia, respectively. These data suggest that therapeutic manipulation of peripheral opioid receptors with HSV-1 may provide an innovative strategy for enhancing peripheral opioid analgesia in humans.

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