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Injury-specific promoters enhance herpes simplex virus mediated gene therapy for treating neuropathic pain in rodents

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Abstract

Chronic neuropathic pain is often difficult to treat with current pain medications. Gene therapy is currently being explored as a therapeutic approach for the treatment of neuropathic and cancer pain. In this study we sought to use an injury-specific promoter to deliver the mu opioid receptor (MOR) transgene such that expression would only occur during the injured state in response to release of injury-specific galanin. To determine whether an injury specific promoter can produce neuron-specific MOR expression and enhanced antinociception we compared animals infected with a galanin promoter virus (galMOR) or a human cytomegalovirus (CMV) promoter virus. (cmvMOR). In behavioral assays, we found an earlier onset and a larger magnitude of antinociception in animals infected with galMOR compared to cmvMOR. Immunohistochemistry of dorsal root ganglion (DRG) neurons revealed a significant increase in MOR positive staining in cmvMOR and galMOR treated mice. Spinal cord sections from galMOR treated mice showed a greater increase in density but not area of MOR positive staining. These results suggest that using injury-specific promoters to drive gene expression in primary afferent neurons can influence the onset and magnitude of antinociception in a rodent model of neuropathic pain and can be used to upregulate MOR expression in populations of neurons that are potentially injury specific.

Perspective—An injury specific promoter (galMOR) was used to drive MOR expression in a population- and injury- specific manner. GalMOR increased antinociception and density of MOR staining in spinal cord. This article presents evidence that promoter selection is an important component in successful gene expression in an injury- and population-specific manner.

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Keywords

galanin; mu opioid receptor; mechanical allodynia; thermal hyperalgesia; nerve injury

Introduction

Chronic neuropathic pain often decreases quality of life, incurs high medical costs^{3, 16} and is difficult to treat. The efficacy of current treatments varies across individuals and is associated with adverse side effects. Alternative treatments such as gene therapy are being explored in animal models of neuropathic pain^{13–15, 23, 32} and in human cancer pain^{9, 11}. The use of recombinant herpes simplex virus type-1 (HSV-1) as a means to deliver genetic material to a targeted region in the peripheral nervous system is particularly beneficial because of its natural propensity for afferent neurons. Studies to date using HSV-1 as a gene therapy vector have produced changes in gene expression in the overall afferent neuron population with antinociceptive effects. The promoter influences the duration of changes in gene expression as well as the afferent population expressing the transgene. The cytomegalovirus (CMV) promoter is commonly used to drive gene expression as it is constitutively active and results in high levels of expression in a wide range of tissues²⁵. The use of tissue specific promoters in gene therapy has been explored to treat liver carcinomas^{10, 34}, cardiac ischemia²⁹, and neurological disorders⁴. However, using tissue and injury specific targeting of transgenes to distinct afferent populations for the treatment of pain has not been explored. Galanin is normally present in low levels in dorsal root ganglia and the spinal cord. After injury that results in neuropathic pain-like behavior, galanin is highly upregulated proportionate to the extent of constriction injury and is used as a marker of nerve injury^{5, 8, 22, 28}. In the present study, we investigated the use of HSV-1 and an injury-specific galanin promoter to express mu opioid receptors specifically in injured primary afferent neurons. To determine whether an injury specific promoter can enhance antinociception and drive expression of MOR in specific populations of afferent neurons as opposed to constitutive expression with a non-specific promoter, we compared animals infected with the galanin-promoter virus (galMOR) to those infected with the CMV-promoter virus (cmvMOR).

Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Efforts were made to reduce the number of animals used and to minimize discomfort. Female Swiss Webster mice (5–6 weeks old, 20–25g, Harlan) were group housed on 12-h light/dark cycle with food and water available *ad libitum*.

L5 Transection

For L5 transection surgeries, animals were anesthetized with isoflurane and all surgical procedures were performed under sterile conditions as previously described²⁰. An incision over L5-S1 was made, the L6 transverse process was partially removed and the L5 nerve was isolated and transected. In sham animals, an identical procedure was followed but the

L5 nerve was not transected. The fascia was closed with 3.0 polyester sutures. Seven days after L5 transection surgery, the mice were infected with virus as previously described³¹.

Viral Constructs and Infection

The following viral vectors have been described³⁶: hsvCON, the control virus expresses β -galactosidase (*E. coli lacZ*) that is driven by the CMV immediate-early enhancer promoter, previously called SGZ, cmvMOR expresses MOR and is driven by the CMV immediate-early enhancer promoter, previously called SGMOR. A newly constructed virus, galMOR expresses MOR under the control of the galanin promoter (Fig. 1). Animals were anesthetized with isoflurane and 10 μ l of the HSV-1 constructs (1×10^7 tu/ μ l) were administered into the left plantar hindpaw (n=5–6/group).

Behavioral Testing

Animals were acclimated to the experimenter and testing environment prior to testing as previously described²¹. Mice were placed in plastic boxes over a mesh floor and their responses to mechanical stimuli were quantified as the total number of paw withdrawals to an ascending series of calibrated Von Frey filaments (VF) (0.16, 0.4, 1.0, 2.0, and 4.0g) applied to the plantar surface of the left hind paw. A positive response was withdrawal of the hindpaw from the filament. Each VF was applied 5 consecutive times at approximately 3 second intervals and the maximum possible withdrawal was 25 responses. Paw withdrawal latency (PWL) to radiant heat stimuli was measured using a plantar stimulator analgesia meter as the time it took until the animal removed its paw. Mice were placed in Plexiglas enclosures on a heated glass surface. Radiant heat was applied from below to the plantar surface of the left hindpaw with a cutoff time of 20 seconds to prevent tissue damage. Baseline behavioral responses were measured prior to surgery and prior to infection with HSV-1 vectors. On the last day of testing (day 22), mice were deeply anesthetized and transcardially perfused for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed as previously reported³⁶. Briefly, 2 weeks after virus infection, mice were deeply anesthetized with isoflurane then transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. Vertebral columns were removed and post-fixed in 4% paraformaldehyde solution for 24 hours followed by isolation of lumbar spinal cords and DRGs then stored in 30% sucrose solution in 0.1M phosphate buffer until sectioning. Lumbar spinal cords (20 μ m) and DRGs (12 μ m) were serially sectioned. DRG sections were thaw-mounted onto slides for staining. Immunohistochemistry was performed using the avidin-biotin method for MOR expression and immunofluorescence was used to examine galanin expression. Free-floating spinal cord sections and slide mounted DRGs were washed in Tris-buffered saline (TBS) then quenched for endogenous peroxidase activity. Spinal cord and DRG sections were rinsed 3 times (10 min each) in TBS then blocked in TBS with 0.2% Triton X100 (TBS+) and normal horse serum for 20 min. Primary antibody incubation was done in TBS+ overnight (4°C for spinal cords and room temperature in a humidifier box for DRGs). After overnight incubation in primary antibody, samples were rinsed three times with TBS+ then incubated for 1.5 hr at

room temperature in biotinylated affinity-purified donkey anti-rabbit immunoglobulin G (1:1,000; Jackson ImmunoResearch, West Grove, PA). Following three washes with TBS+, the samples were incubated for 1 h at room temperature in horseradish peroxidase–conjugated streptavidin (1:1,600; Jackson ImmunoResearch) in TBS+. Substrate–chromogen reaction using 3,3'-diaminobenzidine (Sigma, St. Louis, MO) was performed following four washes with TBS+. For immunofluorescence, samples were incubated in primary antibody overnight followed by 3 washes in TBS then incubation in secondary antibody (donkey anti-goat Alexa Fluor 594, Invitrogen, 1:200). Spinal cord sections were then mounted onto slides and both DRGs and spinal cords were coverslipped. MOR and galanin expression was examined in both spinal cord and DRG sections. A minimum of 3 sections per animal were scored under each condition. The following primary antibodies were used: MOR (Neuromics, RA10104, 1:1000) and galanin (Santa Cruz, N20, sc-16411, 1:500).

Image Analysis

Digital images were taken and density and area analysis was performed on the spinal cord sections using ImageJ software National Institutes of Health, Bethesda, MD. To examine MOR expression levels after L5 transection and virus infection, the L5 transected groups were normalized to the uninjured groups to get the percent MOR positive area and density. In dorsal root ganglia, the number of MOR immunoreactive or galanin immunoreactive cell bodies were counted and categorized by size (small, medium, or large). The total number of small, medium, and large cell bodies was counted and the percentages of MOR positive cells and galanin positive cells were calculated.

Statistical Analysis

Significant differences between groups were assessed using one way ANOVA with Bonferroni post test or Student's t tests. All statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A $P < 0.05$ was considered significant.

Results

Galanin was up-regulated in the ipsilateral dorsal horn of L5 transected (Fig. 2A, B, C) but not sham surgery mice (Fig. 2D). Infection with cmvMOR or galMOR did not alter galanin expression in the ipsilateral or contralateral spinal cord in sham or nerve injured mice (Fig. 2E).

Neuropathic Pain Behavior

L5 nerve transected animals displayed an increase in total number of paw withdrawals to mechanical stimuli and a decrease in paw withdrawal latency to heat on day 7 post-transection. Mice were randomized to 3 groups and infected on day 7 with hsvCON, cmvMOR, or galMOR. L5 nerve transected mice that received the control virus did not show a decrease in nociceptive behaviors. Those that received either the cmvMOR (cmvMOR vs. hsvCON, *, $p < 0.05$, day 18 and 22) or galMOR virus (galMOR vs hsvCON, ** $p < 0.01$, day 12 and 14; *** $p < 0.001$ day 18 and 22; galMOR vs cmvMOR, ° $p < 0.05$, day 18 and 22), in contrast, exhibited a significant decrease in paw withdrawals compared to the control virus group. The mice that received the galMOR virus, however, displayed the

greatest and most sustained reduction in mechanical allodynia (Fig. 3A) and thermal hyperalgesia (galMOR vs hsvCON, * $p < 0.05$ day 14, ** $p < 0.01$ day 12 and 18, *** $p < 0.001$ day 22; galMOR vs cmvMOR, $p < 0.05$) (Fig. 3B).

Spinal Cord and DRG Immunohistochemistry

Immunohistochemistry of spinal cords from the L5 transected control virus group revealed a significant decrease (Fig. 4A, B, C; $P < 0.05$) in MOR positive staining in the dorsal horn of the lumbar spinal cord sections compared to the uninjured group (Fig. 4A, B, D). Spinal cord sections from L5 transected cmvMOR treated animals showed an increase in the area and density of MOR positive staining throughout the dorsal horn (Fig. 4A, B, E) while sections from L5 transected galMOR treated mice revealed a significant increase in the density ($p < 0.001$) but not the area of MOR positive staining in the superficial lamina of the dorsal horn compared to the control virus infected L5 transected mice (Fig. 4A, B, F). These results suggest that the galanin promoter drives gene expression in a specific population of neurons, particularly those in the superficial lamina of the dorsal horn. In examining the dorsal root ganglia, no significant differences in galanin staining were observed in any of the different treatment groups or between sizes of the DRG cells (Fig. 4G). Mice treated with the cmvMOR virus exhibited a higher degree of MOR staining in all sizes of DRG cells compared to the hsvCON group (*, $p < 0.05$) while galMOR treated mice had a greater degree of MOR staining only in small and medium sized DRG neurons compared to the hsvCON group (*, $p < 0.05$) (Fig. 4H).

Discussion

This study explored whether the use of an injury specific promoter to drive MOR would produce greater anti-nociception and greater specificity of expression to nociceptive afferents than a non-specific global promoter. Behaviorally, use of the galanin promoter to drive MOR expression produced a significant and sustained reduction in nociceptive behaviors compared to the non-specific CMV promoter. Importantly, viral vectors did not alter galanin expression in DRG or spinal cord. Hence, changes in nociceptive behavior correlate with viral vector induced changes in MOR expression without changing injury-induced galanin expression.

Galanin has been shown to be involved in pain modulation. Following peripheral nerve injury, galanin is upregulated and there is increased transport to the dorsal horn of the spinal cord^{6, 7}. In terms of future translation of these studies to humans, it has been reported that in monkey DRG neurons, galanin is the only neuropeptide that is found to be strongly up-regulated after nerve injury³⁷. When increased, galanin potentiates the analgesic effects of endogenous^{17, 33, 38} and exogenous opioids^{27, 30}.

Immunohistochemical analysis of MOR expression in the spinal cord suggests a more limited pattern of transgene expression across the lamina of the spinal cord with the galanin promoter compared to the CMV promoter. Coupled with the more limited anatomical profile of transgene expression the galanin promoter appears to drive higher levels of transgene expression compared to the CMV promoter using immunohistochemistry. While immunohistochemical studies are necessary to provide data on the localization of induced

gene expression these same studies are limited on providing quantitative measures of overexpression. Future studies using immunoblotting would be valuable to confirm whether the two promoters change the amount of over-expression of MOR or just the localization of expression.

HSV-1 infects both A δ and C afferent fiber types and it appears that presynaptic mu-opioid receptors contribute more to C-fiber mediated behavioral responses rather than A δ mediated responses¹⁸. In a similar manner, galanin is primarily produced by C fibers. Our previous studies with the *cmvMOR* construct have shown up-regulation of expression of MOR in small, medium, and large diameter DRG neurons. This increased expression of MOR across all DRG neuronal populations underlies the expanded area of MOR expression into deeper dorsal horn lamina. In parallel with this change in MOR afferent expression patterns there is a development of increased mechanical and thermal thresholds. In the current study, the use of the galanin promoter to drive MOR expression resulted in a more limited increase in MOR in only small and medium diameter DRG neuronal populations. This likely correlates with the reduced area of MOR overexpression to the superficial lamina when compared to the CMV promoter construct.

Since this study only examined MOR expression at a single time point it cannot be determined if the different time course and degree of pain reversal is due to only the differential distribution that is observed on the final day of the behavioral analysis. Alternatively, the differences in time course of reversal may be due to differential timing of virally induced MOR up-regulation. Similarly the increased degree of pain reversal in the *galMOR* infected animals may be due to increased upregulation of MOR expression in DRG neurons at both peripheral and central terminals. Future studies are needed to assess these possibilities.

Based on the involvement of galanin in opioid analgesia and its co-localization with endogenous opioids in the superficial layers of the dorsal horn of the spinal cord, it is likely that the galanin promoter drove MOR gene expression in a specific population of neurons since a reduced area and an increased density were observed. Morphine or naloxone application to spinal cord neurons have revealed that deeper wide dynamic range (WDR) neurons respond more to high frequency stimulation compared to more superficial neurons suggesting a greater role for deeper neurons in neuronal sensitization than the response to acute noxious inputs³⁵. Similarly, in MOR knockout mice, neuronal sensitization is greatly enhanced in deep WDR neurons while naloxone precipitation of a wind up response in wild type mice is significantly enhanced in both deep and superficial WDR neurons¹². These findings suggest that there are differential effects of MOR activation on spinal nociceptive transmission that is dependent on the depth of MOR containing neurons in the dorsal horn of the spinal cord.

The benefit of using specific promoters over more general promoters is that gene delivery can be restricted to a localized region. Currently, only tissue and cell type specific promoters have been studied extensively^{1, 19, 24, 26}. While effective, transgene expression is indiscriminately turned on, even in the absence of injury; overexpression of the transgene in the absence of injury would produce analgesia which could lead to an increased risk of

damage. By using an injury specific promoter such as the galanin promoter, the premise is that transgene expression will only occur in neurons and tissues relevant to nociception and will be turned on in the presence of injuries that result in pain.

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Disclosures

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Highlights

- A galanin promoter was used to drive MOR expression in an injury specific manner
- The CMV promoter was used as the control promoter in the HSV vector
- GalMOR infected mice exhibited greater antinociception than cmvMOR infected mice
- A greater increase in density was found in spinal cords of galMOR infected mice
- A promoter can be used to drive gene expression in an injury specific manner

FIGURE 1.

Schematic diagram of PZGalMORW (galMOR) recombinant herpes vector. Expression of β -galactosidase (*E. coli lacZ*) is driven by the hCMV immediate-early enhancer-promoter. Expression of the rat mu opioid receptor is driven by the mouse 4.6-kb galanin promoter². This transcription cassette contains a woodchuck hepatitis virus element (WPRE) to enhance RNA stability. PA, polyadenylation signal; IR, internal repeat; L, long; S, short.

FIGURE 2.

Galanin is similarly up-regulated in the ipsilateral (i) dorsal horn of the spinal cord in uninfected mice (A), galMOR infected mice (B), and cmvMOR infected mice (C) following L5 spinal nerve transection. Galanin is not upregulated in the contralateral (c) dorsal horn of L5 spinal nerve transected mice infected with cmvMOR (D). Galanin is up-regulated only in the ipsilateral dorsal horn of L5 spinal nerve transected mice and is not altered by viral infection (E). *,***p<0.05, 0.001.

FIGURE 3.

L5 spinal nerve transection produced mechanical allodynia (A) and thermal hyperalgesia (B) that was differentially reversed by infection with galMOR and cmvMOR viral constructs on day 7 post-injury. Using the galanin promoter to drive expression of the MOR (galMOR) produced a rapid and robust reversal of allodynia and hyperalgesia. In contrast, using the CMV promoter to drive expression of MOR produced a partial reversal of allodynia and hyperalgesia that was slower in on-set compared to the galMOR vector. *, **, *** $p < 0.05$, 0.01, 0.001 cmvMOR or galMOR versus hsvCON control. ° $p < 0.05$ between galMOR and cmvMOR.

FIGURE 4.

Decreased MOR positive density was observed in sectioned spinal cords from L5 transected mice given the control (hsvCON) virus compared to the uninjured controls while less of a decrease is seen in sections from the cmvMOR virus group and no decrease is observed in the galMOR group (A). Similarly, a decrease in MOR positive area was observed in all of the L5 transected animals compared to uninjured controls with less of a decrease seen in the cmvMOR virus group (B). Data is reported as %MOR positive density (A) or %MOR positive area (B) $\pm$ S.D. Statistical values represent differences between uninjured control

and hsvCON L5 transected or hsvCON L5 transected and galMOR transected animals as indicated by the lines over the bars on the graph. The pictures below the graphs are representative images for the different treatment groups: hsvCON L5 transected (C), hsvCON not L5 transected (D), cmvMOR L5 transected (E), galMOR L5 transected (F). There were no significant changes in galanin expression in the DRG of any of the treatment groups compared to the control group (G). cmvMOR was shown to be significantly increased in all sizes of DRG neurons while galMOR was predominately increased in small and medium DRG neurons compared to the control virus treated animals (H) *, $p < 0.05$.