Herpes simplex virus-mediated RNA interference targeting vesicular glutamate transporter 3 attenuates tactile allodynia in mice (Postprint)

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Abstract

Objective To construct a replication-deficient herpes simplex virus type 1 (HSV-1) vector for delivering short hairpin RNA (shRNA) targeting vesicular glutamate transporter 3 (VGLUT3) and to investigate its effect in alleviating allodynia in mice. Methods The recombinant HSV-1 vector carrying shRNA targeting Vglut3 (HSV-1-shvglut3) was constructed and inoculated into the sciatic nerve in a mouse model of mechanical allodynia to test its analgesic effect. Mechanical allodynia and thermal hyperalgesia in mice were tested using von Frey filaments and the Hargreaves test, respectively. VGLUT3 expression in the dorsal root ganglion (DRG) was evaluated by immunohistochemistry and Western blotting. Results Following inoculation into the sciatic nerve, the HSV vector HSV-1-shvglut3 was retrogradely transported to the DRG. Mechanical withdrawal thresholds in the mouse models receiving HSV-1-shvglut3 inoculation were reversed to nearly baseline levels, and VGLUT3 expression in the DRG was downregulated 2 weeks after vector inoculation. The analgesic effect lasted for over 2 weeks in these mice without obvious systemic side effects or changes in thermal hyperalgesia threshold. Conclusion Vglut3 in the DRG is a promising therapeutic target for alleviating mechanical allodynia, and HSV-1 vector-mediated RNA interference is safe and efficient for inducing long-lasting analgesia after peripheral inoculation of the vector.

Full Text

Preamble

Herpes Simplex Virus-Mediated RNA Interference Targeting Vesicular Glutamate Transporter 3 Attenuates Tactile Allodynia in Mice

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Abstract:
Objective To construct a replication-deficient herpes simplex virus type 1 (HSV-1) vector delivering a short hairpin RNA (shRNA) targeting vesicular glutamate transporter 3 (VGLUT3) and observe its effect in alleviating allodynia in mice.
Methods The recombinant HSV-1 vector carrying shRNA targeting Vglut3 (HSV-1-shvglut3) was constructed and inoculated in the sciatic nerve in a mouse model of mechanical allodynia to test its analgesic effect. Mechanical allodynia and heat hypersensitivity were tested using von Frey filaments and Hargreaves' test, respectively. VGLUT3 expression in the dorsal root ganglion (DRG) was evaluated by immunohistochemistry and Western blotting.
Results Following sciatic nerve inoculation, the HSV vector HSV-1-shvglut3 was retrogradely transported to the DRG. Mechanical withdrawal thresholds in mouse models receiving HSV-1-shvglut3 inoculation were reversed to near baseline levels, and VGLUT3 expression in the DRG was down-regulated 2 weeks after vector inoculation. The analgesic effect lasted for over 2 weeks without obvious systemic side effects or changes in heat hypersensitivity threshold.
Conclusion Vglut3 in the DRG is a promising therapeutic target for alleviating mechanical allodynia, and HSV-1 vector-mediated RNA interference is safe and efficient for inducing long-lasting analgesia after peripheral vector inoculation.

Key words: RNA interference; vesicular glutamate transporter 3; hyperalgesia; herpes simplex virus 1

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INTRODUCTION

Tactile allodynia is the most refractory symptom of neuropathic pain, which along with hyperalgesia and spontaneous pain seriously affects patients' quality of life. As factors associated with the onset and recovery of tactile allodynia are poorly understood, conventional treatments have only limited effects with fre-

quent undesirable side effects. Novel therapeutic strategies are being explored, including gene knockdown and knock-in techniques targeting specific ion channels, signal molecules, receptors, and transmitters, some of which have achieved encouraging effects.

Evidence from gene knockout mice shows that mechanical allodynia is transmitted almost exclusively by a specific subset of dorsal root ganglion (DRG) neurons expressing vesicular glutamate transporter 3 (VGLUT3), highlighting the importance of VGLUT3-positive neurons in the DRG as a promising therapeutic target for relieving mechanical allodynia. However, considering the wide distribution of VGLUT3 expression in the nervous system (including the ventral cochlear nucleus, hippocampus, olfactory tubercle, and dorsal and medial raphe nuclei), systemic Vglut3 gene knockout is associated with high risks of adverse neurological effects such as hearing loss and disorders in learning and memory. Despite reports of reliable analgesic effects in both inflammatory and neuropathic pain models, systemic knockdown does not appear feasible in clinical settings.

Specific RNA interference (RNAi) targeting Vglut3 in the DRG may provide an alternative approach. Several types of carriers, including viral vectors, have been tested for delivering RNAi constructs to the DRG, among which herpes simplex virus (HSV)-derived vectors have shown particular promise. HSV vectors possess not only high tropism for peripheral sensory neurons but also the capacity to establish lifelong persistence in host cells in a latent state of infection. Natural uptake of HSV virion by nerve terminals and its rapid retrograde axonal transport toward nerve cell bodies offers a unique possibility for peripheral, noninvasive vector administration. Recent studies demonstrated that replication-deficient HSV type 1 (HSV-1) was capable of mediating delivery of RNAi constructs targeting pain-related genes from peripheral nerves to the DRG, resulting in highly effective and specific gene silencing in DRG neurons. Therefore, we hypothesize that selective Vglut3 knockdown in the DRG mediated by HSV-1-based RNAi can attenuate mechanical allodynia without causing systemic side effects.

In this study, we constructed a recombinant replication-deficient HSV-1 vector carrying a short hairpin RNA (shRNA) targeting Vglut3, inoculated the vector in the sciatic nerve in a mouse model of mechanical allodynia (without heat allodynia) established by spared nerve injury (SNI), and explored the efficiency of targeted Vglut3 knockdown in the DRG and its analgesic effects in vivo.

MATERIALS AND METHODS

Animal Preparations

Sixty SPF male ICR mice weighing 20–25 g (SLRC Laboratory Animal, Hunan, China) were housed at room temperature (24 ± 1 °C) on a 12 h light-dark cycle

with free access to laboratory chow and water. All experimental procedures and animal care were approved by the Animal Care and Use Committee of Tongji Hospital in accordance with International Association for the Study of Pain (IASP) Guidelines for the Use of Animals in Research.

Nerve Injury Surgery

Spared nerve injury (SNI) of the sciatic nerve was performed as previously described. Briefly, mice were anesthetized with an intraperitoneal dose of ketamine (10 mg) and xylazine (0.5 mg/100 g body weight). The left sciatic nerve and its three terminal branches were carefully exposed. The common peroneal and tibial nerves were tightly ligated with 5.0 silk, and a 2–4 mm segment distal to the ligation was removed. The sural nerve was carefully avoided to ensure its integrity. Muscle and skin were closed in layers. Mice receiving sham operation underwent exposure of the sciatic nerve and its branches without ligation or nerve injury.

Construction of HSV-1 Vectors

Two recombinant HSV-1 vectors were constructed: HSV-1-shvglut3-0 carrying the shRNA targeting Vglut3 and HSV-1-shvglut3-1 carrying a negative control shRNA sequence not predicted to target any known vertebrate gene. Both viral vectors contained a red fluorescent protein (RFP) gene inserted upstream of the two shRNA cassettes to facilitate purification. The shRNA sequences were under the control of a U6 promoter.

Based on the Vglut3 coding gene sequence (GenBank Accession NM_182959.3), the shvglut3-1 sequence was designed and combined with a U6 promoter. The synthesized shvglut3-1 sequence was: CAAGCTTAAG-
GTCGGGCAGGAAGAGGGCCTATTTGCATATAGATAATTAGCAAA-
GATATTGAAAGTAATATTTTAAAATTATATGCTTACATTTCTTGGCGACGAAACACACAACAGAGCA

The shvglut3-0 control sequence was created by disrupting the order of the established shRNA sequence against Vglut3 and selecting a sequence sharing no homology with any vertebrate gene: GCTGTTAGACCTATAGTAACC. The U6-shvglut3-0 fragment (334 bp) with a U6 promoter was synthesized and verified by gene sequencing. PCR identification confirmed the synthesized plasmid contained a 334 bp sequence consistent with Vglut3.

The non-replicating HSV-1 vector backbone (Sino GenoMax, Beijing, China) was constructed by deleting virulent genes ICP27, ICP4, and ICP34.5 using genetic engineering methods. Plasmids pNX-shvglut3-0 and pNX-shvglut3-1 were obtained by inserting U6-shvglut3-0 and U6-shvglut3-1 sequences, respectively, between Hind III and Xba I sites. Replication-deficient HSV-1 vectors were generated by calcium phosphate transfection of complementing OG cells (Vero cell lines with stable expression of ICP27 and ICP4 proteins) with the intermediate plasmids and HSV backbone. Genome structures were confirmed by PCR followed by sequencing. Transfection and interference effects of HSV-1-shvglut3-0

(RFP) and HSV-1-shvglut3-1 (RFP) were evaluated by detecting fluorescence expression and Western blotting. Positive viral plaques were subsequently amplified.

Transfection of OG Cells In Vitro

One day before transfection, cultured OG cells that stably expressed HSV ICP4 and ICP27 proteins (from Sino GenoMax Co., Ltd) were plated on 6-well plates at a density of 1.5 × 10 cells per well in 2 mL DMEM supplemented with 10% fetal bovine serum to ensure 90% cell confluence. The virus vector DNA of non-replicating HSV-1 was co-transfected into OG cells with pNX-shvglut3-1 (RFP) plasmid using calcium phosphate coprecipitation method. Three to six days later, cell lysate was harvested when cytopathic effects occurred. Several red fluorescent plaques were picked for cloning and used to transfect OG cells again. Transfection was repeated several times until the virus was purified.

HSV-1 Vector Inoculation in Mice

Male ICR mice were randomly divided into four groups: sham-operated group, SNI + normal saline (NS) group, SNI + HSV-1-shvglut3-0 (HSV-NC) group, and SNI + HSV-1-shvglut3-1 (HSV-shvglut3) group. Three days after SNI, mice in the HSV-shvglut3, HSV-NC, and NS groups were anesthetized with isoflurane. Five microliters of HSV-1-shvglut3 (1 × 10 pfu/mL), HSV-1-shvglut3-0 (1 × 10 pfu/mL), or normal saline were administered through a microsyringe into the left sciatic nerve near the previous incision. Vectors were infused over 5 min and the syringe was kept in place for an additional 1 min to allow viral diffusion and absorption.

Assessment of Tactile Allodynia

Mice were acclimated to the testing environment by exposure to testing chambers for 20 min on three separate days prior to preoperative testing. Behavioral tests were conducted on the day of SNI surgery (2 days before vector inoculation) and at 0, 4, 7, 14, 21, and 28 days after vector inoculation. All tests were performed at approximately the same time each day (10:00 am to 6:00 pm) by experimenters blinded to the treatment groups. Paw withdrawal threshold (PWT) was measured using the up-down testing paradigm with a series of von Frey filaments (0.008, 0.06, 0.1, 0.4, 0.6, 1.0, 1.4, and 2.0 g) delivering approximately logarithmic incremental forces. Each filament was applied perpendicularly to the lateral side of the paw innervated by the sural nerve, starting with the 2 g filament.

Assessment of Heat Hyperalgesia

Heat hyperalgesia was measured using Hargreaves' plantar tester (model 7372, UGO BASILE, VA, Italy). Briefly, mice were placed in individual plexiglass containers on a glass floor. After 30 min acclimation, perpendicular radiant heat

stimulation was applied through the glass floor to the lateral plantar surface of the hind paw. Paw withdrawal latencies were measured with heat intensity adjusted to produce a baseline latency of 10 s. A cutoff time of 20 s was applied to avoid tissue damage.

Detection of HSV-1 Vector Distribution After Inoculation

On days 4, 7, and 14 after HSV-1 inoculation, two mice from each group were anesthetized with isoflurane and decapitated. The spine was quickly dissected on ice. The L4/5 DRG and lumbar spinal cord enlargement were isolated, wrapped in tissue freezing medium, rapidly transferred to a cryostat microtome, and sectioned at 15–20 m thickness. RFP fluorescence indicating HSV vector transport was monitored with a fluorescent microscope (Zeiss Axiovert 100, Germany) following retrograde axonal transport.

Western Blot Analysis

The L4/5 spinal cord segment was quickly removed and stored at -80 °C. Frozen tissues were homogenized in ice-cold RIPA lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, and complete mini EDTA-free protease inhibitor cocktail (Beyotime, China). Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad) at 200 mA for 2 h. Nonspecific binding sites were blocked for 1 h with 5% nonfat milk in PBS containing 0.1% Tween-20 at room temperature. Blots were incubated overnight at 4 °C with primary antibodies: rabbit anti-VGLUT3 antibody (1:500, Abcam, USA) or mouse anti- -actin antibody (1:400, Boster, China). Membranes were then incubated with HRP-conjugated secondary antibodies (goat anti-mouse, 1:10,000, Abcam, USA) for 1 h at room temperature. Images were captured and analyzed with a Licor Odyssey scanner. Relative protein expression was calculated as the ratio of signal density to -actin density and normalized to the NS group.

Immunohistochemistry

To distinguish tissue autofluorescence from virus fluorescence, tissues were examined under a fluorescence microscope before staining to ensure complete fading of viral fluorescence. At 14 days after viral administration, mice were anesthetized with ketamine (10 mg) and xylazine (0.5 mg/100 g) and transcardially perfused with 20 mL physiological saline followed by 40 mL 4% paraformaldehyde (PFA) in PBS (0.1 mol/L, pH 7.4) at 10 mL/min. PFA perfusion was performed rapidly for the first 20 mL then at a constant reduced rate. The L4/5 spinal cord and DRG were removed, fixed in 4% PFA overnight at 4 °C, and cryoprotected in 30% sucrose in PBS (0.01 mol/L) for 24 h.

Spinal cords and DRGs were sectioned coronally at 20 m and 10 m thickness, respectively, using a freezing microtome. After rinsing in PBS (3 × 5 min), sections were permeabilized with 0.3% Triton X-100 in PBS for 30 min, then

incubated for 24 h at 4 °C with monoclonal mouse anti-VGLUT3 antibody (Abcam; 1:100 in PBS) with PBS as negative control. After rinsing and deactivation of endogenous peroxidase with 3% H O , spinal sections were incubated for 90 min at room temperature with biotinylated goat anti-mouse IgG antibody, then immersed in streptavidin-biotin complex (SABC) for 60 min. DAB staining was performed according to manufacturer' s instructions (Boster, China). VGLUT3 protein expression was observed microscopically. DRG sections were incubated with fluorescent secondary antibody (goat anti-mouse IgG) for 2 h and examined under a Zeiss Axiovert 100 fluorescence microscope equipped with a Hamamatsu CCD digital camera.

Statistical Analysis

All data are presented as mean ± SE and were analyzed by one-way ANOVA followed by post-hoc Bonferroni test using SPSS 12.0 software. A P-value less than 0.05 was considered statistically significant.

RESULTS

Transfection of OG Cells In Vitro and Tissue Targeting Effect of the Virus In Vivo

Red fluorescent protein (RFP) gene was inserted upstream of the two shRNA cassettes to facilitate purification of the vectors. The intermediate plasmid pNX-shvglut3-0 or pNX-shvglut3-1 was co-transfected with HSV-1 backbone in OG cells. The negative virus did not emit fluorescence while the positive virus exhibited strong red fluorescence under a fluorescent microscope at 546 nm [Figure 1: see original paper]. After purification and amplification, the reconstructed HSV vectors were inoculated in the sciatic nerve of SNI mice. On days 4, 7, and 14 after inoculation, strong red fluorescence was detected in the L4/5 DRG [Figure 1: see original paper] but not in the spinal cord or other regions of the nervous system, indicating that the reconstructed vectors possess DRG targeting properties.

VGLUT3 Expression in the DRG and Spinal Dorsal Horn Following Vector Inoculation

Immunofluorescence histochemistry revealed strong VGLUT3 protein expression in the L4/5 DRG after SNI, which was fully reversed by sciatic nerve inoculation of HSV-1-shvglut3-1 [Figure 2: see original paper]. Immunohistochemistry detected similar changes in the L4/5 spinal dorsal horn [Figure 2: see original paper]. In the NS group, the number of VGLUT3-positive cells increased significantly in the superficial zone of the ipsilateral spinal dorsal horn compared with the sham-operated group ($P < 0.01$). Fourteen days after viral administration, VGLUT3-positive cells decreased markedly in the HSV-1-shvglut3-1

group compared with the NS group ($P < 0.05$) and were similar to those in the sham-operated group ($P > 0.05$). No significant difference was found between HSV-NC and NS groups ($P > 0.05$). This downregulation of VGLUT3 was further confirmed by Western blot analysis. Compared with the sham-operated group, VGLUT3 expression increased significantly in the ipsilateral dorsal horn of the lumbar spinal cord enlargement in the NS group ($P < 0.01$), and this SNI-induced upregulation was fully reversed by HSV-1-shvglut3-1 at 2 weeks after inoculation [Figure 2: see original paper].

HSV-1-shvglut3-1 Inoculation Reverses SNI-Induced Tactile Allodynia

Throughout the study, no motor disturbance or other abnormal activities were observed in any mice. SNI, but not sham surgery, produced significant tactile allodynia [Figure 3: see original paper]. Sciatic nerve inoculation of HSV-1-shvglut3-1, but not HSV-1-shvglut3-0, significantly relieved tactile allodynia, and this effect persisted until the end of the 28-day observation period. No obvious changes were observed in heat hyperalgesia [Figure 3: see original paper].

DISCUSSION

In this study, we found that the recombinant HSV-1-shvglut3 vector inoculated in the sciatic nerve effectively downregulated VGLUT3 expression in DRG neuronal cell bodies and their central terminals in the superficial lamina of the spinal dorsal horn, producing a strong and persistent analgesic effect in mice with mechanical allodynia induced by spared nerve injury.

For gene therapy of pain, researchers have tested natural pain-relieving molecules, neurotransmitter receptors, or ion channels whose expression changes during neuropathic pain. However, because neurotransmitters and voltage-gated ion channels are widely distributed in the nervous system, selectively targeting pain-related pathways remains challenging. Since mechanical hypersensitivity is transmitted exclusively by VGLUT3-positive DRG neurons, Vglut3 knockdown in the DRG serves as a promising target. shRNA delivered by pseudo-latent recombinant HSV, transported through unmyelinated C-fibers to dorsal horn laminae I and II, reduced VGLUT3 expression and produced strong, persistent analgesia against mechanical allodynia 7 days after HSV vector inoculation without observable side effects.

Consistent with previous studies using recombinant HSV-1 to deliver interfering RNA to the DRG following peripheral inoculation, we successfully delivered shRNA (HSV-shvglut3) to the DRG after sciatic nerve vector inoculation. These peripherally inoculated replication-deficient HSV-1 vectors reached target DRGs via retrograde axonal transport in a pseudo-latent state and inhibited target gene expression to modulate nociceptive neurotransmission from afferent nerve terminals to the spinal dorsal horn. We observed no side effects from HSV

inoculation; indeed, studies suggest that herpes vector-mediated gene transfer can alleviate pain without systemic side effects or tolerance induction and can be used in combination with standard pain treatments. Although we assessed analgesic effects within only 4 weeks, we assume that transgene expression driven by the human cytomegalovirus immediate-early gene promoter can persist for several weeks and be reestablished by vector reinoculation.

In RNAi techniques, nonspecific silencing may result from non-sequence-specific effects, off-target effects, or interferon response induction. Our results showed that the negative control vector (HSV-1-shvglut3-0) had no effect on pain thresholds or target gene expression, excluding potential nonspecific effects induced by the HSV vector backbone.

This study primarily examined the analgesic effect of HSV-shvglut3 on neuropathic pain. As VGLUT3-positive fibers reportedly contribute in a cause-dependent manner to mechanical and cold hypersensitivity, further work is required to elucidate the mechanism underlying the analgesic effect of Vglut3 knockdown.

CONCLUSION

Vglut3 in the DRG is a promising therapeutic target for alleviating mechanical allodynia, and RNAi mediated by an HSV-1 vector inoculated in peripheral nerves is a safe and efficient strategy for achieving persistent analgesia in mice.

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Note: Figure translations are in progress. See original paper for figures.