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 (HSV-1) for 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 the shRNA targeting Vglut3 (HSV-1-shvglut3) was constructed and inoculated in the sciatic nerve in a mouse model of mechanical allodynia to test its analgesia effect. Mechanical allodynia and heat hypersensitivity of the mice were tested by von Frey filaments and Hargreaves' test, respectively. VGLUT3 expression in the dorsal root ganglion (DRG) was down-regulated 2 weeks after vector inoculation. The analgesic effect lasted for over 2 weeks in these mice without obvious systematic 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 inoculation of the vector.

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

INTRODUCTION

Tactile allodynia is the most refractory symptom of neuropathic pain, which, along with hyperalgesia and spontaneous pain, seriously affects the quality of life of the patients. As the factors associated with the onset and recovery of tactile allodynia are poorly understood, conventional treatments have only limited effects with frequent undesirable side effects. Novel therapeutic strategies are being explored, including gene knockdown and knock-in techniques and therapy targeting specific signal molecules, ion channels, transmitters, and receptors, and some of these strategies have achieved encouraging effects.

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

Cell type-specific RNA interference (RNAi) may provide an alternative approach to Vglut3 knock-down in the DRG. Several types of carriers, including viral vectors, have been tested for delivery of RNAi constructs to the DRG, and among these carriers, herpes simplex virus (HSV)-derived vectors have shown the potential as promising DRG-targeting vectors. HSV possesses not only high tropism for peripheral sensory neurons but also the capacity to establish a life-long persistence in host cells (latent state of infection) in the intranuclear episomal form. Natural uptake of HSV virion by the nerve terminal and its rapid retrograde
axonol transport toward the nerve cell bodies offers a unique possibility of peripheral, noninvasive vector administration. Recently, studies demonstrated that replication-deficient HSV type 1 (HSV-1) was capable of mediating the delivery of RNAi constructs targeting pain-related genes from the peripheral nerve to the DRG of mice in vivo, which resulted in highly effective and specific gene silencing in DRG neurons. Therefore, we hypothesize that selective knockdown of Vglut3 in the DRG by HSV-1-mediated RNAi can attenuate mechanical allodynia without causing systemic side effects.

In this study, we constructed a recombinant replication-defective HSV-1 vector carrying a short hairpin RNA (shRNA) that targeted Vglut3, and 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 Vglut3 knockdown in the DRG and its analgesic effects in the mice.

**MATERIAL AND METHODS**

**Animal preparations**

Sixty SPF male ICR mice weighing 20-25 g (SLRC laboratory animal, Hunan, China) were kept at room temperature (24 ± 1 °C) on a 12 h light-dark cycle and given free access to laboratory chow and water. The experimental procedures and care of the animals were approved by the Animal Care and Use Committee of Tongji Hospital in line with the International Association of the Study of Pain (IASP) Guidelines for the Use of Animals in Research.

**Nerve injury surgery**

SNI of the sciatic nerve was performed as previously described. Briefly, the mice were anesthetized with an intraperitoneal dose of ketamine (10 mg) and xylazine (0.5 mg/100 g body weight), and the left sciatic nerve and its 3 terminal branches were exposed cautiously. The common peroneal and the tibial nerves were tightly ligated with 5.0 silk, and a 2 to 4 mm segment distal to the ligation was removed. Touching or stretching the sural nerve was avoided to ensure its integrity. The ligation was removed. Touching or stretching the sural nerve was avoided to ensure its integrity. The muscle and skin were closed in turn. The mice receiving sham operation were subjected to exposure of the sciatic nerve and its branches without ligation or any injury to the nerve.

**Construction of HSV-1 vectors**

Two recombinant HSV-1 vectors were constructed, namely HSV-1-U6-shvglut3-1 that contained red fluorescence protein gene (rfp) and the shRNA targeting Vglut3 (shvglut3-1), and HSV-1-U6-shvglut3-0 carrying rfp gene and a negative control shRNA sequence (shvglut3-0) that is not predicted to target any known vertebrate gene. Both of the viral vectors were under the control of a U6 promoter. According to the coding sequence of vglut3 gene (GenBank Accession NM_182959.3), shvglut3-1 was designed (underlined sequence) and combined with a U6 promoter:

CAAGCTTAAAGTCGGGCGAGCAGAGGAGCT
ATTTCCCATGATTTCCCTATAGTGCTTCTAT
AGCATACAGGCAGCTTTAGAGATTAAG
AATTAATTTGACTGTAAACAACAAAGATATT
CGTAACTTGAAGTATTTCGATTCTTGGC
TTTATATATCTTTTGTTACTATAGGTCTAAAGCTTTTCTC
AGCG.

Based on the established shRNA sequences against Vglut3, we disrupted its order and selected a sequence that shares no homology with Vglut3 gene as the control shRNA sequence: (CGCTTTAGCTTATACTGACC). Vglut3-0-shRNA (334 bp) with a U6 promoter was synthesized and verified by gene sequencing. Results of PCR identification showed that the synthesized plasmid contained a 334-bp sequence consistent with Vglut3-0-shRNA. The sequence of U6-shvglut3-0 was otherwise identical with U6-shvglut3-1 except for the 271-335 fragments (CGGGCTGTTAGACCTATA GTACC). Non-replicative HSV-1 vector backbone (Sino-GenoMax, Beijing, China) was constructed by deleting the virulent genes ICP27, ICP4, and ICP34.5 with genetic engineering methods. The plasmids pNX-U6-shvglut3-1 and pNX-U6-shvglut3-0 were obtained by inserting U6-shvglut3-1 and U6-shvglut3-0 sequences, respectively, to pNX (RFP) intermediate plasmid between Hind III and Xho I sites. Replication-defective HSV-1 vectors were generated by calcium phosphate co-transfection of complementing OG cells (Vero cell lines with stable expression of ICP27 and ICP4 proteins) with the intermediate plasmids described above and the HSV backbone. The genome structures were confirmed by PCR followed by sequencing. Transfection and interference effects of HSV-1-U6-shvglut3-0 (RFP) and HSV-1-U6-shvglut3-1 (RFP) were evaluated by detecting fluorescence expression and Western blotting. The positive viral plaques of HSV-1-U6-shvglut3-0 (RFP) and HSV-1-U6-shvglut3-1 (RFP) were subsequently amplified.

**Transfection of OG cells in vitro**

One day before transfection, cultured OG cells that stably expressed ICP4 and ICP27 proteins of HSV-1 (from Sino Geno Max Co., Ltd) were plated on a 6-well culture plate at the density of 1×10^4 to 1.5×10^5 cells in 2 mL DMEM supplemented with 10% culture medium to ensure a cell fusion rate of 90%-95%. The virus vector DNA of non-replicative HSV was co-transfected in OG cells with pNX-U6-Vglut3-1 (RFP) plasmid using calcium phosphate coprecipitation method, and 3 to 6 days later, the cell sap was harvested when a lesion occurred. Three days after transfection of OG cells with
the cell sap, several red CPE were picked for cloning to transfet OG cells again. The transfection was repeated several times until the virus was purified.

**HSV-1 vector inoculation in mice**

Male ICR mice were randomly divided into 4 groups, namely the sham-operated group, SNI + normal saline (NS) group, SNI + HSV-1-U6-shvglut3-0 (HSV-1-NC) group and SNI + HSV-1-U6-shvglut3-1 (HSV-1-shvglut3) group. Three days after SNI, the mice in HSV-1-shvglut3, HSV-1-NC, and NS groups were anesthetized with isoflurane and 5 μL of HSV-1-U6-shvglut3-1 (1×10⁸ pfu/mL), HSV-U6-shvglut3-0 (1×10⁶ pfu/mL), and normal saline, respectively, were administered through a 10-μL microsyringe into the left sciatic nerve near the previous incision. The vectors were infused over a period of 5 min and the injection syringe was kept in place for an additional 1 min to allow for viral diffusion and absorption.

**Assessment of tactile allodynia**

The mice were accommodated to the testing environment by exposure to the testing chambers for 20 min on 3 separate days prior to the preoperative testing. Behavioral tests of the mice 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. The tests were performed at approximately the same time points ranging from 6:00 am to 6:00 pm each day. All the tests were done by experimenters blinded to the surgery that the mice had received. Paw withdrawal threshold (PWT) was measured using the up-and-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), which delivered approximately logarithmic incremental forces with the starting filament of 2 g. Each filament was applied perpendicularly at the lateral side of the paw innervated by the sural nerve.

**Assessment of heat hyperalgesia**

Heat hyperalgesia was measured by Hargreaves' test using a plantar tester (model 7372, UGO BASILE, VA, Italy). Briefly, the mice were placed in individual plexiglass containers on a glass floor. After 30 min of acclimation, percutaneous radiant heat stimulation was applied through the glass floor to the lateral side of the planter surface of the hind paw, and paw withdrawal latencies were measured. The heat intensity was adjusted to produce a baseline latency of 10 s. A cut-off time of 20 s was applied to avoid tissue damage.

**Detection of HSV-1 vector distribution after inoculation**

On days 4, 7 and day 14 after HSV-1 inoculation, 2 mice from each group were anesthetized with isoflurane and decapitated. The spine of the mice was quickly separated using tissue scissors on ice. The L₅₆ DRG and lumbar enlargement of the spinal cord were isolated, wrapped with tissue-freezing medium, rapidly transferred into the cryostat microtome, and sliced into sections 15 or 20 μm in thickness. The RFP fluorescence, which indicated the location of the HSV-1 vectors following retrograde axonal transport, was monitored with a fluorescent microscope (Zeiss Axiouvert 100, Germany).

**Western blot analysis**

The L₅₆ segment of the spinal cord was quickly removed at 0-4 °C and stored at -80 °C before use. The frozen tissues were homogenized in ice-cold RIPA lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% (V/V) Triton X-100, 1% sodium deoxycholate, 1% SDS and complete, mini, EDTA-free protease inhibitor cocktail (Beyotime, China). Proteins were separated with 10% SDS-PAGE and then transferred to PVDF membranes (Bio-Rad) at 200 mA for 2 h. Nonspecific binding sites were blocked for 1 h with 0.1% (PBS-T) containing 5% nonfat milk at room temperature. The blots were incubated overnight at 4 °C with a primary antibody [mouse anti-VGLUT3 antibody (1:500, Abcam, USA) or mouse anti-β-actin antibody (1:400, Boster, China)]. The membranes were then incubated with the secondary antibody (HRP-conjugated goat anti-mouse, 1:10 000, Abcam, USA) for 1 h at room temperature. Images of the bands on the membranes were photographed and analyzed with a Licor odyssey scanner (Li-cor Biotechnology, USA). The relative expression of each protein was calculated as the ratio of signal density to β-actin density and was normalized by NS group.

**Immunohistochemistry**

To distinguish fluorescent staining of the tissue from virus fluorescence, tissues were observed under a fluorescence microscope before staining to ensure full fading of the virus fluorescence. At 14 days after viral administration, the mice were anesthetized with ketamine (10 mg) and xylazine (0.5 mg/100 g) followed by transcardial perfusion with 20 mL physiological saline and then with 40 mL 4% paraformaldehyde (PFA) in PBS (0.1 mol/L, pH 7.4; 10 mL/min); PFA perfusion was carried out at a fast rate for the first 20 ml and then at a constant lowered rate. The L₅₆ spinal cord and DRG were removed from the spine, fixed in 4% PFA overnight at 4 °C, and then cryoprotected in a solution containing 30% sucrose in PBS (0.01 mol/L) for 24 h. The spinal cords and DRGs were sectioned in the coronal plane (20 μm and 10 μm in thickness, respectively) with a freezing microtome. After being rinsed in PBS for 3 times (5 min each time), the sections were permeablized with 0.3% Triton X-100 in PBS for 30 min, incubated for 24 h at 4 °C with monoclonal mouse anti-VGLUT3 antibody (Abcam; 1:100 in PBS) with PBS as the negative control. After rinse with 0.1% PBS-T and deactivation of endogenous peroxidase with 3% H₂O₂, the spinal sections were incubated for 90 min...
at room temperature with a biotinylated goat-anti-mouse IgG antibody, and then immersed in SABC for 60 min. Subsequent DAB staining was conducted following the manufacturer’s instructions (Boster, China). The expression of VGLUT3 protein was observed microscopically. The DRG sections were incubated with a fluorescent secondary antibody (goat-anti-mouse IgG-Cy3) for 2 h and the expression of VGLUT3 were examined under a Zeiss Axiovert 100 fluorescence microscope equipped with a Hamamatsu CCD digital camera.

**Statistical Analysis**

All the data were presented as Mean±SE and 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 to indicate a statistically significant difference.

**RESULTS**

**Transfection of OG cells in vitro and tissue targeting effect of the virus**

Red fluorescent protein (RFP) gene was inserted at upstream of the two shRNA cassettes to facilitate purification of the vectors and verification of their DRG-targeting efficiency in vitro. The intermediate plasmid pNX-U6-shvglut3-1 or pNX-U6-shvglut3-0 was co-transfected with HSV-1 backbone in OG cells. The negative virus did not emit any fluorescence while the positive virus exhibited strong red fluorescence under a fluorescent microscope at 546 nm (Fig.1). After purification and amplification, the reconstructed HSV-1 vectors were inoculated in the sciatic nerve in SNI mice. On days 4, 7 and 14 after the inoculation of the HSV-1 vectors, strong red fluorescence was detected in the L4/5 DRG (Fig.1), but not in the spine or any other region of the nervous system. These results indicate that the reconstructed vectors possess DRG-targeting property.

**VGLUT3 expression in the DRG and spinal dorsal horn following vector inoculation**

Immunofluorescence histochemistry revealed strong VGLUT3 protein expression after SNI in the DRG at L4/5 segments, which was fully reversed by inoculation of HSV-1-U6-shvglut3-1 in the sciatic nerve (Fig.2). Immunohistochemistry detected similar change in the spinal dorsal horn at the L4-5 segments (Fig.2). In NS group, the number of VGLUT3-positive cells increased significantly following SNI in the superficial zone of the ipsilateral spinal dorsal horn as compared with that in the sham-operated group (n=3, P<0.01). Fourteen days after the viral administration, the number of VGLUT3-positive cells decreased obviously in HSV-1-shvglut3-1 group as compared with that in NS group (n=3, P<0.05) and was similar with that in the sham-operated group (n=3, P>0.05). No significant difference was found between HSV-1-NC and NS groups (n=3, P>0.05). This down-regulation of VGLUT3 was further
confirmed by Western blot analysis. Compared with that in sham-operated group, VGLUT3 expression increased significantly in the ipsilateral dorsal horn of the lumbar enlargement of the spinal cord in NS group (n=3, P<0.05), and this SNI-induced up-regulation of VGLUT3 expression was fully reversed by HSV-1-U6-shvglut3-1 at 2 weeks after its inoculation (Fig.2).

**DISCUSSION**

In this study, we found that the recombinant HSV-1 vector (HSV-1-U6-shvglut3-1) inoculated in the sciatic nerve effectively down-regulated VGLUT3 expression in the DRG neuron body and its central terminal in the superficial lamina of the spinal dorsal horn, and produced a strong and persistent analgesic effect in hypersensitivity after SNI or inoculation of HSV-1-U6-shvglut3-1.

**HSV-1-U6-shvglut3-1 inoculation reverses SNI-induced tactile allodynia**

Throughout the study, no motor disturbance or other abnormal activities were found in any of the mice. SNI, but not sham surgery, produced significant tactile allodynia (Fig.3). Inoculation of HSV-1-U6-shvglut3-3, but not HSV-1-U6-shvglut3-0, in the sciatic nerve significantly relieved tactile allodynia, and this effect persisted till the end of the observation period of 28 days. No obvious changes were observed in heat
mice with mechanical allodynia induced by spared nerve injury.

For gene therapy for pain, researchers have tested natural pain-relieving molecules as the therapeutic targets that access endogenous antinociceptive circuits in rodent models of neuropathic pain. But because of the wide distribution of neurotransmitter receptors, or ion channels, whose expression changes in the pathological process of neuropathic pain, it is difficult to selectively target pain-related pathways, even when using a DRG-targeting HSV vector. As by mechanical hypersensitivity is transmitted exclusively VGLUT3-positive DRG neurons, Vglut3 serves as a promising target for knockdown. Vglut3 knockdown in the DRG by shRNA delivered by pseudo-latent recombinant HSV-1, which was transported through unmyelinated C fibers to the dorsal horn lamina I and II, resulted in reduced expression of VGLUT3 and produced a strong and persistent analgesic effect on mechanical allodynia 7 days after HSV-1 vector inoculation without observable side effects.

Consistent with a previous study using recombinant HSV-1 to deliver interfering RNA to the DRG following its peripheral inoculation, we successfully delivered shRNA (HSV-1-U6-shvglut3-1) to the DRG after inoculation of the vector in the sciatic nerve. These peripherally inoculated non-replicating recombinant HSV-1 vectors reached the target DRG by retrograde axonal transport in a pseudo-latent state, and inhibited the expression of the target gene to modulate nociceptive neurotransmission from afferent nerve terminals to the dorsal horn of the spinal cord.

We did not observe any side effects of HSV-1 inoculation; in fact, studies suggest that herpes vector-mediated gene transfer can alleviate pain without systemic side effects or inducing tolerance and can be used in combination with standard pain treatments. Although we assessed the analgesic effects of HSV-1 vector inoculation within only 4 weeks, we assumed that the effect of such HSV vectors, in which transgene expression is driven by the promoter of immediate early gene of human cytomegalovirus, can persist for several weeks and can be reestablished by reinoculation of the vector.

In RNAi technique, non-specific silencing may result from a non-sequence-specific effect caused by the virus, sequence-specific off-target effects, or induction of the interferon response. Our results showed that the negative control vector (HSV-1-U6-shvglut3-0) had no effect on pain threshold or target gene expression in the model mice, which could exclude any potential non-specific effects induced by the HSV vector backbone.

This study was conducted to examine primarily the analgesic effect of HSV-1-shvglut3 on neuropathic pain. As VGLUT3-positive fibers were reported to contribute in a cause-dependent manner to the development of mechanical and cold hypersensitivity, further work is required to elucidate the mechanism underlying the analgesic effect of Vglut3 knockdown.

Conclusion

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

REFERENCES: