HHS Public Access

Author manuscript

Gene Ther. Author manuscript; available in PMC 2018 January 17.

Published in final edited form as:

Gene Ther. 2013 July ; 20(7): 742–750. doi:10.1038/gt.2012.90.

Comparative effectiveness of antinociceptive gene therapies in animal models of diabetic neuropathic pain

Y Wang[1], **MO Nowicki**[1], **X Wang**[2], **WD Arnold**[3], **SA Fernandez**[4], **X Mo**[4], **J Wechuk**[5], **D Krisky**[5], **J Goss**[5], **D Wolfe**[5], **PG Popovich**[2], **S Lawler**[1], and **EA Chiocca**[1]

[1]Department of Neurological Surgery, The Ohio State University Wexner Medical Center, Dardinger Laboratory for Neurooncology and Neurosciences, Columbus, OH, USA

[2]Department of Neuroscience and Center for Brain and Spinal Cord Repair, Columbus, OH, USA

[3]Division of Neuromuscular Medicine, Department of Neurology, Columbus, OH, USA

[4]Center for Biostatistics, The Ohio State University Wexner Medical Center, Columbus, OH, USA

[5]Dyamid, Inc., PA, Pittsburgh, USA

Abstract

Peripheral neuropathic pain is one of the most common and debilitating complications of diabetes. Several genes have been shown to be effective in reducing neuropathic pain in animal models of diabetes after transfer to the dorsal root ganglion using replication-defective herpes simplex virus (HSV)1-based vectors, yet there has never been a comparative analysis of their efficacy. We compared four different HSV1-based vectors engineered to produce one of two opioid receptor agonists (enkephalin or endomorphin), or one of two isoforms of glutamic acid decarboxylase (GAD65 or GAD67), alone and in combination, in the streptozotocin-induced diabetic rat and mouse models. Our results indicate that a single subcutaneous hindpaw inoculation of vectors expressing GAD65 or GAD67 reduced diabetes-induced mechanical allodynia to a degree that was greater than daily injections of gabapentin in rats. Diabetic mice that developed thermal hyperalgesia also responded to GAD65 or endomorphin gene delivery. The results suggest that either GAD65 or GAD67 vectors are the most effective in the treatment of diabetic pain. The vector combinations, GAD67 + endomorphin, GAD67 + enkephalin or endomorphin + enkephalin also produced a significant antinociceptive effect but the combination did not appear to be superior to single gene treatment. These findings provide further justification for the clinical development of antinociceptive gene therapies for the treatment of diabetic peripheral neuropathies.

Keywords

herpes simplex virus; diabetes; neuropathic pain

Correspondence: Dr EA Chiocca, Department of Neurological Surgery, The Ohio State University Wexner Medical Center, Dardinger Laboratory for Neurooncology and Neurosciences, Wiseman Hall, 400 West 12th Avenue, Columbus, OH 43210, USA. EA.Chiocca@osumc.edu.

CONFLICT OF INTEREST

The remaining authors declare no conflict of interest.

INTRODUCTION

Neuropathic pain is the most common complication of diabetes and is recognized as one of the most difficult types of pain to treat.[1] Conventional analgesics, tricyclic antidepressants, anticonvulsants, such as pregabalin and gabapentin, selective serotonin/noradrenaline reuptake inhibitors, channel blockers and opioids are partially effective or even ineffective for some patients, and 'off-target' adverse effects of systemic administration limit their utility.[2] More recently, herpes simplex virus (HSV)-based gene transfer with specific targeting of primary sensory neurons in dorsal root ganglia (DRGs) has been used to treat neuropathic pain in animal models.[3] Based on the natural neurotropism of HSV1, peripheral intradermally injected HSV1 vectors expressing antinociceptive genes are taken up by sensory nerve endings and transported in a retrograde manner to DRGs where the antinociceptive gene products are produced. These products are released in the dorsal horn of the spinal cord (SC) where they modulate nociceptive neurotransmission between primary nociceptors and second-order neurons.[4]

Several studies have demonstrated antinociceptive effects in animal models of neuropathic pain using HSV vectors expressing a variety of gene products. Gene transfer to DRGs with an enkephalin precursor expressing HSV-based vector has been shown to produce robust pain relief in inflammatory pain, cancer pain and diabetic neuropathic pain.[5–7] Recently, a phase I clinical study showed that intradermal injection of an HSV-based vector expressing human preproenkephalin provided relief from intractable cancer pain.[5] An HSV vector expressing human endomorphin 2 has been used to reduce pain caused by nerve injury in rats[8] and similar results were observed for glutamic acid decarboxylase (GAD)67, an isoform of the g-aminobutyric acid (GABA)-producing enzyme.[9] Both enkephalin and endomorphin are endogenous opioid agonists that primarily bind to d- or m-opioid receptors, respectively, and like GABA, are considered inhibitory neurotransmitters. Recent studies showed that HSV-mediated enkephalin or GAD67 expression inhibits nociceptive behavior in rats with streptozotocin (STZ)-induced diabetes.[6,10] Also, anti-inflammatory cytokines such as interleukin-4,[11] soluble tumor necrosis factor receptor[12] and growth factors such as neurotrophin-3[13] have been shown to have analgesic effects in animal models of pain.

Therefore, overexpression of various gene products appear to provide effective analgesia in different models of pain. However, there has been no systematic comparison of the relative effectiveness of these different genes in a single model. In this study, utilizing a rodent model of STZ-induced diabetic neuropathic pain, we sought to examine and compare the effectiveness of four antinociceptive genes delivered by HSV vectors: enkephalin (NP2), endomorphin (NE2), GAD65 or GAD67.

RESULTS

Vector detection and transgene expression in DRGs after hindpaw injection

HSV-based vectors expressing GAD67, GAD65, endomorphin 1 and 2 (NE2), preproenkephalin (NP2) or green fluorescent protein (GFP) (Figure 1) were used. Identical transcriptional regulatory elements were utilized to minimize possible differences in transgene expression. One week after the subcutaneous inoculation of each vector into the

plantar surface of rat hind paws, HSV-specific DNA was detected in pooled L4–L6 DRGs, but not in corresponding lumbar SC (Figure 2a). Vector DNA persisted in DRGs for least 12 weeks after injection (Figure 2b).

As a correlate for transgene expression, GFP mRNA was present for at least 12 weeks in lumbar DRGs after injection of a GFP-expressing control vector in the rat hindpaw (Figure 2c). Immunohistochemistry confirmed the presence of HSV-1 ICP0 antigen and GFP antigen in DRGs 1 week after hindpaw injection but not in DRGs of vehicle-injected animals (Figure 2d). Transgene expression in DRGs was colocalized within cells expressing the neuron-specific marker NeuN. These data indicate that HSV vector injection in rat hind paws causes persistence expression of vector DNA and delivered transgenes in lumbar DRG neurons.

Target gene expression in DRGs and SC

We next verified that an antinociceptive gene delivered by the HSV vector was expressed in primary sensory neurons (in DRGs) and in their terminals in the SC dorsal horn. One week after hindpaw injection of either GAD67 or GAD65 vector, transgene expression within lumbar DRG neurons was observed by immunohistochemistry and it colocalized with NeuN-positive neurons (Figure 3a). Similarly, western blotting also showed a multifold increase of GAD67 expression in the lumbar DRGs of the GAD67 vector-injected rats (Figure 3b). Furthermore, expression of GAD67 and GAD65 mRNAs was significantly increased in DRGs at the 1- and 2-week time points (Figure 3c). In addition, 1 week after subcutaneous injection of GAD67-expressing HSV vector, GAD67 was increased in the ipsilateral side of the SC when compared with the contralateral side where the contralateral hindpaw was injected with phosphate-buffered saline (PBS: Figure 3d). A significant increase in GAD65 and GAD67 mRNAs was observed within the dorsal horn of the SC, that is, the terminal field of the DRG neurons (Figure 3e), supporting the hypothesis that local gene expression occurs in the nerve terminal.[14] In rats injected with NP2 vectors, immunohistochemistry revealed increased expression of enkephalin in the lumbar DRGs and SC (Supplementary Figures S1a and b) when compared with PBS-injected rats. For NE2-injected rats, quantitative reverse transcription-PCR showed that mRNA expression was induced 165-fold in DRGs of the NE2 vector-injected side compared with the PBS-injected contralateral side (Supplementary Figure S1c).

Behavioral analyses in diabetic models

Type 1 diabetes was induced by intraperitoneal (i.p.) injection of 50 mg kg^{-1} STZ in adult Wistar rats. Four weeks after STZ injection, diabetic rats exhibited increased mechanical allodynia (MA), manifested as reduced paw withdrawal threshold measured by the von Frey test, which lasted for at least 10 weeks (data not shown). Diabetic neuropathy is also characterized by a reduction in neuropeptide content in sensory ganglia and sensory nerve terminals in the SC dorsal horn.[15] Four weeks after STZ injection, the amount of calcitonin gene-related peptide immunoreactivity in the DRG was significantly reduced in animals when compared with animals not injected with STZ (Supplementary Figures S2c–e).

Three independent experiments were performed to determine the effect of four single antinociceptive genes (GAD65, GAD67, NP2 and NE2) and six antinociceptive gene combinations (GAD65 + GAD67, GAD65 + NP2, GAD65 + NE2, GAD67 + NP2, GAD67 + NE2 and NP2 + NE2). Figure 4a shows the effect of two individual GAD vectors, a NE2 vector and gabapentin on diabetes-induced MA. The two GAD-expressing constructs (GAD65 and GAD67) were significantly more effective in reversing allodynia compared with NE2- or PBS-injected rats. Daily injection of gabapentin, a drug widely used to treat neuropathic pain, produced significantly less relief compared with either the GAD65 or GAD67 vectors, reducing allodynia only at the 3-week time point. Not surprisingly, the combination of GAD67 + NE2 or GAD67 + NP2 also produced a significant antinociceptive effect but this was not superior to that of GAD65 or GAD67 alone. Surprisingly, the combination of GAD65 + GAD67 did not appear to produce very effective pain relief (Figure 4b), and while the NP2 vector did demonstrate some effectiveness and the NE2 vector did not; the combination of the two appeared to be more effective than either vector alone. In general, observed antinociceptive effects from the single injection lasted 4 weeks and started to wane afterwards (Figure 4b and data not shown). The data were also analyzed by assessing the statistical significance of an effect before and after treatment, confirming the significant effectiveness of GAD65 and GAD67, but also of NP2, GAD67 + NP2, NP2 + NE2 and GAD67 + NE2 (Figure 4b). Overall, single injections of GAD65, GAD67 or NP2 and the combined injections of GAD67 + NP2, NP2 + NE2 and GAD67 + NE2 reduced MA, for at least one time point after treatment, when compared with pretreatment.

Consistent with previous reports,[16] we did not observe thermal hyperalgesia in STZ-injected rats. Therefore, we used C57BL/6 mice to test the effects of HSV vectors on diabetes-induced thermal pain.[17] Two weeks after STZ injection, diabetic C57BL/6 mice displayed increased thermal sensation, manifested as reduced paw withdrawal latency that lasted 2 weeks as measured by the hot plate test. Development of thermal pain was measured every 2 days for 4 weeks after vector injection. The data indicate that GAD65 and NE2 vectors significantly increased thermal paw withdrawal latency on days 15 and 19 post vector injection, respectively, when compared with the mice injected with PBS (Figure 5).

Additional analyses were carried out to determine whether antinociceptive gene transfer led to further alterations in the autonomic and sensory innervations displayed by diabetic rats by using the sudomotor test and sensory nerve conduction studies, respectively. However, none of the tested antinociceptive genes or gabapentin was found to alleviate autonomic or sensory nerve abnormalities in diabetic rats (Supplementary Figure S3).

Taken together, these results thus showed that several antinociceptive genes or gene combinations could be useful therapeutically for diabetic neuropathic pain, although the antinociceptive effects of single GAD65 or GAD67 appeared to be of a higher magnitude and longer lasting than all other treatments, including the current standard of care consisting of oral gabapentin.

Toxicology and biodistribution studies

To assess the possible acute toxicity of HSV vectors, four groups of 12 adult Wistar rats were injected with 30 ml of 10^7p.f.u. HSV vectors expressing GFP (or PBS; n = 3) into their

hind paws then at 1 h, 1 day or 1 week post injection rats were euthanized and differences in complete blood counts with differential, electrolytes and liver function were analyzed. No differences were observed between vector- and PBS-injected rats at any time (data not shown). Various tissues and organs (brain, DRG, SC, liver, heart, spleen, lung, gonad and paw) also were collected for pathology and histological analyses. Again, no signs of pathology were evident in tissues from vector or control-injected animals (data not shown). There was also no elevation of acute inflammatory cytokines in response to GFP-HSV vector inoculation, including interleukin-6, tumor necrosis factor-a and interferon-g (Supplementary Figure S4). However, a mild but significant increase of circulating anti-HSV antibodies was noted in rats inoculated with HSV vectors (Figure 6).

To evaluate cellular immune responses in DRGs after HSV vector inoculation, CD11b immunohistochemistry was performed for mononuclear cells (macrophages) in DRGs. There was an increase in CD11b-expressing cells in the DRG of diabetic rats (12 weeks post STZ injection) compared with control nondiabetic rats as reported in the literature.[18] However, HSV-GAD67 inoculation did not lead to a significant increase in CD11b cells in the DRG of diabetic rats compared with PBS-treated diabetic animals (Supplementary Figure S5).

HSV-based vectors preferentially infect neuronal tissues and are therefore considered ‘targeted’ delivery vectors. To determine whether there was ‘off-target’ distribution of the injected vectors, organs harvested in the toxicology studies were examined for vector distribution. Vector DNA was examined by PCR using vector-specific primers in tissue samples from eight organs as well as the injection site at 1 day and 1 week post vector inoculation. Vector DNA was detected in samples from the injection site and associated DRG but not in other tissues (Supplementary Figure S6a). Reverse transcription-PCR was performed to detect the presence of the GFP transcript from injected vector in organs and injection site. The results indicated that the GFP transcript was present at the injection site (paw) at 1 h, 1 day and 1 week after vector injection and in the DRG at 1 week after injection but not in other organs (Supplementary Figure S6b). These experiments show that HSV vector-mediated gene expression preferentially distributes to neurons in the DRG. Some transcript was initially detected at the site of injection (paw); however, no vector-mediated gene expression was detectable in other organs. There was also no evidence of immune responses against the vector or transgene.

DISCUSSION

This study offers the first detailed comparison of the efficacies of four therapeutic HSV-based vectors, alone and in combination, in animal models of diabetic neuropathic pain. Vectors expressing GAD65 or GAD67 showed the most significant combined (thermal and mechanical) antinociceptive effects among all tested treatments. Although combinations of GAD67 + NE2, NP2 + NE2 or GAD67 + NP2 also produced significant antinociceptive effects, they were not superior compared with GAD65 or GAD67. Our study is consistent with previous reports that vector-mediated expression of enkephalin or GAD67 in DRG neurons reduces pain-related behaviors in models of painful diabetic neuropathy.[6,10] In

addition, we showed that HSV-1-based vector-mediated gene transfer of a therapeutic gene to DRGs produced few off-target or systemic effects.

GAD65 and GAD67 are two forms of glutamate decarboxylase encoded by two differentially regulated genes.[19,20] Both forms of GAD exist in GABA-producing neurons and GAD65 also presents in pancreatic islet cells. In the mature neuron, GAD67 is localized in both terminals and the cell body, where it preferentially synthesizes cytoplasmic GABA. GAD65, on the other hand, is primarily localized at nerve terminals to produce synaptic GABA for vesicular release.[19,21] Selective GABAergic inhibition, caused by the loss of GAD65-producing neurons in the ipsilateral dorsal horn, has been well documented in various pain models.[22,23] Introduction of either GAD65 or GAD67 to the DRG has been shown to effectively relieve neuropathic pain symptoms.[10,24–26] For diabetic pain, it has been reported that transfection of DRG neurons with an HSV vector expressing GAD67 results in constitutive release of GABA[9], which provides analgesia[10] by functioning as an inhibitory neural transmitter and by downregulating sodium channels (NaV1.7) through activation of $GABA_B$ receptors. However, there is no study that has compared the analgesic efficacy of GAD65, GAD67 or both under identical experimental conditions. In this study, we show that subcutaneous inoculation of GAD65- or GAD67-expressing HSV vectors produced similar antinociceptive effects in STZ-induced diabetic rats. However, the combination of GAD65 and GAD67 produced no analgesic effect. For this combination and other combinations, a relatively simple explanation may relate to the fact that animals in the combined treatment groups received half the amount of each vector. This was due to limitations on volumes that could be injected in a paw and on maximum available titers for the vectors. However, as both GAD65 and GAD67 singly produced an effect whereas their combination did not, additional reasons may be responsible for the lack of effect of the combination. One possibility may be that GABA release at the synapse after single GAD65 or GAD67 vector injection activates $GABA_BR$, which downregulates NaV1.7 and reduces secondary sensory neuron excitability. However, high level of GABA release caused by gene transfer of combined GAD65 and GAD67 at the DRG may activate both $GABA_AR$ and $GABA_BR$. Activation of the $GABA_A$ receptor can result in a depolarizing or excitatory current in spinal lamina I neurons of animals with neuropathic pain.[27,28] The reason for the lack of anti-allodynia effects after overexpression of both GAD65 and GAD67 may thus be due to competing effects from excess GABA release. In the mouse diabetic pain model, GAD65 was more effective than GAD67 in alleviating thermal pain. It has been shown that thermal and mechanical pains are mediated by different molecular pathways.[29] GAD65 knockout mice showed significant reduction in response latency measured by the hot plate test but there was no genotype-specific difference when measured by the von Frey test.[30]

It was somewhat surprising that the antinociceptive effect of GAD65 and GAD67 was present at the first time point measured after vector injection (day 1). We believe that this is a real phenomenon and not an effect of temporary altered sensation from volume injection in the paw, because the day 1 antinociceptive effect did not occur with NP2 or NE2. It is possible that vector entry into the paw's sensory fibers or other cells in the paw may have raised peripheral expression of GABA by vector expression of GADs. It has been shown that peripheral GABA can arise from primary afferent fibers that contain glutamate[31–33] that

could have been converted locally to GABA by GAD expression in locally infected cells in the paw.

We also observed that paw inoculation of the enkephalin-expressing NP2 vector produced an anti-allodynia effect in STZ diabetic rats, consistent with a previous study[6] in which activation of the pre-synaptic d-opioid receptor by enkephalin produced the antinociceptive effect in diabetic rats by preventing the increase of neuronal NaV1.7 in DRG. Unlike the d-opioid receptor agonist enkephalin, endomorphin 1 and 2 primarily bind to m-opioid receptors to initiate signaling cascades via G-protein-activated inwardly rectifying potassium channels, calcium channels, kinases and adenylcyclases resulting in potent analgesia.[34] Local delivery of synthetic endomorphin has been shown to be analgesic in several animal models of pain.[35–38] Although the analgesic effects of the endomorphins are significant, these effects are transient because of the activity of peptidases that rapidly degrade endogenous opioid peptides including endomorphins.[39,40] In our case, we did observe a trend for anti-allodynic effects mediated by NE2 at 3 and 7 days post vector injection but not at later time points. The vector combinations GAD67 + NP2, GAD67 + NE2 and NE2 + NP2 also showed anti-allodynic effects in diabetic animals but these were not more effective than the single vector treatment. The overall finding that the GAD65 and GAD67 vectors performed better than the opioid-related NP2 and NE2 vectors agrees with clinical observations that opiate drugs have poor efficacy in the treatment of diabetic neuropathic pain.

Our study also seems to indicate that a single injection of the GAD65 or GAD67 vectors produced allodynia for 4 weeks with waning of the response afterwards and that this was superior to daily gabapentin injection. This significantly reduced administration schedule may thus be beneficial if confirmed in humans. The waning of the antinociceptive response in either the rat or mouse models with the extrachromosomal HSV vector is likely due to transgene silencing, but additional experimentation would be required to formally prove this.

For the comparative analysis of the study, we utilized a method of selecting a fixed dose and schedule for each vector.[41] Another method to perform comparisons would be to first study each vector for maximum expression of the protein in the DRG and then adjust dose and schedule for each vector based on this before comparing effects. Although this is doable in animals, it would be more difficult to perform in humans and the relevance of maximum expression in a rat DRG to a human DRG is uncertain. The backbone of the vectors used in this study is engineered to eliminate the potential for homologous recombination resulting in replication competent recombinants and thus it is more appropriate for human use than previous vectors.[42] In addition, by using identical vector backbones with identical transcriptional regulatory signals, we minimized the possibility that differences in gene expression from differential transcript processing could account for observed differences. With the same vector backbone, the clinical grade replication-defective HSV-1 vector expressing human preproenkephalin has been used in clinical trials for cancer pain.[5] This clinical study suggested the vector treatment was well tolerated with no study agent-related serious adverse events observed during the study.[5] In this study, we showed that vectors specifically targeted DRGs but not other organs after paw inoculation and produced few immune responses to the vector.

In summary, our study compared the efficacy of four HSV-1-based vectors and six vector combinations in treating diabetic neuropathic pain. The result suggested that gene transfer of GAD65 or GAD67 expressing HSV-1-based vectors to DRG by paw inoculation was the most effective for treating diabetic neuropathic pain in both rat and mouse STZ models. NP2-expressing vectors also exhibited an anti-allodynia effect, at least at one time point post injection. Injection with vector combinations of GAD65 + NE2, GAD67 + NE2 or NE2 + NP2 effectively treated diabetic mechanic pain but was not better than single vector treatment in our study. In C57BL/6 mouse diabetic modells, GAD65 vector inoculation most significantly relieved thermal pain. NE2 also showed an anti-hyperalgesia effect at day 19 post injection. The toxicology study indicated single paw inoculation of HSV-1-based vectors is safe with very few off-target side effects.

MATERIALS AND METHODS

GAD65, GAD67, NE2, NP2 and GFP constructs

All constructs are replication-defective HSV-1-based vectors deleted for the essential immediate early genes ICP4 and ICP27 with a human cytomegalovirus immediate early promoter-driven expression cassette inserted into both ICP4 loci (Figure 1). The deletions of the ICP4 loci also remove the promoters for the ICP22 and ICP47 genes. The vectors were produced by infection of an ICP4 and ICP27 complementing Vero cell line.[43] The infected cultures were harvested, salt treated, clarified and purified on ion exchange chromatography columns.

Diabetic animal models

Male Wistar rats, with an average weight of 200–250 g (Harlan Laboratories, Inc., Madison, WI, USA), were housed two per cage on ALPHA-dri soft bedding (Shepherd, Chicago, IL, USA) with free access to food and water, and were maintained on a 12:12, light:dark schedule at 21 1C and 60% humidity. Eight-week-old female C57BL/6 mice weighting 20–25 g (Charles River, Wilmington, MA, USA) were housed in sterile environments. Animals were allowed to habituate in the colony room for 1 week before experimental manipulations.

Type 1 diabetes was induced by i.p. injection of 50 mg kg^{-1} STZ (Sigma-Aldrich, St Louis, MD, USA) in sodium citrate buffer (pH 5.0) in Wistar rats. Average serum glucose elevated to 500 mg dl^{-1} the first week after STZ injection and remained at this level for the duration of the experiment (4 months; Supplementary Figure S2a). Serum glucose levels were measured weekly, and about 85% of rats with glucose 4300 mg dl^{-1} were selected for further studies. Weight was monitored weekly and diabetic rats showed growth retardation as expected (Supplementary Figure S2b) with an average 15% weight loss by the end of the experiment (3 months after STZ injection). For C57BL/6 mice, 92% developed stable hyperglycemia with blood glucose 4300 mg dl^{-1} after two consecutive daily doses of 100 mg kg^{-1} STZ (i.p.). Overall, they experienced an average of 10% body weight loss during the 5 weeks of the experiment. Animals that were not overtly ill and did not require insulin supplementation were included in the final analysis. Animals with 25% or greater weight loss were euthanized as per veterinary recommendations.

For the rat diabetic model, development of MA and thermal hyperalgesia was assessed weekly for 3 months utilizing the von Frey and Hargreaves et al.[44,45] tests, respectively. Three weeks after STZ administration, rats developed significant MA manifested as reduced withdrawal threshold in the von Frey test that lasted for at least 10 weeks. However, thermal hyperalgesia measured by the Hargreaves test did not occur in rats (data not shown), as reported by others, possibly due to strain variation or difference of the breeding source.[16] Five weeks after STZ treatment, when MA was confirmed, animals were randomly assigned to experimental groups (n = 10–12 rats per group) and were injected with either a total of 30 ml of 1×10^9 plaque-forming units (p.f.u.) ml^{-1} of the HSV-based vector or 15 ml of 1×10^9p.f.u. ml^{-1} of each vector for the combination study, subcutaneously into both hind paws. The dose of HSV-based vector was chosen to produce sufficient analgesic effect based on previous studies..[6,8,9] Volume and titers limited the ability to increase the dose of each vector in the combination study.

Three controls were included in all experiments: (1) a group of normoglycemic animals served as nondiabetic control, (2) a group of diabetic rats were treated with an injection of PBS in their hindpaw and (3) a group of diabetic rats were treated with daily i.p. injections of 50 mg kg^{-1} gabapentin. The dose of gabapentin selected for this study has been reported to be sufficient for pharmacological activity with daily i.p. injection or subcutaneous mini-pump in diabetic rats.[46,47] The four therapeutic genes were GAD67, GAD65, endomorphin (NE2, expressing both human endomorphin 1 and 2) and preproenkephalin (NP2, expressing human enkephalin). The six vector combinations included GAD67 + GAD65, GAD65 + NE2, GAD67 + NE2, GAD65 + NP2, GAD67 + NP2 and NP2 + NE2. A separate group of healthy rats were injected with a HSV-based GFP control vector in a similar manner, killed 1 h, 1 day and 1 week post vector injection, and blood serum and organs were harvested for toxicology studies.

We also used mouse diabetic models to study the effects of antinociceptive gene transfer in diabetic thermal hyperalgesia. Diabetic C57BL/6 developed thermal hyperalgesia, 2–3 weeks after STZ injection that lasted for 1–2 weeks assessed by the hot plate test, as previously reported.[17] One week after STZ injection when hyperglycemia was confirmed, mice were randomly assigned into experimental groups (n = 13–15 mice per group) and were subcutaneously injected with 10 μl of 1×10^9 p.f.u. ml^{-1} of the HSV-based vector expressing one of the four therapeutic genes (GAD67, GAD65, NE2 or NP2) or PBS alone into both hind paws. A group of normoglycemic mice served as nondiabetic controls.

Behavioral analysis

Behavioral testing for MA in rats was performed between the hours of 0800 and 1600, once per week. All animals were randomized and observers were blinded to the treatments. Rats were placed inside acrylic chambers on top of a wire mesh grid, which allowed access to the paws, and were left to habituate for B10 min before testing. The von Frey filaments (Stoelting, Chicago, IL, USA) were applied from underneath the grid floor perpendicular to the plantar surface until slight buckling occurred, and held for B2–3 s. A positive response was noted if the paw was sharply withdrawn. Flinching immediately upon removal of the hair was also considered a positive response. Ambulation was considered an ambiguous

response, and in such cases the stimulus was repeated. The 50% withdrawal threshold was determined using the up–down method.[48] A testing regime described by Chaplan et al.[44] was used. Ten von Frey filaments with bending forces at approximately equal logarithmic increments were chosen (von Frey numbers: 3.61, 3.84, 4.08, 4.17, 4.31, 4.56, 4.74, 4.93, 5.18, 5.46 and 5.88; equivalent to 0.4, 0.6, 1, 1.4, 2.0, 4, 6, 8, 10, 15, 26 and 60 g, respectively) starting with filament 4.31. In the event of a positive response, the next weaker stimulus was chosen for the next measurement. In the absence of response, a stronger stimulus was presented. This consecutive way of applying filaments was continued until six responses were obtained. The resulting sequence of positive and negative responses was used to calculate the 50% withdrawal threshold. Based on the observations on normal controls, the cutoff of a 40-g hair (B10% of the body weight of rats) was selected as the upper limit for testing. Thermal hyperalgesia in rats was measured with a Plantar Test (Hargreaves et al.[45] method) apparatus (IITC life Science, Woodland Hills, CA, USA) as described previously.

Thermal pain sensation in mice was assessed by a hot plate test every other day for 4 weeks. Mice were placed on a metal plate (IITC life Science) heated to 52 1C with gradually increasing temperature at a rate of 1 1C min^{-1}. Withdrawal latency was measured in seconds up to the time the animals lifted their paw from the plate or licked their paw. Twenty seconds was used as the cutoff time to avoid harm.[49]

Immunocytochemistry

Lumbar SCs and DRGs (L4–6) were removed 1 week post vector paw inoculation in a subset of animals, fixed with 4% paraformaldehyde overnight and then transferred into 30% sucrose in PBS for 2 days. In all, 20-mm cryostat sections were collected on poly-D-lysine-coated slides, washed with 0.1% Triton X-100 in PBS and incubated with blocking solution (PBS with 4% normal donkey serum and 0.1% Triton X-100) for 1 h. The sections of DRG and SC were incubated overnight at 4 1C with primary antibodies either anti-HSV-1 ICP0 (1:10 000, Eastcoast Bio, North Berwick, ME, USA), anti-GFP (1:200, Clontech, Mountain View, CA, USA), anti-calcitonin gene-related peptide (1:200, Calbiochem, Darmstadt, Germany) anti-GAD67 (K-87 1:1000, Abcam Inc., Cambridge, MA, USA) or anti-GAD65 (GAD-6 1:1000, Abcam Inc.) followed by washing four times. Anti-neuronal nuclei alexa fluor488-conjugated antibody (anti-NeuN 1:200, Millipore, Temecula, CA, USA) was used to label neurons. After incubation in the specific secondary fluorescent Dylight649-conjugated AffiniPure Donkey antibody (1:500, Jackson Laboratories, Bar Harbor, ME, USA) for 1 h at room temperature, the specimens were washed three times and mounted with VECTASHIELD mounting medium (Vector Lab, Burlingame, CA, USA) with a cover slip. Images were taken with a Nikon Eclipse Ti florescence microscope (Nikon, Melville, NY, USA).

PCR detection of the viral genome

Total DNA was isolated from DRG, SC, brain, paw, heart, liver, spleen and reproductive organs using the phenol/chloroform extraction method.[50] Tissue was first digested with genomic digestion buffer (Invitrogen, Carlsbad, CA, USA) with 2 mg ml^{-1} proteinase K at 55 1C overnight. PCR was performed for 40 cycles (94 1C for 45 s, 57 1C for 45 s and 72

1C for 45 s) using primers for the HSV (HSV-F: 50-GAGCAGCGCACGATGGA-30; HSV-R: 50-CCCGAAACAGCTGATTGATACA-30). The PCR products were separated by 1% agarose gel and visualized with ethidium bromide.

Reverse transcription-quantitative

To assess transgene expression, lumbar DRGs and SCs were removed 1 and 2 weeks post vector inoculation, and total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was transcribed from the mRNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and quantitative PCR was performed on an Applied Biosystems 7500 real-time PCR system using the predesigned TaqMan Gene expression assay for GAD65 (Hs01100982_m1, ABI, Foster City, CA, USA) or GAD67 (Hs01100982_m1, ABI). For GFP mRNA detection, Sybr Green gene expression assay (ABI) was used. The PCR primers for amplify GFP were 50-TGACCCTGAAGTTCATCTG CACC-30 and 50-TCTTGTAGTTGCCGTCGTCCTTG-30.

Western blotting

Total protein from lumbar DRGs was denatured and extracted with urea buffer (8 M urea, 5 mM dithiothreitol, 150 mM NaCl and 50 mM Tris-Cl pH 7.5) and 50 mg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Antibodies used for western blotting were mouse anti-GAD67 (K-87 1:1000, Abcam Inc.) and peroxidase-conjugated secondary antibodies (Jackson Laboratories). Blots were then stripped and reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (1:1000, Abcam Inc.) as control for loading.

Enzyme-linked immunosorbent assay

Serum was collected from rats at 1 h, 1 day, 1 week post HSV vector inoculation, and animals injected with PBS was used as control. Serum levels of HSV immunoglobulin G were measured with rat HSV-1 immunoglobulin G enzyme-linked immunosorbent assay kit (Calbiotech, Spring Valley, CA, USA) according to the manufacturer's recommendations.

Quantitative and statistical analyses

Biostatistical input was utilized to design all animal experiments in terms of number of animals per group and time points. The mechanical withdrawal threshold determined by the 'up–down' method represents the percentage of maximum possible effect (% MPE) calculated according to the formula: %MPE = withdrawal threshold of diabetic animal/withdrawal threshold of control animal multiplied by 100. The threshold of diabetic rats before vector treatment was found to be B10–20 g, and the cutoff value was 40 g. The thermal pain withdrawal latency was used to calculate the MPE using the formula: % MPE = withdrawal latency of diabetic animal/withdrawal latency of control animal multiplied by 100. With the transformations to % MPE, endpoint had a value in a continuous scale. Two types of statistical analyses were used for the behavioral studies. After checking the overall distribution of the two endpoints: mechanical pain and thermal pain for normality, a two-way analysis of variance was used to compare the % maximal response among groups. This analysis was performed to provide a test for the 'overall' treatment effect. In a more detailed

analysis, mixed effect models were used to study trends across time. This analysis incorporates the repeated measurements for each rat to compare overall trends pre- and post-vector injection. As the differences between the left and right foot were quite similar and the variability was small compared with the variability across time, the two feet measurements for each rat were averaged before fitting the mixed linear models. This analysis used data points from 1 to 4 weeks post vector injection as the largest antinociceptive effect occurred during this time window. Data points after 1 month post vector inoculation were not considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by USAF/AFMC FA 7014-10-2-0002, Clinical Gene Therapy.

JW, DK, JG and DW are employees of Diamyd Inc., where HSV1-based vectors used in this study were manufactured.

REFERENCES

1. Luft D. Neuropathic pain in diabetic nephropathy—update on analgesic strategies. Nephrol Dial Transplant. 1999; 14:2285–2288. [PubMed: 10528644]
2. Ziegler D. Painful diabetic neuropathy: treatment and future aspects. Diabetes Metab Res Rev. 2008; 24(Suppl 1):S52–S57. [PubMed: 18395890]
3. Kumar S, Ruchi R, James SR, Chidiac EJ. Gene therapy for chronic neuropathic decarbox-pain: how does it work and where do we stand today? Pain Med. 2011; 12:808–822. [PubMed: 21564510]
4. Glorioso JC, Fink DJ. Herpes vector-mediated gene transfer in the treatment of chronic pain. Mol Ther. 2009; 17:13–18. [PubMed: 18841093]
5. Fink DJ, Wechuck J, Mata M, Glorioso JC, Goss J, Krisky D, et al. Gene therapy for pain: results of a phase I clinical trial. Ann Neurol. 2011; 70:207–212. [PubMed: 21796661]
6. Chattopadhyay M, Mata M, Fink DJ. Continuous delta-opioid receptor activation reduces neuronal voltage-gated sodium channel (NaV1.7) levels through activation of protein kinase C in painful diabetic neuropathy. J Neurosci. 2008; 28:6652–6658. [PubMed: 18579738]
7. Goss JR, Mata M, Goins WF, Wu HH, Glorioso JC, Fink DJ. Antinociceptive effect of a genomic herpes simplex virus-based vector expressing human proenkephalin in rat dorsal root ganglion. Gene Ther. 2001; 8:551–556. [PubMed: 11319622]
8. Wolfe D, Hao S, Hu J, Srinivasan R, Goss J, Mata M, et al. Engineering an endomorphin-2 gene for use in neuropathic pain therapy. Pain. 2007; 133:29–38. [PubMed: 17395375]
9. Liu J, Wolfe D, Hao S, Huang S, Glorioso JC, Mata M, et al. Peripherally delivered glutamic acid decarboxylase gene therapy for spinal cord injury pain. Mol Ther. 2004; 10:57–66. [PubMed: 15233942]
10. Chattopadhyay M, Mata M, Fink DJ. Vector-mediated release of GABA attenuates pain-related behaviors and reduces NaV1.7 in DRG neurons. Eur J Pain. 2011; 15:913–920. [PubMed: 21486703]
11. Hao S, Mata M, Glorioso JC, Fink DJ. HSV-mediated expression of interleukin-4 in dorsal root ganglion neurons reduces neuropathic pain. Mol Pain. 2006; 2:6. [PubMed: 16503976]
12. Peng XM, Zhou ZG, Glorioso JC, Fink DJ, Mata M. Tumor necrosis factor-alpha contributes to below-level neuropathic pain after spinal cord injury. Ann Neurol. 2006; 59:843–851. [PubMed: 16634039]

13. Yeomans DC, Lu Y, Laurito CE, Peters MC, Vota-Vellis G, Wilson SP, et al. Recombinant herpes vector-mediated analgesia in a primate model of hyperalgesia. Mol Ther. 2006; 13:589–597. [PubMed: 16288901]
14. Giuditta A, Chun JT, Eyman M, Cefaliello C, Bruno AP, Crispino M. Local gene expression in axons and nerve endings: the glia-neuron unit. Physiol Rev. 2008; 88:515–555. [PubMed: 18391172]
15. Jiang Y, Nyengaard JR, Zhang JS, Jakobsen J. Selective loss of calcitonin gene-related Peptide-expressing primary sensory neurons of the a-cell phenotype in early experimental diabetes. Diabetes. 2004; 53:2669–2675. [PubMed: 15448099]
16. Morrow TJ. Animal Models of Painful Diabetic Neuropathy: The STZ Rat Model. Current Protocols in Neuroscience. John Wiley & Sons: Somerset. 2004:9.18.1–9.18.11.
17. Pabbidi RM, Yu SQ, Peng S, Khardori R, Pauza ME, Premkumar LS. Influence of TRPV1 on diabetes-induced alterations in thermal pain sensitivity. Mol Pain. 2008; 4:9. [PubMed: 18312687]
18. Wodarski R, Clark AK, Grist J, Marchand F, Malcangio M. Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats. Eur J Pain. 2009; 13:807–811. [PubMed: 18977160]
19. Pinal CS, Tobin AJ. Uniqueness and redundancy in GABA production. Perspect Dev Neurobiol. 1998; 5:109–118. [PubMed: 9777629]
20. Karlsen AE, Hagopian WA, Grubin CE, Dube S, Disteche CM, Adler DA, et al. Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. Proc Natl Acad Sci USA. 1991; 88:8337–8341. [PubMed: 1924293]
21. Soghomonian JJ, Martin DL. Two isoforms of glutamate decarboxylase: why? Trends Pharmacol Sci. 1998; 19:500–505. [PubMed: 9871412]
22. Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci. 2002; 22:6724–6731. [PubMed: 12151551]
23. Castro-Lopes JM, Tavares I, Coimbra A. GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. Brain Res. 1993; 620:287–291. [PubMed: 8369960]
24. Hao S, Mata M, Wolfe D, Huang S, Glorioso JC, Fink DJ. Gene transfer of glutamic acid decarboxylase reduces neuropathic pain. Ann Neurol. 2005; 57:914–918. [PubMed: 15929041]
25. Kim J, Kim SJ, Lee H, Chang JW. Effective neuropathic pain relief through sciatic nerve administration of GAD65-expressing rAAV2. Biochem Biophys Res Commun. 2009; 388:73–78. [PubMed: 19643087]
26. Lee B, Kim J, Kim SJ, Lee H, Chang JW. Constitutive GABA expression via a recombinant adeno-associated virus consistently attenuates neuropathic pain. Biochem Biophys Res Commun. 2007; 357:971–976. [PubMed: 17466264]
27. Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, et al. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature. 2003; 424:938–942. [PubMed: 12931188]
28. Jolivalt CG, Lee CA, Ramos KM, Calcutt NA. Allodynia and hyperalgesia in diabetic rats are mediated by GABA and depletion of spinal potassium-chloride co-transporters. Pain. 2008; 140:48–57. [PubMed: 18755547]
29. Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, et al. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci USA. 2009; 106:9075–9080. [PubMed: 19451647]
30. Kubo K, Nishikawa K, Ishizeki J, Hardy-Yamada M, Yanagawa Y, Saito S. Thermal hyperalgesia via supraspinal mechanisms in mice lacking glutamate decarbox-pain: ylase 65. J Pharmacol Exp Ther. 2009; 331:162–169. [PubMed: 19571163]
31. Carlton SM, Zhou S, Coggeshall RE. Peripheral GABA(A) receptors: evidence for peripheral primary afferent depolarization. Neuroscience. 1999; 93:713–722. [PubMed: 10465455]
32. Zhou S, Komak S, Du J, Carlton SM. Metabotropic glutamate 1alpha receptors on peripheral primary afferent fibers: their role in nociception. Brain Res. 2001; 913:18–26. [PubMed: 11532243]

33. Sawynok J. Topical and peripherally acting analgesics. Pharmacol Rev. 2003; 55:1–20. [PubMed: 12615951]
34. Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, et al. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature. 1996; 383:819–823. [PubMed: 8893006]
35. Fichna J, do-Rego JC, Mirowski M, Costentin J, Janecka A. Binding of endomorphin-2 to mu-opioid receptors in experimental mouse mammary adenocarcinoma. J Pept Res. 2005; 65:459–464. [PubMed: 15813894]
36. Obara I, Przewlocki R, Przewlocka B. Local peripheral effects of mu-opioid receptor agonists in neuropathic pain in rats. Neurosci Lett. 2004; 360:85–89. [PubMed: 15082185]
37. Tseng LF. The antinociceptive properties of endomorphin-1 and endomorphin-2 in the mouse. Jpn J Pharmacol. 2002; 89:216–220. [PubMed: 12184724]
38. Soignier RD, Vaccarino AL, Brennan AM, Kastin AJ, Zadina JE. Analgesic effects of endomorphin-1 and endomorphin-2 in the formalin test in mice. Life Sci. 2000; 67:907–912. [PubMed: 10946850]
39. Okada Y, Tsuda Y, Bryant SD, Lazarus LH. Endomorphins and related opioid peptides. Vitam Horm. 2002; 65:257–279. [PubMed: 12481550]
40. Janecka A, Kruszynski R, Fichna J, Kosson P, Janecki T. Enzymatic degradation studies of endomorphin-2 and its analogs containing N-methylated amino acids. Peptides. 2006; 27:131–135. [PubMed: 16087275]
41. Robinson, DS., Schooler, NR. Clinical Study Design—Critical Issues. New York, New York: Raven Press; 1995.
42. Wolfe D, Goins WF, Yamada M, Moriuchi S, Krisky DM, Oligino TJ, et al. Engineering herpes simplex virus vectors for CNS applications. Exp Neurol. 1999; 159:34–46. [PubMed: 10486173]
43. Grant KG, Krisky DM, Ataai MM, Glorioso JC 3rd. Engineering cell lines for production of replication defective HSV-1 gene therapy vectors. Biotechnol Bioeng. 2009; 102:1087–1097. [PubMed: 18828174]
44. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994; 53:55–63. [PubMed: 7990513]
45. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain. 1988; 32:77–88. [PubMed: 3340425]
46. Ortiz MI, Ponce-Monter HA, Fernandez-Martinez E, Macias A, Rangel-Flores E, Izquierdo-Vega JA, et al. Pharmacological interaction between gabapentin and glibenclamide in the formalin test in the diabetic rat. Proc West Pharmacol Soc. 53:49–51. [PubMed: 22128454]
47. Chen SR, Samoriski G, Pan HL. Antinociceptive effects of chronic administration of uncompetitive NMDA receptor antagonists in a rat model of diabetic neuropathic pain. Neuropharmacology. 2009; 57:121–126. [PubMed: 19422840]
48. Dixon WJ. Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol. 1980; 20:441–462. [PubMed: 7387124]
49. Aloe L, Moroni R, Angelucci F, Fiore M. Role of TNF-alpha but not NGF in murine hyperalgesia induced by parasitic infection. Psychopharmacology. 1997; 134:287–292. [PubMed: 9438678]
50. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987; 162:156–159. [PubMed: 2440339]

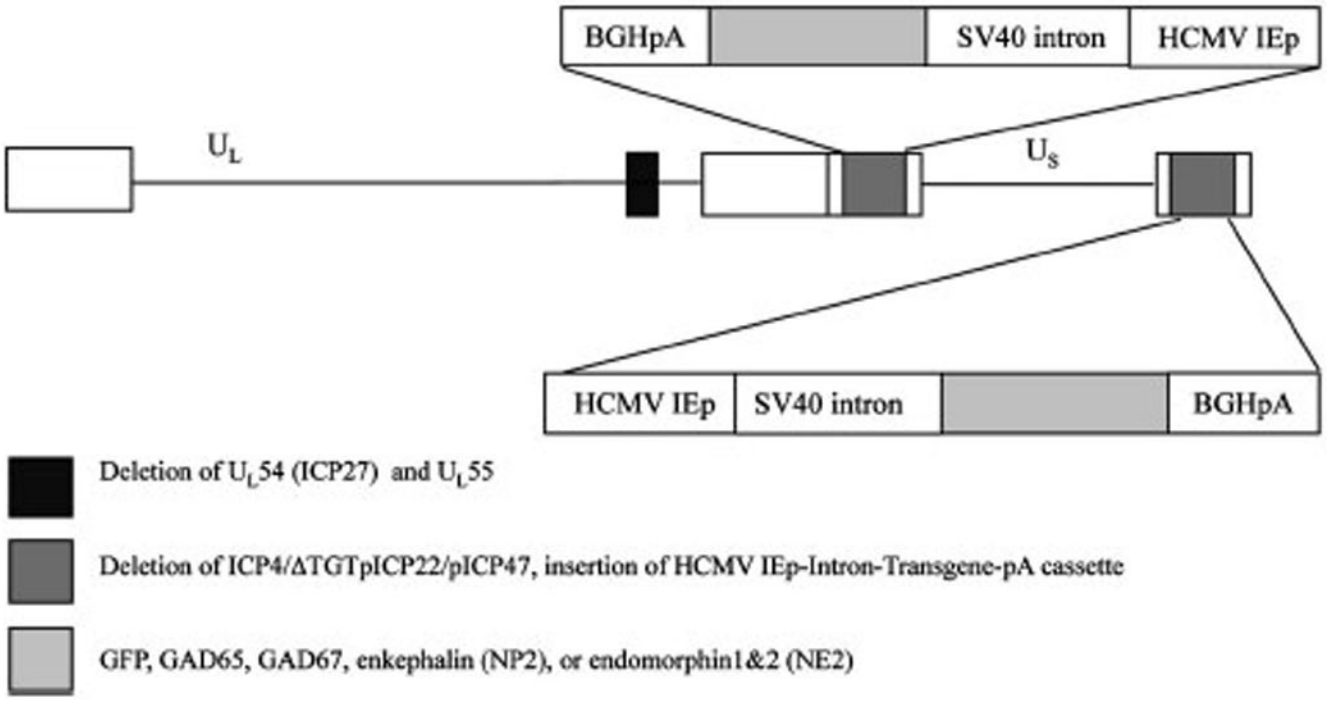

Figure 1.

Schematic structures of four vectors. Diagrams of the modifications engineered into the replication-defective HSV-1-based vectors are shown. The backbone and transcriptional regulatory elements were identical with the only differences being in the transgene for each vector.

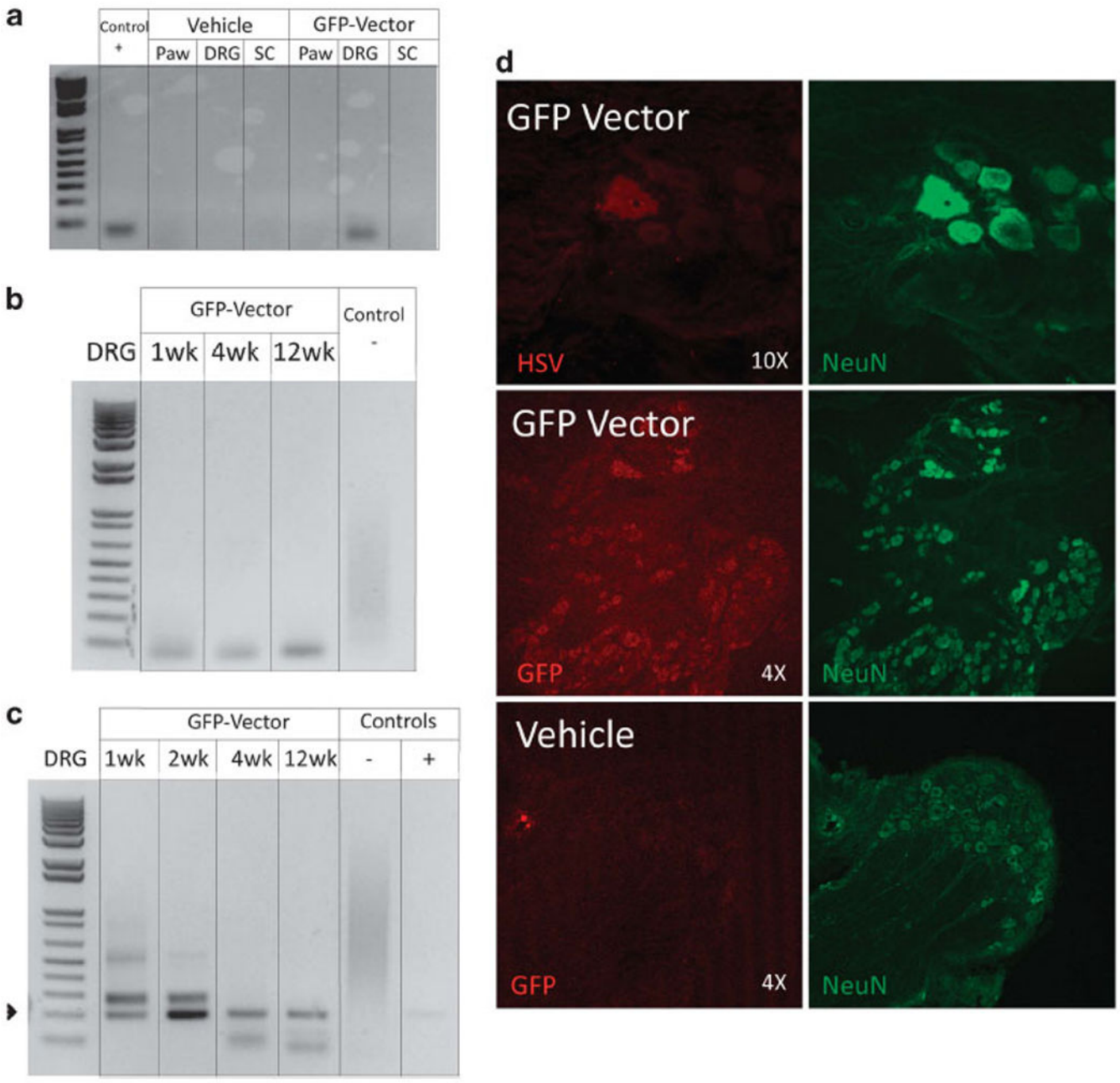

Figure 2.

Detection of vector in DRG. The presence of vector in DRG was confirmed by PCR with specific primers at 1 week after vector injection (a). The DNA of HSV-based vector was detected at least 12 weeks after footpad injection in DRG (b). The HSV PCR product size was 64 bp. GFP mRNAs was detected in DRG by reverse transcription-PCR with specific GFP primers at 1, 2, 4 and 12 weeks after hindpaw vector injection (c). The GFP PCR product size was 197 bp (band marked with arrow). Vector was also detected by immunohistochemistry (IHC) with anti-HSV antibody (red) or anti-GFP antibody (red) (d). Two weeks after hindpaw injection of HSV-based GFP vector, DRGs from injected side

(lumbar region) were dissected and fixed for IHC. DRGs were stained with antibodies for HSV antigen (red), GFP (red) and neurons (anti-NeuN antibody; a neuronal marker; green).

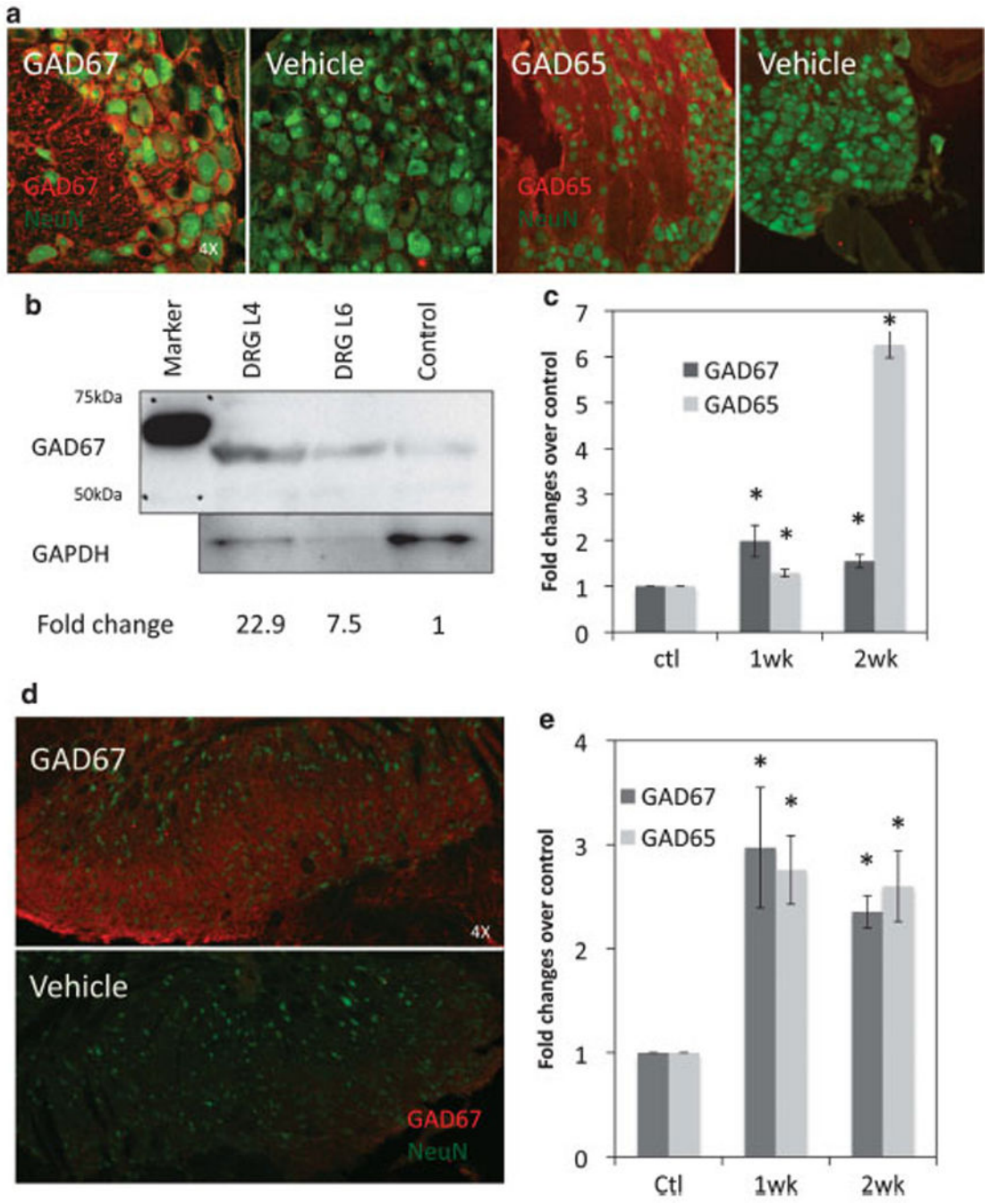

Figure 3.

HSV-GAD vector injection into the footpad increased GADs expression in DRG and lumbar SC. Thirty microliters containing 3×10^7p.f.u. of vector was injected into the hind paws on day 0. One week post injection of GAD65 or GAD67 vector, GAD67 (red) and GAD65 (red) immunostaining is increased in L5 DRG (double labeling with the neuronal marker NeuN; green) compared with DRG from vehicle-injected rat (a). Western blot shows that GAD67 protein is increased in lumbar DRGs from rats injected 1 week earlier with GAD67 vector when compared with vehicle-injected controls (b). Expression of GAD65 and GAD67

mRNA also was significantly increased in lumbar DRGs at 1 and 2 weeks after HSV-GAD65 or GAD67 injection in hind paws. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is included as a control for loading and densitometric scanning was carried out to semiquantitatively determine the fold change in GAD67 after normalizing to GAPDH. (c). Increased GAD67 immunostaining was also seen in the substantia gelatinosa of the dorsal horn of lumbar SC at the vector-injected side, when compared with vehicle-injected contralateral side (d). mRNA levels for GAD65 and GAD67 were also increased in in SC after HSV-GAD65 or GAD67 injection in hind paws, as shown after quantification (e). Data shown as means±s.e.m. N = 3 *Po0.05 t-test.

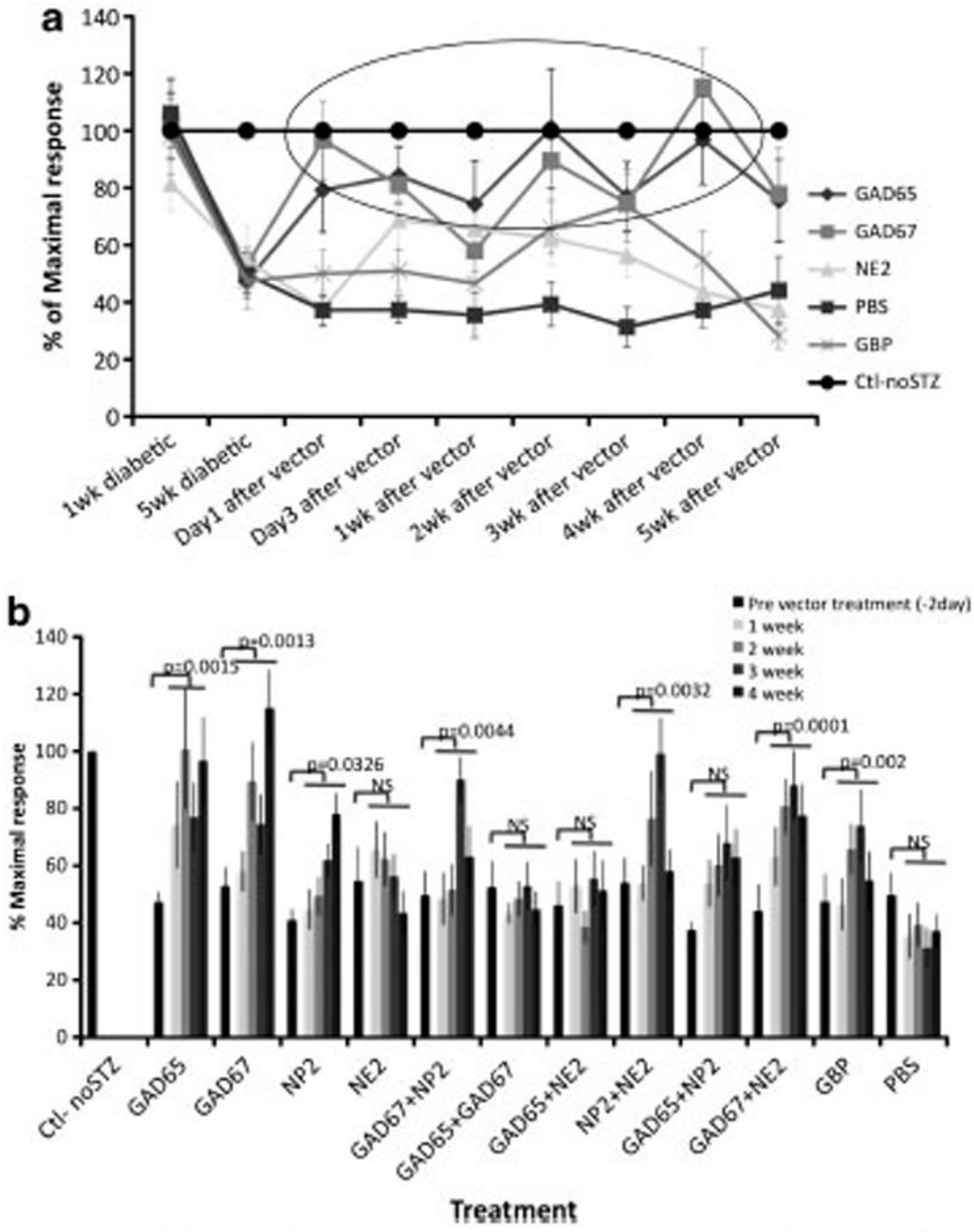

Figure 4.

Effects of HSV-vector injection on diabetes-induced mechanical pain. MA was measured in diabetic and control rats using von Frey monofilaments. Approximately 4 weeks after STZ treatment, diabetic rats started to exhibit MA. Five weeks post STZ injection, a dose of 3×10^7p.f.u. of single vector or combination was injected into hind paws. Vehicle (PBS) was injected into diabetic rats as a negative control. Gabapentin (GBP) was administered daily at a dose of 50 mg kg^{-1} (i.p.). Data are normalized to control nondiabetic animal (Ctl-no STZ) and are presented as mean±s.e.m. of % maximal response. Normalized withdrawal threshold

was measured weekly after STZ injection (shown as 1–5 weeks) and after vector inoculation (shown as day 1–5 weeks after vector). Data points within the black oval = Po0.05 (two-way analysis of variance was used to compare the % maximum response among groups) (a). Data were collected before and 1–4 weeks after vector injection and analyzed with trend statistical analysis to compare pre- and post-virus effects (from 1 to 4 weeks post virus inoculation) on allodynia (b). Po0.05; n = 8–10 per group; NS, not significant.

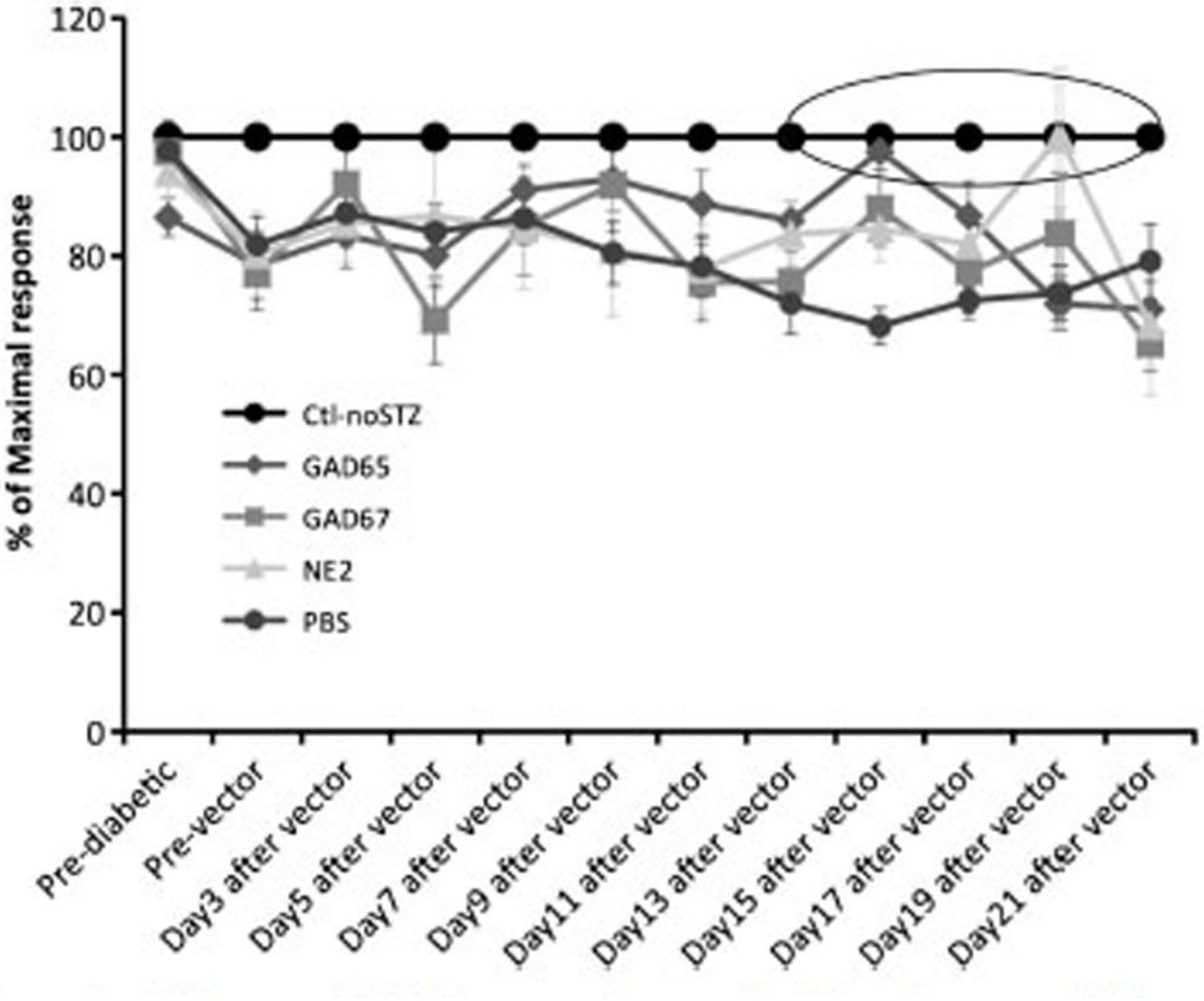

Figure 5.

Effects of HSV-vector footpad injection on diabetes-induced neuropathic thermal pain in mice. Diabetic and control C57BL/6 mice were tested for development of thermal pain and effect of single vector (GAD65, GAD67 and NE2) with the hot plate test by measuring the paw withdrawal latency. Vehicle (PBS) was injected into control diabetic rats (PBS) as a negative control. The data are normalized to control nondiabetic animals (Ctl-no STZ) and is presented as the mean±s.e.m. of % maximal response. n = 10–14 for C57BL/6. Data points within the black oval = Po0.05 (two-way analysis of variance was used to compare the % maximum response among groups).

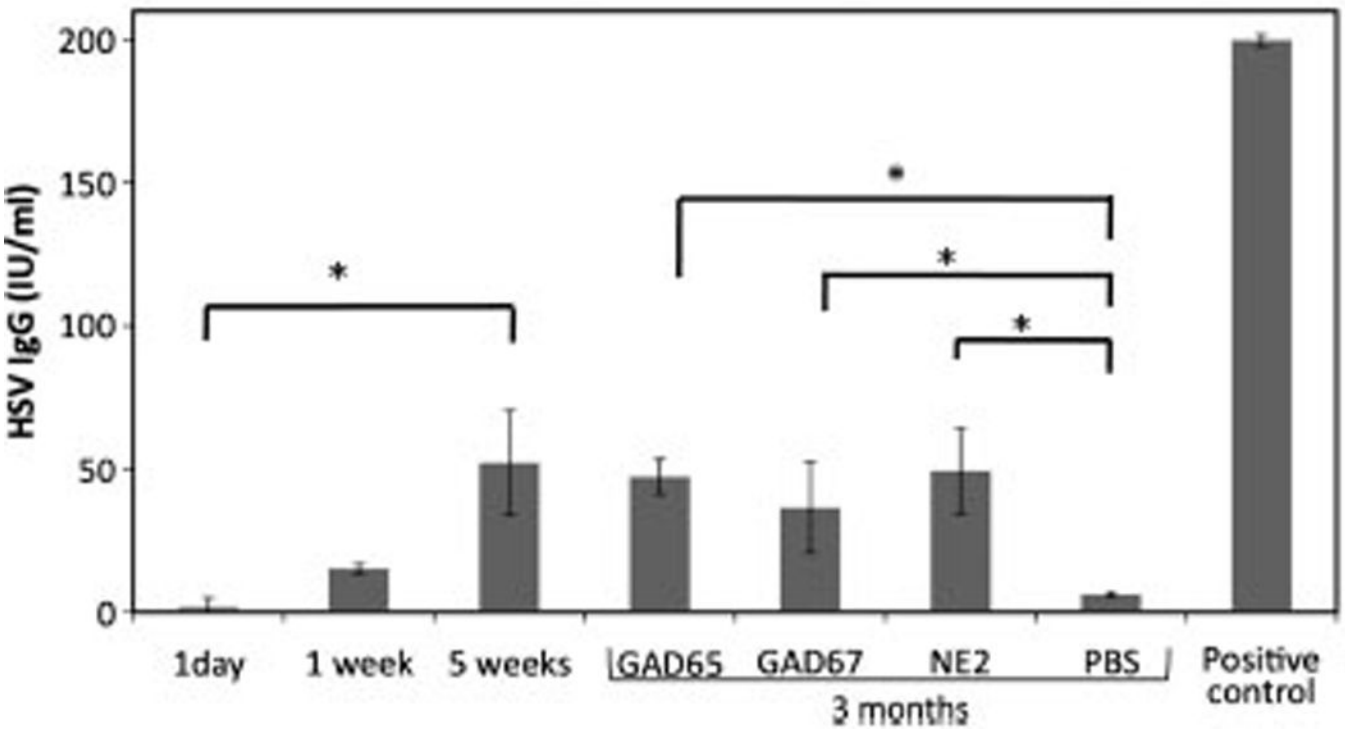

Figure 6.

Detection of anti-HSV immunoglobulin G (IgG) antibodies in the serum of HSV-vector-injected animals. Enzyme-linked immunosorbent assay was used to detect the level of anti-HSV antibodies in serum collected at indicated time points after HSV vector injection. Samples marked 0 h are negative controls from rats that have not been injected with HSV vectors. The GAD65, GAD67, NE2 and PBS groups represent samples collected from diabetic rats at 3 months after corresponding vectors or PBS treatment. Data are shown as the mean±s.e.m. n = 3 *Po0.05 Student's t-test.